Roles of Cytomegalovirus and *Chlamydia trachomatis* in the Induction of Cervical Neoplasia in the Mouse

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ABSTRACT

Cytomegalovirus and *Chlamydia trachomatis* are prevalent sexually transmissible pathogens. They produce persistent infections of the cervix and have been associated with cervical neoplasia. Cytomegalovirus has also been shown to induce transformation of cells in culture. Because of the high prevalence of genital infections with these pathogens and evidence that they may have oncogenic effects on the cervix, cytomegalovirus (strain AD-169) and *C. trachomatis* (serovar LGV-2) were tested for oncogenicity in a mouse model in which induction of cervical neoplasia by repeated exposure to inactivated herpes simplex viruses has been demonstrated previously. Cotton tampons, saturated with UV-inactivated cytomegalovirus, *C. trachomatis*, or corresponding control fluids, were inserted into the vaginas of virgin C57 mice 3 times a week. Smears of vaginal aspirates for cytological examination were obtained every 5 weeks. After 75-90 weeks of exposure, the mice were sacrificed and serial sections of their reproductive tracts were examined. Cervical dysplasia was detected by histological examination in 51% and cervical carcinoma in 10% of mice exposed to cytomegalovirus. In control mice, in contrast, dysplasia developed in 3% and carcinoma in none. The progression from normal cervical epithelium to dysplasia to carcinoma observed with cytomegalovirus exposure was similar to that observed previously in this model after exposure of mice to herpes simplex virus types 1 and 2. The frequencies of cervical abnormalities in mice exposed to *C. trachomatis* or corresponding control fluid were low, and differences between the two groups were not statistically significant. These data indicate that strain AD-169 of cytomegalovirus is oncogenic for the mouse cervix and suggest that the LGV-2 serovar of *C. trachomatis* is not.

INTRODUCTION

The observation that cervical carcinoma has the epidemiological characteristics of a venereal disease (1) suggested that genital infections may have a role in the etiology of this neoplasm. On the basis of seroepidemiological evidence, HSV-2 (2) was among the first of the sexually transmissible pathogens to be implicated in the pathogenesis of cervical cancer (2, 3). However, not all seroepidemiological studies support this association (4), and, in those that do, up to 50% of women with cervical carcinoma had no antibodies against HSV-2 (2, 4, 5). This suggests that other sexually transmissible organisms may be oncogenic for the cervix. Among those that have also been implicated are the papillomaviruses (6-8), cytomegalovirus (9, 10), and *Chlamydia trachomatis* (11-13).

Cytomegalovirus is a herpesvirus that, like HSV-2, frequently infects the cervix (14) and produces persistent infections of the genitourinary tract (15). Cytomegalovirus has been isolated from cell cultures derived from biopsies of cervical carcinomas (16) and has been shown to induce transformation of cells in vitro (17, 18). In some seroepidemiological studies, a higher frequency of circulating antibodies against cytomegalovirus has been found in women with cervical carcinoma than in women with other cancers or without malignant disease (9, 10). These observations suggest that cytomegalovirus should be investigated as a possible cause of cervical cancer.

The most prevalent sexually transmitted pathogen in western societies is *C. trachomatis* (19, 20). This organism has been isolated from the cervices of 12-31% of women attending venereal disease clinics (21) and from 3-18% of pregnant women (22). Chlamydial infections are often persistent and apparently can become latent (23). An etiological relationship between chlamydial infection of the cervix and cervical neoplasia has been suggested by reports of higher frequencies of circulating chlamydial antibodies in women with cervical neoplasia than in controls (11-13). Additional support for this hypothesis is provided by reports of a higher frequency of cervical neoplasia in women with chlamydial antibodies than in women without these antibodies (24) and a higher rate of isolation of *C. trachomatis* from the cervices of women with cervical neoplasia than from controls (25).

A mouse model in which cervical carcinoma can be induced *in vivo* by repeated exposure of the cervix to inactivated HSV-1 or HSV-2 has been described previously (26, 27). Inactivated, noninfectious HSV was used to avoid the inflammatory response associated with HSV infections that might interfere with detection of neoplasia and because of the high mortality rate of HSV infection in mice. Repeated exposure resulted in prolonged contact of cervical epithelial cells with the constituents of HSV-1 or HSV-2. This mouse model was used in the present study to test two other sexually transmissible pathogens, cytomegalovirus and *C. trachomatis*, for oncogenic effects on the mouse cervix *in vivo*.

