Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 4520–4524, June 1989
Cell Biology

Induction of cervical neoplasia in the mouse by herpes simplex virus type 2 DNA

(*in vivo* transfection/viral carcinogenesis)

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Communicated by Lester O. Krampitz, March 17, 1989 (received for review November 12, 1988)

ABSTRACT **Induction of cervical neoplasia in the mouse cervix by herpes simplex virus types 1 (HSV-1) and 2 (HSV-2) has been reported. The present study was done to determine if transfection with DNA of HSV-2 can induce carcinogenesis in this animal model. Genomic HSV-2 DNA was isolated from infected HEp-2 cells and separated from host cell DNA by cesium chloride density gradient centrifugation. The DNA was applied to mouse cervix for periods of 80–100 weeks. Experimental controls were treated with uninfected genomic HEp-2 cell DNA or with calf thymus DNA. Vaginal cytological preparations from all animals were examined monthly to detect epithelial abnormalities. Animals were sacrificed and histopathology studies were done when cellular changes indicative of premalignant or malignant lesions were seen on vaginal smears. Cytologic and histologic materials were coded and evaluated without knowledge of whether they were from animals treated with virus or control DNA. Premalignant and malignant cervical lesions similar to those that occur in women were detected in 61% of the histologic specimens obtained from animals exposed to HSV-2 DNA. The yield of invasive cancers was 21% in animals treated with HSV-2 DNA. No cancers were detected in mice treated with either HEp-2 or calf thymus DNA. Dysplasia was detected in only one of these control animals.**

Previous studies have demonstrated that the induction of premalignant and malignant lesions in the mouse cervix occur after prolonged exposure to formalin- or ultraviolet (UV)-inactivated herpes simplex virus types 1 or 2 (HSV-1 or -2, respectively) (1–3). In these earlier experiments, repeated exposure of the mouse cervix resulted in a sequence of pathological changes analogous to those that occur during human cervical carcinogenesis. The data obtained in this *in vivo* model support conclusions derived from the *in vitro* transformation of rodent cells by HSV-1 and -2 (for review, see refs. 4 and 5). However, a consensus has not been attained concerning a direct role for these viruses in carcinogenesis. There has been an inability to delineate a unique fragment of HSV DNA that morphologically transforms cells (6–13). Furthermore, evidence that *in vitro*-transformed cells remain morphologically transformed in the absence of detectable herpesvirus DNA sequences has contributed to the hypothesis that DNA of the virus is not essential to maintain the transformed cell state (5, 14–16). In addition, a high spontaneous rate of transformation relative to induced transformation has generated healthful skepticism concerning the interpretation of cellular transformation data obtained in *in vitro* experiments. Nucleotide sequences of HSV RNA complementary to HSV DNA that induce *in vitro* transformation have been detected by *in situ* hybridization in 35–67% of cervical carcinomas (17, 18). Viral DNA sequences homologous to the morphological transforming regions of HSV-1 and -2 have been detected in 10–30% of human cervical neoplasia (19–24). These findings, in light of an inability to detect HSV DNA sequences in serially passaged but stably transformed cells, have posed further questions concerning the interpretation of data obtained from experiments in rodent cells. Nevertheless, the studies concerning *in vitro* transformation by HSV have provided an invaluable basis upon which to assemble studies concerning the role and mechanism of HSVs in cervical carcinogenesis.

The present study was undertaken to determine if *in vivo* carcinogenesis can be induced with whole HSV-2 DNA across cervical cell membranes. The demonstration of this process should provide a foundation for molecular studies concerning the mechanism of carcinogenesis in the mouse model.

MATERIALS AND METHODS

Propagation of Virus and Isolation of HSV-2 and Cellular DNA. HSV-2 (strain ATCC VR-734, Roizman) was obtained from the American Type Culture Collection. Monolayer cultures of HEp-2 cells (ATCC CCL23, human epidermoid carcinoma) grown in 75-cm^2 plastic tissue culture flasks in Dulbecco's modified Eagle's medium containing 2% (vol/vol) fetal calf serum were inoculated with HSV-2 and incubated at 37°C. Uninoculated cultures for preparation of control HEp-2 cell DNA were incubated in the same way. When 75% of the cells in inoculated cultures showed viral cytopathic effects, cells and fluids were harvested from both virus-inoculated and control cultures as described (1) and stored in 30-ml aliquots at −70°C.

