INFECTION AND IMMUNITY, Oct. 1977, p. 196-202

Vol. 18, No. 1
Printed in U.S.A.

Transplacental Transmission of Polyoma Virus in Mice

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Received for publication 17 May 1977

When pregnant mice were inoculated on day 1 of gestation with polyoma, some of them exhibited total resorption or reduced litter size, the extent depending on the dose of virus. Virus was detected in 4 out of 11 mouse embryo fibroblast (MEF) cultures made from infected mothers. After maternal infection on day 5 or 10 of gestation, virus titers of up to 10^7 50% tissue culture infectious doses ($TCID_{50}$)/g of fetus were found in all pools of fetuses tested 5 days later, with the titers falling by day 6. Hemagglutination-inhibiting antibodies against polyoma appeared in maternal serum by day 6 and rose to a maximum by day 14. Immunoglobulin G class antibodies were detected by day 7, with titers rising rapidly to a maximum at day 14. After maternal infection later in gestation (day 15), one out of three litters of newborn mice was found to have 10^5 $TCID_{50}$ polyoma virus per g in pooled kidney samples.

Intrauterine infections by various viruses in humans and animals have been well documented (3, 7). The effect of these infections on the fetus varies depending on such parameters as the virus and the stage of gestation at which infection occurs (8).

The papovaviruses have been implicated in intrauterine infections, and, in a recent study (17), a new papovavirus of stumptailed monkeys was found in fetal and neonatal kidney cultures after they had been maintained for a minimum of 6 weeks in vitro and after at least one tissue culture passage. Polyoma, a papovavirus whose natural host is mice, is found in many different tissues after infection of both adults and neonates (15; D. J. McCance, unpublished data), but the evidence concerning transplacental transmission is not convincing (C. P. Li and W. G. Jahnes, Fed. Proc. **19**:101, 1960). In one study, female mice were infected with polyoma just before or after mating, embryo cell cultures were made at a later stage, and virus was detected in seven out of ten experiments (Li and Jahnes, Fed. Proc. **19**:101, 1960). In this paper, we describe intrauterine infections with polyoma in its natural host, mice, and discuss possible implications for man with respect to recently discovered human papovaviruses.

MATERIALS AND METHODS

Animals. Specific pathogen free CD-1 mice bred in our animal house were used. Mice were mated in the evenings, and observations for vaginal plugs were carried out 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 (MEM; Wellcome Reagents Limited, Beckenham, 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. Polyoma virus, large-plaque strain, was a gift of L. Mallucci from this department. Mice were inoculated intraperitoneally (i.p.) when they were less than 24 h old, and the kidneys were harvested 10 days later, homogenized, and used as the main stock virus (10^{10} 50% tissue culture infectious dose [$TCID_{50}$]/g of kidney). This stock virus (diluted 10^{-3}) was used to produce a pool in secondary MEF (10^{10} $TCID_{50}$/ml). Pregnant mice were inoculated i.p. with 5×10^8 $TCID_{50}$ of this MEF pool at various times after plugging.

Extraction of maternal and fetal tissues. Tissues were removed, weighed, placed in MEM supplemented with 15% fetal calf serum and stored at −70°C until assayed. Blood was taken in heparin-coated syringes by cardiac puncture.

During the first 8 days of pregnancy the entire conceptus was removed intact containing uterus, fetus, and placenta. Fetal tissues could be separated from the uterus and placenta from day 9 of pregnancy. The fetuses were removed (by a method demonstrated by M. Smith, School of Anatomy, University of New South Wales) with fine forceps and scissors under a dissecting microscope. They were washed three times in MEM (serum free), weighed, and then stored in MEM supplemented with 15% fetal calf serum at −70°C until assayed.

Virus assays. Secondary MEFs were seeded into multiwell dishes (Falcon) at a concentration of 2×10^5 cells per well and incubated overnight, after which monolayers were confluent.

