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Reactivation of Polyoma Virus in Kidneys of Persistently Infected Mice During Pregnancy

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Female mice infected at birth with 10^7 50% tissue culture infective doses of polyoma virus were mated when at least 6 weeks old. Polyoma was not detected in any tissues of 27 female mice before mating except for trace amounts in the kidneys of 2 mice, but late in gestation polyoma virus could be found in the kidneys of 21 of 38 mice with titers of $10^{3.7}$ to $10^{6.2}$ 50% tissue culture infective doses per gram of kidney. The virus was not detected in the brain, salivary gland, lung, liver, spleen, ovaries, placenta, or fetuses during gestation. Nonpregnant females were injected with female sex hormones over a period of 17 days, and polyoma was then detected in kidneys of 4 of 18 mice. Treatment of cultures of mouse embryo fibroblasts with either sex hormones or a glucocorticosteroid resulted in approximately a threefold increase in the rate of infection of cells with polyoma virus.

The papovavirus polyoma can be transmitted transplacentally to the fetus when the mother is infected at various stages throughout gestation (7). There is a new papovavirus of the stump-tailed macaque which is recoverable from the kidney of normal monkey fetuses (15). Evidently, this virus also is transmitted to the fetus, possibly via the placental route. Recently Coleman et al. (5) showed that the human papovavirus JC is found in the urine of normal women late in pregnancy, but the virus has not been detected in the fetus (S. D. Gardner, personal communication), although immunoglobulin M antibodies to BK, another human papovavirus, have been found in 9.1% of cord bloods (13).

Polyoma virus in mice, like simian virus 40 in the rhesus monkey, persists at low levels for long periods in organs such as the kidney (12). In this study we tested for the reactivation and transplacental transmission of virus in persistently infected females.

MATERIALS AND METHODS

Animals. Specific pathogen-free CD-1 mice bred in the Guy's Hospital Medical School Animal House were used. Mice were mated in the evening, and observed for vaginal plugs the following morning, which was considered day 1 of pregnancy.

Cells. Primary mouse embryo fibroblasts (MEF) were prepared from 16-day-old fetuses. The fetuses were minced, trypsinized, and seeded in 20-ounce (ca. 0.59-liter) bottles in minimal essential medium (Wellcome Reagents Ltd., London, England), containing 2.5% bicarbonate, 200 U of penicillin per ml, 100 mg of streptomycin per ml, and 5% fetal calf serum.

Virus and inoculations. The polyoma virus used and the preparation of a stock of virus ($10^{9.5}$ 50% tissue culture infective dose [$TCID_{50}$] per ml) in MEF has been previously described (7). One-day-old mice were inoculated intraperitoneally with 10^7 $TCID_{50}$ of polyoma virus.

Virus assays. Various tissues were taken from pregnant mice, sometimes after perfusion through the left ventricle with 20 ml of normal saline. Suspensions were made, and virus was assayed using a cytopathic endpoint in MEF in microtiter plates as previously described (7).

Explant cultures. Kidneys were harvested from mice 7 to 20 weeks after neonatal infection. One kidney was frozen at −70°C for virus assay, whereas the other was minced into approximately 1-mm cubes. At least 36 pieces from each kidney were placed on cover slips and incubated in a drop of minimal essential medium growth medium supplemented with L-glutamine (5.8 mg/ml) and 10% fetal calf serum. After attachment of the tissue fragment, 1 ml of medium was added to the cover slip. By day 7, cells grew out from the explanted tissue, and when cytopathic effect was observed in these cells the cover slips were dried, fixed in acetone for 10 min, and stained for viral antigens by the fluorescence technique (7).

Hormone treatment. (i) In vivo. Female mice infected as neonates were inoculated daily subcutaneously for 17 days with 0.005 μg of estradiol benzoate and 2 mg of progesterone in an oil emulsion of 0.1-ml volume. Control mice were inoculated with phosphate-buffered saline (PBS).