HSV-2 DNA was purified from infected HEp-2 cells essentially as described by Walboomers and Shegget (25), except that banding in cesium chloride was substituted for sodium iodide for the separation of viral from cell DNA. Aliquots of infected or uninfected HEp-2 cells were centrifuged in a Beckman 70.1 Ti rotor at 37,000 rpm for 60 min at 4°C. Pellets were suspended in 2 ml of 10 mM Tris·HCl, pH 7.5/10 mM Na_2EDTA/10 mM NaCl (TNE) containing 0.5% SDS and proteinase K (2 mg/ml) and incubated at 37°C for 3 hr. An additional 5 mg of proteinase K was then added and the incubation continued for a total of 15 hr.

Aliquots of proteinase K digests were centrifuged in CsCl gradients at 48,500 rpm for 40 hr at 20°C in a Beckman 70 Ti rotor. Bands of cellular DNA from uninfected preparations of HEp-2 cells and of viral DNA from infected cultures were collected in an ISCO fraction collecting system. Pooled HSV-2 virus or HEp-2 cellular DNA preparations were dialyzed against several exchanges of 10 mM Tris·HCl, pH 8.0/1 mM EDTA. The HSV-2 DNA was then characterized by *Bgl* II and *Hpa* I restriction endonuclease cleavage patterns on agarose minigels. *Bgl* II- and *Hpa* I-digested

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Abbreviations: HSV, herpes simplex virus; DMSO, dimethyl sulfoxide.

minigel patterns of five preparations of HSV-2 DNA that were ultimately pooled for use in this study are depicted in Fig. 1. Preparations were stored at −20°C.

Final preparations contained HEp-2 cellular DNA or genomic HSV-2 DNA at 0.15 μg/ml in 0.01 M Hepes, pH 7.05/125 mM $CaCl_2$. Additional solutions containing calf thymus DNA (Sigma) (1.8 μg/ml) as carrier plus HSV-2 DNA or calf thymus DNA alone were also prepared.

Animal Treatment and Assessment. C57BL virgin female mice were employed in these studies. After assessing vaginal cytological preparations obtained weekly for 4 weeks to rule out pre-existing abnormalities, sterile cotton pledgets saturated with HSV-2 DNA (experimental), or fluid containing HEp-2 or calf thymus DNA (controls) were inserted intravaginally three times per week. Each pledget absorbed ≈0.1 ml of solution containing HSV-2 or HEp-2 DNA at 0.15 μg/ml or calf thymus DNA at 1.8 μg/ml. Cytologic preparations were made by introducing sterile isotonic saline into the vagina, aspirating, and spreading the fluid onto a glass slide. Slides were then stained by the Papanicolaou method and evaluated by one worker without bias. These preparations were obtained from animals every 4 weeks.

The mice were sacrificed when cytological changes consistent with invasive cancer were identified in animals treated with HSV-2 DNA. The duration of the study was 100 weeks. The reproductive tract was fixed in formalin and sections of the entire tract were stained with hematoxylin and eosin. Sections were reviewed by two workers who were unaware of the cytological interpretations at the time of sacrifice.