Tissues were thawed, transferred to serum-free medium, and homogenized. Dilutions were made, and 0.1 ml of each dilution was placed in three wells of the

Falcon dishes and adsorbed for 1 h at 37°C. After adsorption, 1 ml of maintenance medium (MEM supplemented with 1.5% fetal calf serum) 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 effect (CPE) was recorded at day 14, and the titers were calculated by the method of Reed and Muench (12) and expressed as the number of $TCID_{50}$ per gram of tissue or per millimeter of whole blood.

Immunofluorescence. Hyperimmune anti-polyoma serum was raised in mice by inoculation of 10^9 $TCID_{50}$ of virus i.p. on day 0 and by a booster dose of 10^9 $TCID_{50}$ i.p. on day 14, and the serum was harvested by cardiac puncture 7 days later. The serum had a hemagglutination-inhibition (HAI) titer of 12,800.

Antibodies were precipitated with methanol in the cold, and the precipitate was dissolved to the original volume with normal saline. The antibodies were conjugated with fluorescein isothiocyanate (1:20 ratio of fluorescein isothiocyanate to serum protein) overnight with shaking at 4°C. The conjugate was adsorbed once with rabbit liver powder (100 mg/ml of conjugate) and used at a dilution of 1:5. Tissues were frozen in liquid nitrogen, sectioned in a cryostat, fixed in acetone for 10 min, and stained by the direct method. The indirect method was used to test for immunoglobulin G (IgG) antibodies by using goat anti-mouse IgG fluorescent antibodies (Nordic Immunological Laboratories, Maidenhead, England). Immunofluorescence observations were made with a Leitz Ortholux microscope equipped for epi-illumination with an HBO 200 lamp as light source.

HAI test. Sera were incubated overnight with RDE (Wellcome Reagents Limited, Beckenham, England), heated at 56°C for 1 h to inactivate the enzyme, and adsorbed with 50% (vol/vol) guinea pig erythrocytes at 4°C for 2 h. The tests were carried out by a modified method (14) in Cooke Microtitre 'U' plates with twofold dilutions of treated mouse sera, 4 hemagglutination units of polyoma, and 0.4% guinea pig erythrocytes. HAI antibody against polyoma was not detected in the mouse colony.

RESULTS

Fetal resorption. Mice were inoculated i.p. with 5×10^8 $TCID_{50}$ polyoma virus on day 1 of pregnancy. Generally there was either total resorption (no embryos) or no resorption when mice were examined on day 14 of pregnancy, but, in approximately 10% of mice, partial resorption occurred. When no fetuses were found, pregnancy was judged to have occurred by the thickening and vascularization of the uterine wall and the increase in the size of the ovaries with corpora lutea visible. Figure 1 shows the average number of fetuses in all mothers harvested on day 14 of pregnancy and includes mice showing complete, partial, or no resorption after infection on day 1 of pregnancy with various doses of polyoma virus. A total of 60% of mothers showed total resorption when 5×10^8 $TCID_{50}$ were given, and this was the dose used in nearly all subsequent experiments.