(ii) In vitro. Primary MEF were prepared as previously described (7). Secondary MEF were seeded in ring cultures (3) at 2×10^5 cells per ml and incubated at 37°C in 5% CO_2. When confluent the cells were infected with polyoma (≈0.05 plaque-forming units per cell), and adsorbed for 1 h in 0.1 ml of serum-free medium containing either the two sex hormones, es-

tradiol benzoate (50 pg/ml) and progesterone (40 ng/ml), or the glucocorticosteroid, corticosterone (150 ng/ml). Cells were then incubated for 36 h in 1 ml of maintenance medium (minimal essential medium, supplemented with 2% fetal calf serum) containing the hormones at the above concentrations, after which time the monolayer was washed with PBS, dried, fixed in acetone for 10 min, and stained by direct fluorescence for presence of viral capsid antigens (7).

Secondary MEF were also seeded into multiwell dishes (Falcon Plastics, Oxnard, Calif.) at a concentration of 2×10^5 cells per ml and incubated overnight, after which monolayers were confluent. Dilutions of polyoma virus were made in serum-free medium containing hormones (as above), and 0.1 ml of each dilution was placed in three wells of the multiwell dishes and adsorbed for 1 h. After adsorption, 1 ml of maintenance medium (2% fetal calf serum) plus hormones was added to each well and the dishes were incubated at 37°C for 14 days with a change of medium at day 7. Cytopathic affect was recorded at days 7 and 14, and the titers of virus were calculated as before (7) and expressed as the number of $TCID_{50}$ per milliliter.

Hemagglutination inhibition test. Sera were assayed for anti-polyoma virus antibodies by the hemagglutination inhibition test previously described (7).

Lymphocyte transformation test. (i). Cells and media. Spleens were removed aseptically, and single-cell suspensions were prepared by teasing the spleen apart with a scalpel into RPMI 1640 (Grand Island Biological Co. Bio-Cult, Glasgow, Scotland) supplemented with glutamine (2 mM/ml), penicillin, and streptomycin (as for cell growth medium), *N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid (20 mM, Wellcome Reagents Ltd.), and 10% heat-inactivated fetal calf serum (Bio-cult). Cell clumps and debris were allowed to settle before decanting the cell suspension, which was centrifuged at $180 \times g$ for 10 min and respended in bicarbonate-buffered RPMI 1640 containing 10% fetal calf serum before dispensing.

(ii) Cell culture. The test was based on that of Rosenstreich and Glode (11). A 0.1-ml volume of a spleen cell suspension (3×10^6 cells per ml) and 0.1 ml of concanavalin A (Miles) at 5 μg/ml were added to microtray wells ('U' bottom, Cooke Engineering Co., Alexandria, Va.). All cultures were set up in triplicate and incubated at 37°C in a humidified atmosphere with 5% CO_2 in air. After 48 h of incubation, 20 μg of [^{3}H]thymidine (25 μCi/ml: low-specific-activity thymidine, 2.0 Ci/mmol; Radiochemical Centre, Amersham, England) was added and incubated for a further 24 h, after which the cultures were harvested on filter paper using a multiple harvesting apparatus (Mach II) and washed four times with PBS (pH 7.2). The filter after drying was added to vials containing toluene/butyl PBD [2-(4'-*t*-butylphenyl)-5-(4'-biphenyl)-1,3,4-oxadiazole] scintillation fluid and counted in a Beckman scintillation counter.

RESULTS

Effect of first pregnancy on polyoma virus titers in persistently infected mice. Mice were infected as neonates, and various organs were tested for virus when they were 6 weeks or older. In 2 out of 15 six-week-old mice, there were trace amounts of virus in their kidneys (10^2 $TCID_{50}$/g of tissue). Polyoma virus was not detected in brain, salivary gland, lung, liver, or spleen of 38 male and female mice or the ovaries of female mice tested at 6 weeks to 12 months.

Thirty-eight neonatally infected female mice were mated and tissue tested on day 17 of gestation (Table 1). Virus was present in the kidneys of 100% and 90%, respectively, of mice mated 6 and 7 weeks after neonatal infection, and titers were as high as $10^{6.2}$ $TCID_{50}$/g. Virus was also present in the urine of all of these mice (Table 1), but not in any of the 17 placentas or fetuses even when there was $10^{6.2}$ $TCID_{50}$/g of kidney. Apart from trace amounts of polyoma in the kidneys of 2 six-week-old nonpregnant control mice, virus was not detected in the kidneys of another 16 six- or seven-week-old control mice. No virus was detected in the kidneys of five mice when mated at 6 weeks old and assayed at 9 days of gestation. Of the mice mated 10 to 12 weeks after neonatal infection, one-third had virus in the kidney, but none was detectable in those mated at 23 weeks.