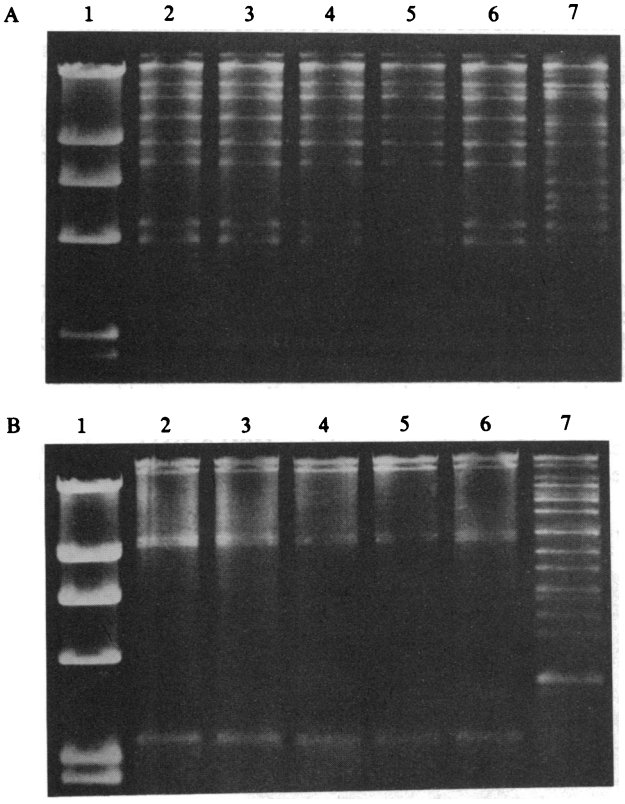


FIG. 1. *Bgl* II and *Hpa* I restriction endonuclease patterns of separate HSV-2 DNA preparations. Prior to combination of individual preparations of genomic DNA, aliquots of each preparation were characterized by analysis of *Bgl* II and *Hpa* I restriction endonuclease cleavage patterns on agarose minigels. In this experiment, aliquots of separately purified preparations of HSV-2 DNA were quantitatively digested with *Bgl* II or *Hpa* I. Buffer specifications and reaction conditions were as set forth by the supplier. After digestion was complete, samples were electrophoresed on a 0.7% agarose gel and stained with ethidium bromide. The electrophoresis patterns of the *Bgl* II (*A*) and *Hpa* I (*B*) digests of five preparations are shown (lanes 2–6). Lanes 1 and 7 in *A* contain *Hin*dIII-digested λ DNA molecular mass markers and a *Bgl* II digest of HSV-1 DNA, respectively. Lanes 1 and 7 in *B* contain *Hin*dIII-digested λ DNA molecular mass markers and a *Hpa* I digest of HSV-1 DNA, respectively.

RESULTS

The frequencies of cytologic lesions induced in the cervix after exposure to HSV-2 DNA are depicted in Fig. 2. Cytological changes consistent with dysplasia were observed in more than 40% of animals by 15–20 weeks. Cellular changes interpreted as microinvasive cancer began to appear by ≈40 weeks. Alterations interpreted to be indicative of invasive cancer were present in >10% of animals at 80 weeks. A decrease in frequency of dysplasia occurred as the process progressed to cancer.

Experiments were terminated and histologic specimens were prepared at 100 weeks when >35% of the animals treated with solutions containing HSV-2 DNA demonstrated the cytological alterations of cancer. The frequencies of cervical abnormalities observed by histologic examination at the termination of these experiments are shown in Table 1. There were a total of 80 mice in experimental groups that were treated with HSV-2 DNA and 80 mice in experimental groups that were treated with various control solutions. A number of mice from each of the groups were not available for pathologic study at the termination of the experiment due to deaths from intercurrent infection and autolysis or cannibalization of tissues. The results were derived only from animals whose tissues were available for microscopic study. The frequency of cervical lesions in mice exposed to control solutions is shown in Table 1, experimental groups 1–4. For these experiments, DNA was administered in 0.01 M Hepes, pH 7.05/125 mM $CaCl_2$ to optimize transfection (26). The addition of dimethyl sulfoxide (DMSO) has been reported by Stowe and Wilke (27) to enhance DNA transport in some *in vitro*-transformation experiments. Treatment of animals with Hepes buffer containing $CaCl_2$ alone or $CaCl_2$ plus DMSO produced no dysplasia or cancer. Treatment with Hepes buffer containing $CaCl_2$ plus HEp-2 DNA produced dysplasia in only 1 of 20 animals and no cancers. Hepes buffer, $CaCl_2$, and calf thymus DNA produced neither dysplasia nor cancer. The frequency of cervical lesions in mice exposed to HSV-2 DNA is shown in Table 1, experimental groups 5 and 6. Thirty