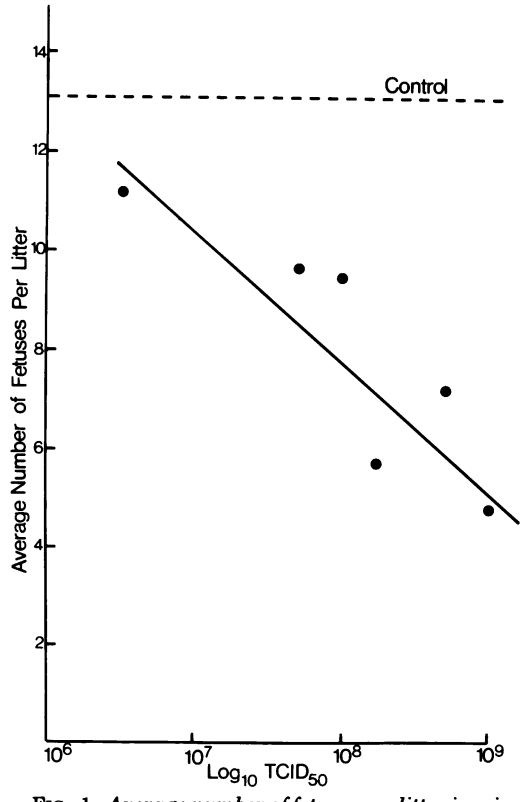

FIG. 1. *Average number of fetuses per litter in mice infected with various doses of polyoma on day 1 of pregnancy. All points are an average of at least 15 mice except those at doses $10^{6.5}$ and $10^{7.7}$ $TCID_{50}$ per mouse, which are averages of 12 and 5 mice, respectively.*

In another four experiments, mice were inoculated 5 and 10 days after plugging. On examination on day 18 of pregnancy, 13 or 8 days after infection, one or two resorbing white-colored fetuses were observed in approximately one-quarter of mice.

Titers of virus in maternal tissues. After infection on day 1 of pregnancy, titers in ovaries and conceptus rose to a peak at days 4 through 6 (Fig. 2). Virus in the blood could not account for virus levels in the tissues because blood titers fell sharply after day 2 and were undetectable by day 8 (Fig. 2). When nonpregnant mice were infected i.p. with the same dose of virus, titers in the ovaries between days 4 and 6 were similar to those in pregnant mice, but, subsequently, the titer fell more rapidly and was undetectable 12 days after infection. Mammary glands of mothers who were infected on day 1 of pregnancy were harvested at parturition, and virus titers of $10^{4.5}$ and $10^{3.2}$ $TCID_{50}$/g of tissue were found in two out of three animals tested.

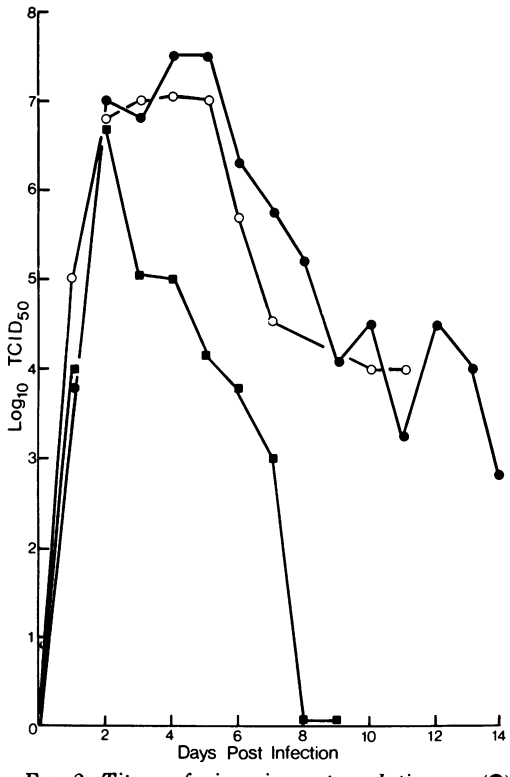

FIG. 2. *Titers of virus in maternal tissues:* (●) *ovaries;* (○) *conceptus; and* (■) *whole blood. Each point is the average of three experiments using two to three mice per experiment.*

Milk taken from the stomachs of infants soon after birth was negative for virus. No virus was found in any of the male reproductive organs.

The mouse placentas developed by day 10 of gestation, and, after infection on day 1 of pregnancy, the placenta titers at day 14 varied between $10^{3.4}$ and $10^{4.8}$ $TCID_{50}/g$. In mice inoculated with the same dose of virus at days 5 and 10 of pregnancy, placental titers were at a high level by day 5 after infection and then fell (Fig. 3).