After inoculation with polyoma virus on the first day of life, mice produced high titers of hemagglutination inhibition antibody. Maximum titers of 10,000 to 20,000 were reached by 3 weeks, after which there was a fall to 320 to 1,280, remaining at this level for the rest of the life of the mouse. Thirteen sera were tested

TABLE 1. *Virus reactivation in kidneys during pregnancy in female mice infected as neonates*[a]

Time between infection and mating (weeks)	No. reactivating/ no. of mice	% Reactivating	Range of kidney titers ($TCID_{50}$/g of wt)	Range of urine titers ($TCID_{50}$/ml)	Hemagglutination inhibition antibody in mice (geometric mean)
6	7/7	100	$10^{3.7}$–$10^{6.2}$	$10^{3.25}$–$10^{4.1}$	740
7	9/10	90	$10^{3.5}$–>$10^{4.5}$	ND[b]	1,280
10–12	5/15	33	$10^{4.5}$; $10^{3.7}$; 10^5 10^3–$10^{3.5}$	ND	1,280
23	0/6	0		ND	640

[a] Tests made on day 17 of pregnancy.
[b] ND, Not done.

separately on day 17 of pregnancy, but there was no evidence for a significant change in antibody levels during pregnancy (Table 1). Antibodies were present in all mice during pregnancy, and the antibody in the blood present in the kidneys as taken might have been expected to partially neutralize virus during the preparation of organ suspensions. However, perfusion with PBS to blanche the kidneys before removal did not increase the titers of virus recovered.

Titers of polyoma during third and fourth pregnancies. Neonatally infected mice were mated when 9 weeks old, and 16 were allowed to have three to four consecutive pregnancies over a period of 15 to 20 weeks. Their kidneys were removed for assay on day 17 of their last pregnancy, but no virus was detected (i.e., $<10^2$ $TCID_{50}$/g of tissue). At this stage the mice were 30 to 31 weeks old.

Presence of virus in explanted tissues. Groups of four unmated female mice were killed 7, 8, 11, 13, and 20 weeks after neonatal infection. One kidney was removed from each mouse and tested for polyoma after explantation (see Materials and Methods). In all kidneys, virus was detected by immunofluorescence (Fig. 1) in the outgrowing cells 2 to 5 weeks after explanting. Medium from these explants was harvested when cytopathic effect appeared, and when placed on susceptible MEF it produced cytopathic effect 7 to 10 days later, with polyoma-specific nuclear antigen detected by immunofluorescence. The other kidneys from these 20 mice were homogenized and directly assayed on MEF, but none of them contained detectable virus (i.e., $<10^2$ $TCID_{50}$/g of kidney).

Effect of hormones on polyoma virus replication. (i) In vivo. Seven-week-old mice infected when newborn were injected subcutaneously over a period of 17 days with daily doses of 0.005 μg of estradiol benzoate and 2 mg of progesterone. On the last day, kidneys were harvested and virus was assayed. Four of 18 mice tested (22%) had detectable virus whereas virus was not detected in the kidneys of 11 control mice who were inoculated with PBS (Table 2).

(ii) In vitro. The effect of estradiol benzoate plus progesterone or of corticosterone on the replication of polyoma was investigated (see Material and Methods). The concentrations of hormones used were equivalent to serum levels found in mice during pregnancy (1, 8). Polyoma replication was determined either by counting cells containing polyoma antigen at 36 h or by