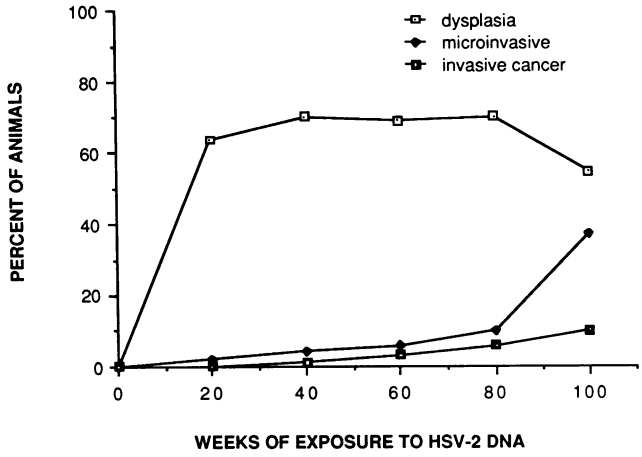


FIG. 2. Cytological alterations after cervical exposure to HSV-2 DNA. Animals were exposed to HSV-2 DNA three times weekly. Cytological preparations were made after various intervals of treatment by introducing sterile isotonic saline into the vagina, aspirating, and spreading the fluid on a glass slide. The preparations were stained by the Papanicolaou method. Smears were evaluated by one worker without bias.

Table 1. Frequency of cervical lesions in C57 mice exposed to control solutions or to HSV-2 DNA

Mice (group)	Treatment	No. of animals	No. of animals with cervical lesions		
			Normal	Dysplasia	Invasive cancer
Control					
1	Hepes buffer	11	11	0	0
2	DMSO	13	13	0	0
3	HEp-2 DNA	20	19	1	0
4	Calf thymus DNA	23	23	0	0
Total		67	66 (98.5%)	1 (1.5%)	0
HSV-2 DNA-treated					
5	HSV-2 DNA	30	11	11	8
6	HSV-2 DNA + calf thymus DNA	31	13	13	5
Total		61	24 (39.4%)	24 (39.4%)	13 (21.2%)

All treatment solutions contained 0.01 M Hepes, pH 7.05/125 mM $CaCl_2$. Group 1, Hepes buffer/$CaCl_2$ alone; group 2, DMSO at a final concentration of 2%; group 3, HEp-2 cell DNA at 1.8 μg/ml; group 4, calf thymus DNA at 1.8 μg/ml; group 5, HSV-2 DNA at 0.15 μg/ml; group 6, HSV-2 DNA at 0.15 μg/ml plus calf thymus DNA at 1.8 μg/ml. The increased frequency of neoplasia among HSV-2 DNA treated animals relative to control treated groups was statistically significant ($P < 0.0001$). Numbers in parentheses are percent of total control or HSV-2 DNA-treated mice.

animals were treated with Hepes buffer/$CaCl_2$ and HSV-2 DNA. Invasive cancer was detected in 8 mice and dysplasia was detected in 11 mice. Thirty-one animals were treated with Hepes buffer/$CaCl_2$ containing HSV-2 DNA and calf thymus DNA. The calf thymus DNA was added in this group as carrier and to possibly reduce degradation of HSV-2 or HEp-2 DNA by proteolytic enzymes present in vaginal secretions. Invasive cancer was detected in 5 mice and dysplasia was detected in 13 of these animals. Differences between the frequencies of neoplastic lesions in animals treated with HSV-2 DNA alone and those treated with HSV-2 plus calf thymus DNA were not statistically significant. Thus, when the two groups are combined, invasive cancers were detected in 21% and dysplasia in 39% of mouse cervices exposed to solutions that contained HSV-2 DNA. In contrast, there were no cancers and dysplasia was detected in only one of the animals exposed to control solutions. The differences between the combined HSV-2 DNA group and the combined control group were statistically significant by the χ^2 test ($P < 0.0001$).