Immunofluorescence in maternal and fetal tissues. Immunofluorescence observations were made on sections of the conceptus and ovaries of mice inoculated i.p. on day 1 and harvested on day 5 of pregnancy. In the ovaries, polyoma antigen was seen in the nuclei of cells in the connective tissue, corpus lutea, and follicles. The cells infected in the follicles were those of the discus proligerus and the theca, but no viral antigens were observed in 50 ova identified in sections (Fig. 4a). In the conceptus, virus antigens were seen in the nuclei of cells in submucosal layers of the uterus, in the serous coat, in connective tissue cells of the muscle, but not in the endometrium (Fig. 4b).

Viral antigens were also observed in the nuclei of cells from blood smear preparations. The cells exhibiting fluorescence were 12 to 15 μm in diameter with large nuclei and small cytoplasm and appeared to be lymphocytes.

Occasional mononuclear fluorescent cells were seen in the pleural cavities of embryos from infected mothers. The fluorescence was thought to be specific since no fluorescence was seen in embryos from uninfected mothers and was quenched when mouse anti-polyoma serum was used followed by conjugated mouse anti-polyoma serum.

Titers of virus in fetal tissues. After an i.p. inoculation on day 1 of pregnancy, fetuses were removed 14 days after infection, and primary MEF cultures were set up. Of 11 such cultures (20 embryos per culture), 4 showed CPE after 14 days in culture. Fluorescent-antibody staining showed that the cells were infected with

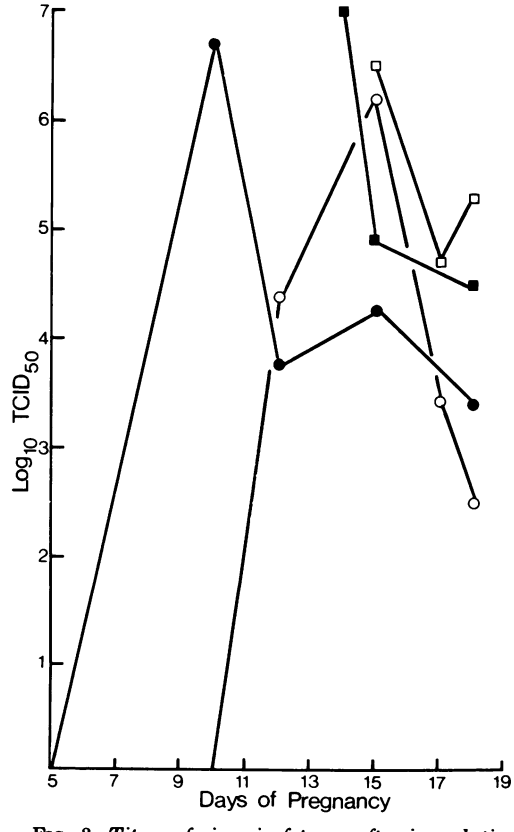

FIG. 3. *Titers of virus in fetuses after inoculation on day 5* (●) *and day 10* (○) *of pregnancy and in placentas after inoculation on day 5* (■) *and day 10* (□) *of pregnancy. Each point is the average of three experiments in which a pool of 25 to 30 fetuses were tested in each experiment.*