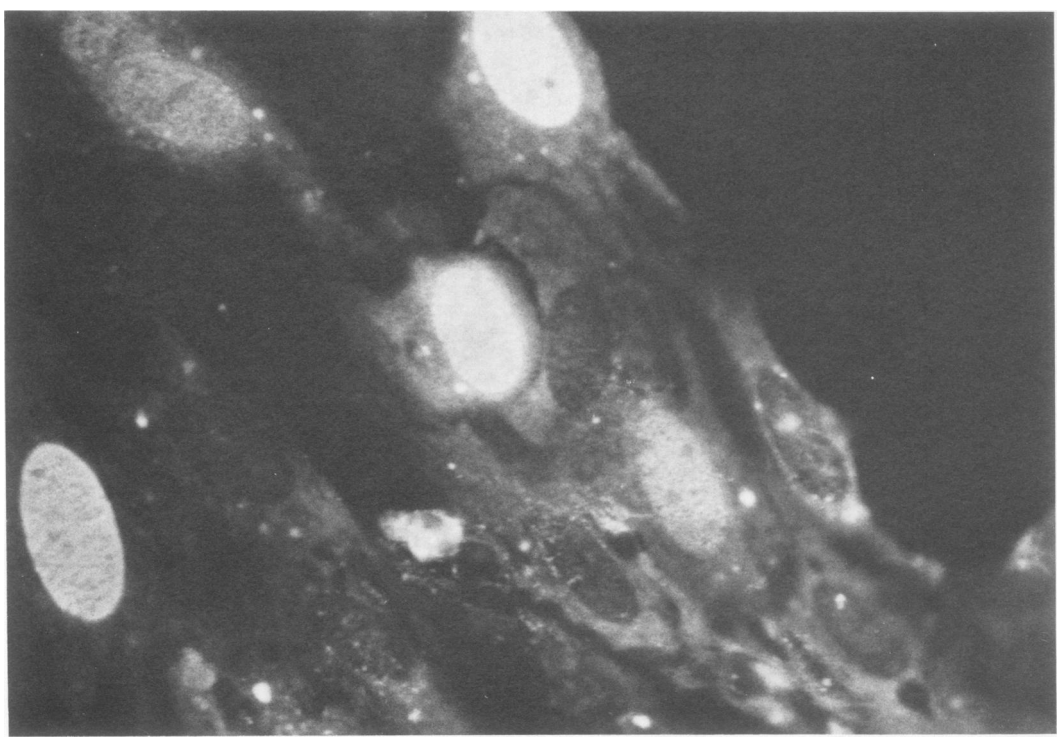

FIG. 1. *Nuclear fluorescence of polyoma antigens in cells growing out from a kidney explant. Magnification, ×420.*

observing for cytopathic effect in an endpoint assay.

The number of cells infected at 36 h (after one cycle of viral growth) was 3.1-fold higher when cells were incubated with sex hormones and 3.3-fold higher with corticosterone (Table 3). In the endpoint assay the treated cells at 7 days still showed a higher titer than the untreated cells, but by day 14 there was no difference (Table 3).

Lymphocyte blastogenesis in persistently infected pregnant and nonpregnant mice. Pregnant and nonpregnant littermates infected at birth were tested at 7 weeks for lymphocyte blastogenesis in response to an optimum stimulating concentration of concanavalin A. Spleen lymphocytes from eight pregnant mice gave a mean of 94,742 ± 35,139 cpm; spleen lymphocytes from seven nonpregnant littermates gave a mean of 42,166 ± 20,773 cpm. Although the difference was not significant, all but one of the pregnant mice had higher counts, perhaps in response to the increase in polyoma virus replication observed in 7-week-old pregnant mice.

DISCUSSION

We have shown previously that polyoma can cross the placenta when mice are infected during gestation (7). In this study we investigated mice infected as neonates and then mated 6 or more weeks later. The isolation of polyoma virus from kidney explants, but usually not by direct testing of kidney suspensions, suggests that the virus can persist in this organ throughout life at low or undetectable levels. This study of persistently infected mice has shown that, during pregnancy, polyoma virus reactivates or increases in titer form from undetectable levels to 10^3 to $10^{6.2}$ $TCID_{50}$/g of tissue during late gestation (day 17) but not during early gestation (day 9). Despite this, virus was not detected in the placentas or fetuses nor in any other organs assayed in the pregnant mouse. The virus present in the kidney of pregnant mice would probably be neutralized by antibodies once it entered the blood, and spread to the placenta and fetuses would be prevented. Similarly, in pregnant guinea pigs persistently infected with cytomegalovirus, virus spread to fetuses is prevented by circulating antibodies and infection of the fetuses is only achieved during acute primary maternal infection (4).

When pregnancy was deferred until 10 to 12 weeks, virus was less likely to appear in the kidney and it was not detected in those becoming pregnant at 23 weeks of age. It is unusual for mice under natural circumstances to have the first pregnancy at the age of 23 weeks, and we therefore tested mice of approximately the same age but who had been pregnant three or four times during this period. Again polyoma virus was not detected in their kidneys. It is concluded that pregnancy reactivation of polyoma virus takes place in young (less than 3-month-old) mice. This could be because the mice are older or because it is a longer period after initial infection.