Representative photomicrographs of tissues obtained from three animals that were treated with Hepes buffer/$CaCl_2$ containing HSV-2 DNA are illustrated in Figs. 3–5. These data are reported in Table 1. Fig. 3 depicts severe dysplasia in a mouse cervix. The epithelium is increased in width with immature cells extending from the basement membrane to the surface. The lower one-third of epithelium consists predominantly of basal type cells. Fig. 4 shows a microinvasive lesion in a mouse cervix with cords of anaplasic cells superficially penetrating the basement membrane. A highly invasive non-keratinizing large cell carcinoma with neoplastic cells extending deeply into the submucosal layer of the mouse cervix is illustrated in Fig. 5. Fig. 5*B* demonstrates the degree of nuclear pleomorphism and nuclear hyperplasia present in this cancer.

DISCUSSION

In this series of experiments, premalignant and malignant lesions of the cervix were produced in C57 mice that were identical to those produced in previous studies utilizing inactivated virions of HSV-1 or -2 (1, 2). The cytologic and histologic characteristics were similar to cervical lesions that occur in women. The differences between groups of mice treated with solutions containing HSV-2 DNA and groups treated with control solutions were statistically significant by

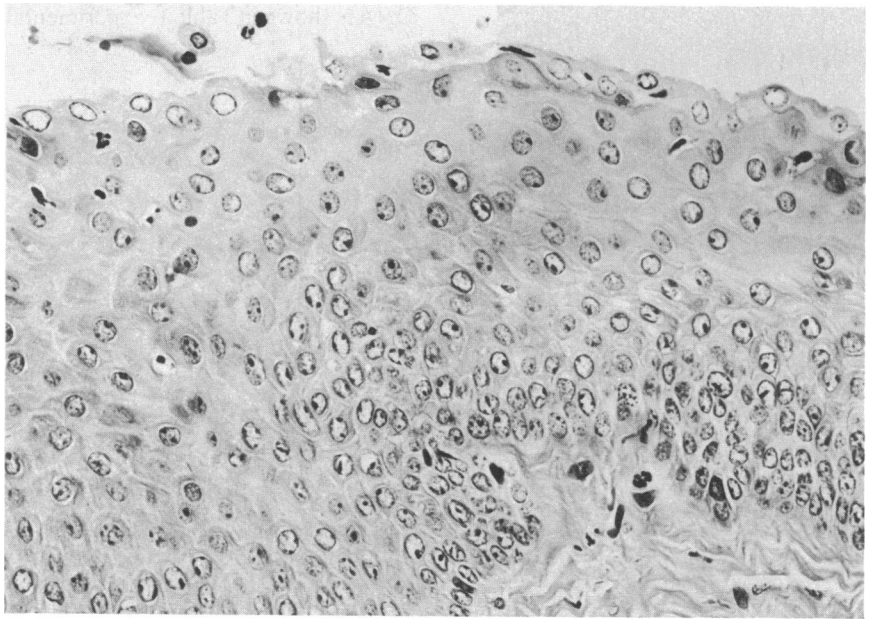


FIG. 3. Dysplasia. The entire epithelium has been replaced by atypical cells. The lower one-third of the epithelium has been replaced by basal type cells with extension of the atypia into the superficial zone. (×350.)