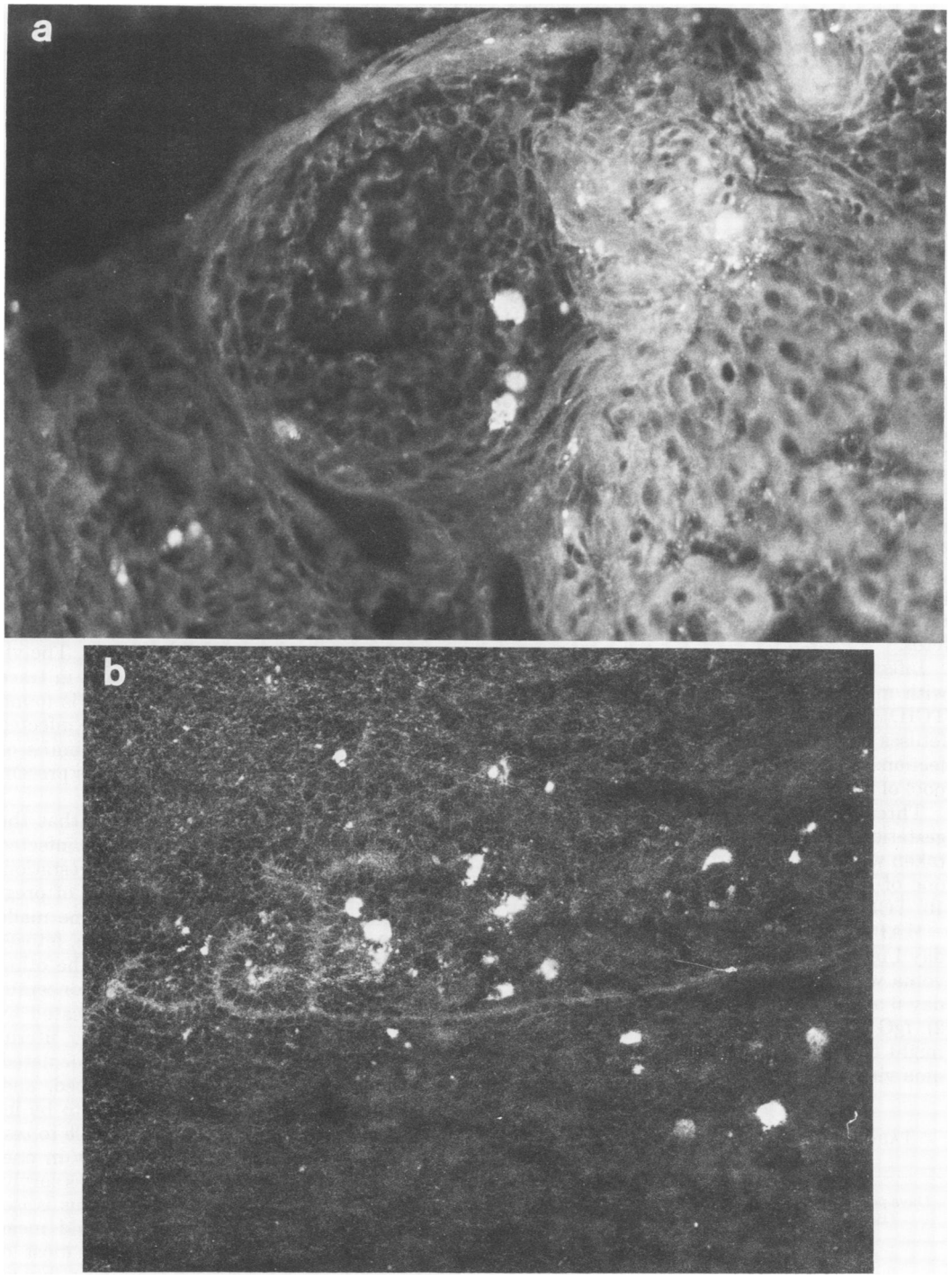

FIG. 4. *(a) Fluorescent cell nuclei are seen in the discus proligerus of a germinal follicle. ×400. (b) Fluorescent cells and cell debris are seen in the submucosal layers of the uterine wall. The endometrium is seen as a folded structure through the center of the photograph. ×200.*

polyoma. Fetuses were also removed between 9 and 14 days after infection and assayed (Table 1). Between 25 and 33% of pools of fetuses assayed contained detectable levels, i.e., $>10^2$ $TCID_{50}$ of virus per g of fetus.