Strain AD-169 of cytomegalovirus was selected for testing because it has been widely used in laboratory investigations and has been used in an attenuated form as a vaccine for human use (28). Although serovars D through K of *C. trachomatis* are the ones most frequently isolated from the genital tract (20), serovar LGV-2 was used in this study because it grows to high titer in cell culture without treatment of cultures with mitotic inhibitors such as cycloheximide (29). Although the more frequently isolated genital serovars will grow in untreated cultures, there is evidence that the highest yields of these organisms are obtained when mitotic inhibitors are used (30-33). Because exposure of the mouse cervix to these potentially toxic chemicals could add a confounding factor to the experiment, LGV-2 was considered to be more suitable than other serovars for preparation of the relatively large quantities of organisms required by this experiment. Moreover, the implication of LGV serovars in the pathogenesis of cancers of the anorectum and external genitalia (34, 35) suggested that LGV-2 would be at least as likely to induce cervical neoplasia as other serovars of *C. trachomatis*.

MATERIALS AND METHODS

Cytomegalovirus. Cytomegalovirus, strain AD-169, was obtained from the American Type Culture Collection, where it had been tested by repeated exposure to inactivated HSV-1 or HSV-2 has been described previously (26, 27). Inactivated, noninfectious HSV was used to avoid the inflammatory response associated with HSV infections that might interfere with detection of neoplasia and because of the high mortality rate of HSV infection in mice. Repeated exposure resulted in prolonged contact of cervical epithelial cells with the constituents of HSV-1 or HSV-2. This mouse model was used in the present study to test two other sexually transmissible pathogens, cytomegalovirus and *C. trachomatis*, for oncogenic effects on the mouse cervix *in vivo*.

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and found to be free of contaminating agents, including Mycoplasma. The virus was propagated in monolayer cultures of HFF that were prepared in 150-sq cm plastic flasks using Eagle’s minimum essential medium containing 10% fetal calf serum for growth and maintenance. Flasks were inoculated with 2.0 ml of stock virus containing $10^5$ TCID$_{50}$ per ml and then agitated gently every 30 min for 2 h at 36°C. Fresh medium was added and the cultures were incubated at 36°C in 5% carbon dioxide. Uninoculated control HFF cultures were incubated in the same way. When 75% of the cells in inoculated cultures showed cytopathic effects induced by cytomegalovirus, cell suspensions from both infected and control cultures were harvested by scraping the cells into the culture fluids. An aliquot of the infected cell suspension was immediately sonicated and assayed for infectivity by inoculation of serial 10-fold dilutions into monolayer cultures of WI-38 cells. The infected and control cell suspensions were then centrifuged at 1000 × g for 10 min. To disrupt cells, the pellet was resuspended in 3 ml of supernatant fluid, frozen and thawed 3 times, and sonicated for 10 min (Fisher Model 300 Sonic Disembrator, output setting of 0.6, cup tip adapter on probe). The disrupted cell suspension was then centrifuged at 1000 × g for 10 min. The pellet of cell debris was discarded and the supernatant fluid was added to the previously saved culture fluids. Cytomegalovirus and control fluids were tested for bacterial sterility by inoculation into thioglycolate broth that was incubated at 37°C for 48 h. For inactivation of infectivity, 90 ml of cytomegalovirus suspension were dispensed into 22- × 34-cm glass dishes and exposed for 12 min at a distance of 20 cm to an UV light source consisting of two G15T8 germicidal lamps (General Electric Co.). Under these conditions, the intensity of UV energy was 100 ergs/s/sq mm. Control fluids were irradiated in the same way. Cytomegalovirus stocks contained approximately $10^9$ TCID$_{50}$ of virus per ml before inactivation. No infectious virus was detected after inactivation, as tested by inoculation of WI-38 cell cultures and observation for 21 days. Inactivated cytomegalovirus and control fluids were stored at −70°C.

C. trachomatis, C. trachomatis, serovar LGV-2 (strain 434), Mycoplasma-free, was used in this study. Stocks were prepared by inoculation of monolayer cultures of McCoy cells in 150-sq cm plastic flasks. Modifications of Eagle’s minimum essential medium (36), containing 10% fetal calf serum but no cycloheximide, were used for growth and maintenance medium. The inoculation, harvesting, and storage procedures for Chlamydiae and control fluids were the same as described for cytomegalovirus, except that cultures were incubated at 35°C for 72 h after inoculation and UV irradiation was done in the dark. Stocks of Chlamydiae were assayed for infectivity before and after inactivation by inoculation of coverslip cultures of McCoy cells (36) and counting the cells containing iodine-stained intracytoplasmic inclusions (37). Chlamydial stocks contained approximately $2 \times 10^5$ IFU/ml before inactivation. No inclusions were detected in cultures inoculated with UV-inactivated Chlamydiae.