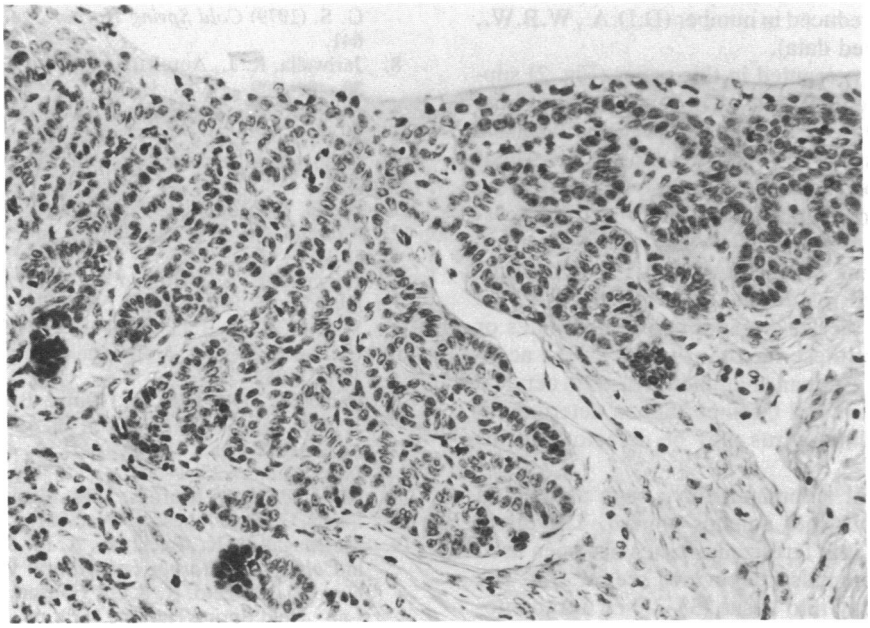
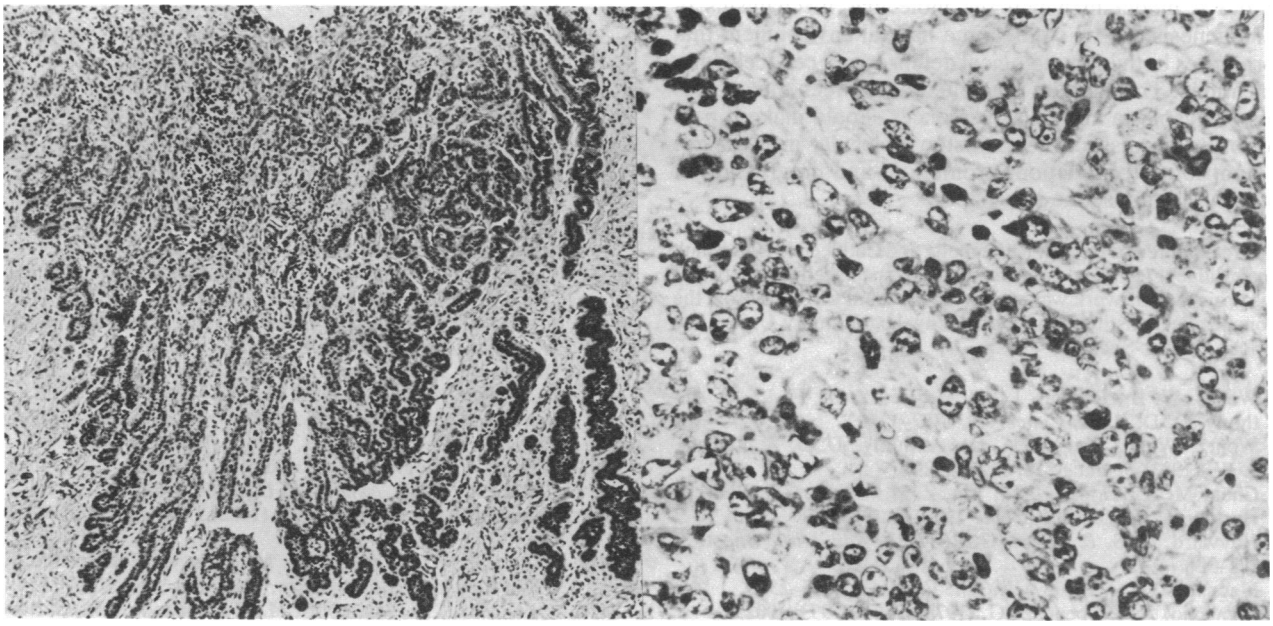


FIG. 4. Microinvasive squamous carcinoma. Tongues of neoplastic squamous epithelium extend from the surface into the superficial underlying stroma. (×175.)

the χ^2 test ($P < 0.0001$). Differences in frequencies of dysplasia alone or cancers alone were also statistically significant ($P < 0.001$).