Pregnant mice were also infected later in gestation with the same dose of virus, and Fig. 3 shows the titers of virus in the fetuses after infection on days 5 and 10 of gestation. Maximum titers were observed in maternal tissues. In contrast to the results when mothers were inoculated on day 1 of pregnancy (Table 1), all pools of fetuses assayed contained virus. The shape of the curves were consistent in each of three experiments, although the titers of virus varied between pools. In another set of experiments, fetuses were also assayed individually for the presence of virus 5 days after infection, and, of 30 tested from 3 mothers infected on day 10 of pregnancy, all were found positive. This indicates that the presence of virus in pools of fetuses is not due to an occasional event, such as vascular breakdown in one placenta, which would lead to infection of only one fetus in the litter. Virus was also present in the white-colored resorbing fetuses at $10^{3.2}$ to $10^{5.25}$ $TCID_{50}$/g of fetus.

Infection of mothers on day 10 of pregnancy with much smaller amounts of virus, i.e., $10^{4.5}$ $TCID_{50}$ per mouse, resulted in infection of the fetuses when they were assayed 5 days after infection. A titer of $10^{2.9}$ $TCID_{50}$/g was found in a pool of fetuses from three mice.

Three mothers were inoculated on day 15 of gestation, and the kidneys of the neonates were taken within 10 h of birth. Pooled kidneys from one of the three litters showed a virus titer of 10^5 $TCID_{50}$/g of kidney.

HAI antibody titers. After inoculation on day 1 of pregnancy, HAI antibodies against polyoma were first detected in maternal serum at day 6 and rose to peak levels by day 14 (Table 2). IgG class antibodies cross the placenta, and might therefore play a part in controlling polyoma virus replication in the fetus. IgG class antibodies were therefore measured by the indirect fluorescence techique (Table 2). They were detectable by day 7 and rose to a peak between days 14 and 16.

TABLE 1. *Titers of virus in fetuses[a] from mice infected on day 1 of pregnancy*

Days postinfection	No. of positive isolations/total no. assayed	Titer[b]
9	2/3	$10^{5.5}$
11	0/4	$<10^2$
13	1/3	$10^{3.5}$
14	1/4	10^3

[a] Pool of 20 to 25 fetuses from 2 pregnant mice for each assay.

[b] $TCID_{50}$ per gram of fetus.

TABLE 2. *HAI antibody titer and immunofluorescent IgG class antibody titer against polyoma in mice after inoculation on day 1 of pregnancy*

Days postinfection	HAI titer	Immunofluorescent IgG titer
6	200	ND[a]
7	64	20
8	200	ND
9	160	ND
10	640	320
12	1,600	ND
14	3,200	800
16	3,200	800

[a] ND, Not done.

DISCUSSION

Mice are the natural host of polyoma virus, and the adults or newborn mice undergo a generalized infection, with virus replicating in a number of different organs including the brain, lungs, liver, spleen, kidneys, and salivary glands (15; D. J. McCance, unpublished data). The virus persists in mice for 2 to 5 months in trace amounts after infection of adults, but is found in higher titers and for longer in mice infected as newborns (15). In mice tested 4 months or more after initial infection, the virus is present mostly in the kidneys (2, 15).

The results presented above show that the virus could be transmitted from the infected mother to the fetuses throughout gestation. When mice were inoculated on day 1 of pregnancy, tests for fetal virus could not be made until day 9 or later because, until then, fetuses were too small to be separated from the other tissues of the conceptus. Titers in the conceptus reached a peak at day 4 and, as indicated by immunofluorescence observations, the uterus was then infected. At the dose inoculated, approximately 60% of mothers resorbed, and, although pools of fetuses were found to be infected at day 9 when it was first possible to test them separately, virus was found only in one-quarter to one-third of the sets of fetuses surviving at days 9 to 14. The fact that virus is isolated from a minority of fetuses could mean either that fetuses are infected, but most of these are then resorbed, or that most fetuses remain infected, but antibody in the fetus is often sufficient to neutralize any virus present. Furthermore, the method of assay would fail to detect titers of less than 10^2 $TCID_{50}$/g of tissue. The first antibody produced in the infected

pregnant mouse is likely to be mainly IgM, which does not cross the placenta, but IgG antibody, which can cross the placenta, was detected by indirect immunofluorescence as early as day 7 and rose to high levels between days 9 and 14 at the times when sets of fetuses were assayed. Presumably any virus present at these times would have been neutralized by antibody after homogenization.