Exposure of the Mouse Cervix to Cytomegalovirus, C. trachomatis, or Control Materials. Cotton tampons, saturated with approximately 0.1 ml of cytomegalovirus suspension that before inactivation contained $10^5$ TCID$_{50}$/0.1 ml, C. trachomatis that before inactivation contained $10^5$ IFU/0.1 ml, or equivalent volumes of corresponding control fluids, were inserted into the vaginas of virgin C57 mice three times a week for 90 weeks. Smears of vaginal aspirates, obtained at 5-week intervals, were examined for cytological abnormalities. Most of the animals were sacrificed at the end of the experiment and histological preparations of their entire reproductive tracts were examined without knowledge of the treatment received. Mice that appeared ill and likely to die at some unpredictable time were sacrificed earlier to prevent loss of tissues from autolysis. The details of these procedures (26, 27) and the criteria on which cytological and histological diagnoses were based (26, 38, 39) have been reported previously.

RESULTS

The frequencies of cytological abnormalities consistent with dysplasia or invasive carcinoma after increasing durations of exposure to inactivated cytomegalovirus or uninoculated HFF culture fluid are presented in Table 1. Exposure to cytomegalovirus and Chlamydiae in Cervical Neoplasia.
CYTOMEGALOVIRUS AND CHLAMYDIA IN CERVICAL NEOPLASIA

The animals exposed to cytomegalovirus. In control mice, in contrast, dysplasia occurred in 3% and carcinomas in none. Photomicrographs of representative histological preparations showing cervical dysplasia, microinvasive carcinoma, and invasive carcinoma in mice exposed to cytomegalovirus are presented in Figs. 1–3, respectively.

Cytological abnormalities consistent with dysplasia were detected in 3–5% of mice during the course of exposure to inactivated C. trachomatis. Differences between the frequencies of these cytological abnormalities in Chlamydia-exposed animals and in controls, however, were not statistically significant. Histological findings in Chlamydia-exposed and control mice at termination of the study are shown in Table 3. The frequencies of abnormalities were low in both control and Chlamydia-exposed groups and differences between the groups were not statistically significant.

Differences between the total numbers of mice available for cytological and histological examinations were related to loss of some animals from death and autolysis and to early sacrifice of other animals that appeared ill and likely to die. In all groups, most of the animals that died or were sacrificed prior to termination of the study had pelvic abscesses and diffuse peritonitis. None of these animals had disseminated cancer.

DISCUSSION

Previous studies in our laboratory have shown that repeated exposure of the mouse cervix to UV- or formaldehyde-inactivated HSV-1 or HSV-2 induces cervical dysplasia and invasive carcinoma (26,27). Pre-exposure immunization with inactivated HSV-2 was found to prevent the oncogenic effects of subsequent exposure of the cervix to this virus (40), a finding that further supports a causal role for HSV-2. The present study indicates that strain AD-169 of cytomegalovirus also is carcinogenic for the mouse cervix. This finding provides in vivo evidence for the oncogenicity of this virus that has been suggested previously by demonstration of transformation of cells in vitro (17, 18). Cytomegalovirus is the third human herpesvirus that has been shown to be oncogenic in this mouse model of cervical neoplasia. The same progression from normal cervical epithelium to dysplasia to invasive carcinoma occurred after repeated cervical exposure to cytomegalovirus, as was observed with HSV-1 and HSV-2 (26,27). This suggests that similar mechanisms may be involved in the induction of cancer by these viruses.

Neoplastic changes were detected by histological examination in only one animal after repeated exposure of the mouse cervix to the LGV-2 serovar of C. trachomatis. In comparison with controls, the frequency of this abnormality was not statistically significant. Therefore, the hypothesis that C. trachomatis is oncogenic for the cervix is not supported by the response of the mouse cervix to the LGV-2 serovar of this organism. Although LGV-2 is antigenically cross-reactive with all of the other serovars of C. trachomatis (41), these observations do not exclude the possibility that other chlamydial serovars may induce cervical neoplasia. It is also possible that Chlamydia-induced oncogenesis may require conditions that are not provided by this mouse model of viral carcinogenesis.

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Table 3

<table>
<thead>
<tr>
<th>Cervical exposure</th>
<th>Normal cervix</th>
<th>Dysplasia</th>
<th>Invasive carcinoma</th>
</tr>
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<tbody>
<tr>
<td>C. trachomatis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control fluid*</td>
<td>68</td>
<td>67 / 67</td>
<td>98 / 97</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>70 / 72</td>
<td>97 / 97</td>
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<tr>
<td></td>
<td></td>
<td>2%</td>
<td>3%</td>
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</table>

* Differences in frequencies between C. trachomatis-exposed and control mice are not statistically significant ($\chi^2$ test).

* Prepared from un inoculated McCoy cell cultures.
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REFERENCES

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