$CaCl_2$ was added to each treatment solution to facilitate transfection. In one of the control groups (experimental group 2), DMSO was added to determine its effects on cervical cells in the event that transfection did not occur with HSV-2 DNA in the presence of $CaCl_2$ alone. However, the occurrence of dysplasia in several animals after 8–10 weeks of treatment with $CaCl_2$ plus HSV-2 DNA (Fig. 2), but not in animals treated with the solution containing Hepes buffer and $CaCl_2$, made it unnecessary to proceed with experimental solutions containing buffer, $CaCl_2$, DMSO, and HSV-2 DNA. The differences in results among each of the four control groups were not statistically significant. In contrast, the differences between each control group and the treatment group containing either HSV-2 DNA or HSV-2 DNA plus calf thymus DNA were statistically significant by the χ^2 test ($P < 0.001$).

The HEp-2 DNA used in these experiments was prepared by methods identical to those used to prepare the HSV-2 DNA. Moreover, HSV-2 DNA was propagated in HEp-2 cells. It is, therefore, unlikely that the neoplastic lesions were produced in the mouse cervix by some factor contaminating HSV but not HEp-2 cell DNA. Experiments utilizing restriction enzyme cleaved fragments of HSV-1 DNA provide further evidence against this possibility. When HSV-1 DNA was cleaved by enzymes that cut within the morphological transforming region 1 (*Bgl* II, fragment I) or morphological transforming region 2 (*Bgl* II, fragment N), the neoplastic



A B

FIG. 5. Invasive squamous carcinoma. (*A*) Nests of malignant squamous epithelium are present deep within the stroma. (×56.) (*B*) Non-keratinizing large cell carcinoma characterized by nuclear pleomorphism and nuclear hyperchromasia. (×225.)

lesions produced were reduced in number (D.D.A., W.B.W., and A.D.H., unpublished data).

The cytological data presented in this paper (Fig. 2) support the results obtained (1, 2) using whole inactivated virus. The protracted interval between the occurrence of dysplasia and invasive cancer is analogous to the long interval that separates the onset of dysplasia and cervical cancer in humans. The multiple molecular events that must be necessary for promotion and progression of carcinogenesis remain speculative. What seems clear, however, is that multiple factors may be required for the induction of cancer in the mouse model and that the DNA of the virus constitutes or provides at least one of these factors. In view of the accumulated data implicating human papilloma viruses in cervical carcinogenesis (28–31), it will be important to determine if HSV and human papilloma virus may be cofactors in this process.

A number of different techniques have been used in an effort to introduce genes into animals *in vivo*. The direct microinjection of DNA into fertilized oocytes has been used to produce transgenic animals (for review, see ref. 32). The injection of DNA directly into tissue (33–37) or intraperitoneally (38) and the intravenous injection of DNA contained within liposomes (39) have also been reported to result in *in vivo* transfection. These methods have generally involved the use of invasive procedures and proteolytic enzymes. Application of DNA in solutions containing calcium or calcium plus DMSO (26, 27, 40) and the technique of electroporation (41, 42) have been used to facilitate transport of DNA across cell membrane barriers in tissue culture. To our knowledge, direct transfection across intact mucous membrane barriers has not been reported. The high frequency with which this appears to occur in the mouse model is somewhat unexpected. It should be pointed out, however, that in these experiments, DNA was applied to the cervix three times weekly over a period of 80–100 weeks. Furthermore, the application of pledgets with each treatment may have been sufficiently traumatic to render the epithelial cell barrier more easily penetrable. Whatever the reason, the ability to induce cervical carcinogenesis *in vivo* with purified viral DNA provides a model in which the molecular mechanisms of carcinogenesis can be studied in a natural environment that incorporates both immunological defenses and cell–cell communications. This and the time interval between the appearances of dysplasia and invasive cancer should make it possible to further dissect the multistep molecular mechanisms that contribute to initiation, promotion, and progression of cancer in the mouse model system.

We are indebted to Lori A. Rusterholtz and Cathleen M. Olenchuk for their diligent technical contributions and special efforts on behalf of this project. This work was supported by Grant Ca-31973 from the National Cancer Institute and by the Cuyahoga County Unit of the American Cancer Society.

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