When pregnant mice were infected later in gestation, fetuses could be isolated and separately tested much sooner after infection. Virus was then isolated from all pools of fetuses at day 5 after infection when viral titers in maternal tissues were at a peak and when antibody levels were undetectable. After day 5, virus titers dropped and, at the same time, antibody levels increased. Although virus was isolated 5 days after infection from every fetus in a sample of three mothers inoculated at days 5 and 10 of gestation, no more than one or two embryos in a few litters were resorbing.

Some of the offspring whose mothers were infected late in gestation had virus in their kidneys when born, and it is possible that these infected mice might remain infected for long periods, as when neonates are infected with polyoma virus (15). It may be that in the natural state transplacental transmission is another way, other than through saliva and urine (15, 16), in which virus can be passed between generations and so maintain polyoma in a colony. Antibodies, however, would tend to prevent or reduce this means of transmission, since there are low titers of virus in adults in an infected colony (16). It is possible that reactivation may occur in pregnancy, resulting in transplacental transmission of infection. It may be noted that all the above assays were for infectious virus, and the immunofluorescence technique was used to identify infected cells as seen by the production of viral antigens. Tests for polyoma deoxyribonucleic acid sequences might give further information.

Other studies have suggested that papovaviruses cause intrauterine infections. In a very brief report (Li and Jahnes, Fed. Proc. **19**:101, 1960) polyoma hemagglutinin was found in the growth media of seven out of ten primary MEF cultures from mothers infected early in pregnancy or just before pregnancy, but few details were given. More recently, a new papovavirus of stumptailed monkeys (13) was found regularly in fetal and neonatal kidney cultures from these monkeys (17). Intrauterine infections due to a papovavirus may have an important consequence for humans since the discovery of a new range of human papovaviruses. The human papovaviruses BK and JC infect a large proportion of the population during childhood and adolescence (1, 4, 9, 11), and JC has been identified as the etiological agent in progressive multifocal leukoencephalopathy (20). BK virus has been isolated from immunologically incompetent or immunosuppressed patients. It is found in the urine of some renal transplant patients (5, 6) in concentrations of up to 10^{14} particles per ml (S. D. Gardner, personal communication) and has also been isolated from the brain of a boy with the Wiskott-Aldrich syndrome (19). The isolation of BK in the renal transplant patient is thought to be due to reactivation rather than to primary infection because 80% of adults have already been infected (4). This persistence of papovaviruses in the human host is similar to that of polyoma in the mouse and simian virus 40 in monkeys. Possible transplacental transmission by BK has recently been reported (18) when 6 women out of 80 tested had a primary BK virus infection during pregnancy as indicated by seroconversion. BK antibody was present in cord blood and appeared to be IgM because there was a significant decrease in titer after treatment with 2-mercaptoethanol. As with polyoma, reactivation of BK to cause an intrauterine infection in normal adults seems unlikely because most pregnant women have high antibody levels to BK, although there is evidence that females are immunosuppressed during pregnancy (10), and this may lead to a drop in antibody titer and reactivation of the virus. Further work is in progress to investigate the frequency with which litters are born infected following maternal infection with polyoma virus and to determine any long-term effects resulting from congenital infection. Studies are also being made of the mechanism of resorption and whether or not this is due to a direct effect of the virus on the egg or fetus.

ACKNOWLEDGMENT

We thank Valerie Finucane for her excellent technical assistance.

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