When kidneys were explanted, virus was detected by immunofluorescence in outgrowing cells from neonatally infected but nonpregnant mice up to 20 weeks of age. It is possible that virus in their kidneys is in an uninfectious form which changes to productive infection when pieces of kidney are explanted in vitro, or, alternatively, infectious virus is present but in amounts too small to be detectable. It has been shown by infectious center assay with trypsinized kidney cells from neonatally infected 4-week-old mice that less than 1 in 10^5 cells contain virus which can initiate infection under those circumstances (D. J. McCance, unpublished data).

The constraints which operate in vivo may be immunological. Since the antibody levels remain

TABLE 2. *Titers of virus in kidneys of persistently infected female mice after daily treatment for 17 days with estradiol benzoate and progesterone*[a]

Mice	Total no. of mice	No. of mice with polyoma virus detected in kidneys	Titers in kidneys ($TCID_{50}$/g of tissue)
Test	18	4	10^4; $>10^4$; 10^5; 10^3
Control	11	0	

[a] Controls were inoculated with PBS.

TABLE 3. *Effect of hormones on the replication of polyoma in MEF*

Hormone added	No. of infected cells in:				Ratio of treated to untreated	Endpoint assay	
	Expt 1	Expt 2	Expt 3	Expt 4		7 days	14 days
Virus alone	43	121	126	130	1	$10^{7.5}$	$10^{10.5}$
Virus + estradiol and progesterone	162	500	221	375	3.1	$10^{8.5}$	$10^{10.25}$
Virus + corticosterone	208	255	371	430	3.3	$10^{8.3}$	$10^{10.5}$

high throughout life and do not change during pregnancy (Table 1), it was thought that the cell-mediated arm of the immune response could be important. Results here show there was no significant difference in T-cell responses to concanavalin A measured by the lymphoblastogenesis assay between pregnant and nonpregnant mice. However the T-cell response still may be important in influencing virus replication since immunosuppression by hydrocortisone acetate and antilymphocyte serum (a treatment that reduces T cells, leaving some antibody responses intact) has been shown to lead to reactivation of polyoma virus in persistently infected mice (D. J. McCance, unpublished data). Alternatively, the constraints may depend on subtle differences between kidney cells in vivo and in vitro. Poliovirus, for instance, fails to replicate in kidneys of infected monkeys, even after direct injection into kidneys, yet kidney cells in vitro provide a standard source of viruses and an assay system.

Another factor causing activation of polyoma during pregnancy could be hormonal. Pregnancy hormones might affect immune responses, but studies on pregnant women have given contradictory results concerning in vitro T-cell responses to various mitogens (2, 9, 16). In this study, the mitotic response of spleen cells to concanavalin A did not change significantly during pregnancy. Alternatively, changing hormone levels during pregnancy might enhance viral replication. Steroids are known to increase the synthesis of mouse mammary tumor virus ribonucleic acid in cell cultures (10, 14). We treated 7-week-old nonpregnant mice with estradiol benzoate and progesterone for 17 days, to give levels equivalent to those found late in pregnancy. Infectious virus was then detectable in kidneys in 22% of the mice, but not in those treated with PBS. In vitro studies showed that both estradiol benzoate plus progesterone and corticosterone alone enhanced the number of cells producing virus capsid antigens at 36 h. A glucocorticosteroid was used because, in mice, these hormones increase dramatically (10-fold) in the latter stages of pregnancy (1). The initial growth cycles were accelerated, and at 7 days the endpoint titer was 10 times greater, but there was no significant change in the final extent of replication at 14 days. It has been shown that mice treated with estrogen 24 h before infection with MM (a member of the encephalomyocarditis group of viruses) showed accelerated viral replication. They developed viremia 48 h before untreated infected mice, but the final titers of virus in organs were similar (6).

Work is in progress to further investigate hormonal effects on polyoma virus replication, especially the effect on cellular deoxyribonucleic acid synthesis. We are also studying the steroids used to immunosuppress transplant patients for effects on immunoresponsiveness and on polyoma virus replication.

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