

Experimental In Utero Infection of Fetal Pigs with a Porcine Parvovirus

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In utero infection of fetuses of six specific-pathogen-free large white sows at 35, 48, 55, 72, 99, and 105 days was studied. The fetuses were infected by direct inoculation of porcine parvovirus into the amniotic sac. The inoculation consisted of 0.25 ml of tissue culture fluid containing $10^{4.5}$ mean tissue culture infective doses per ml of porcine parvovirus strain G10/1. Fetuses of one uterus horn were infected, whereas fetuses in the opposite horn were given 0.25 ml of noninfected cell culture material. No clinical signs of infection were observed; however, all sows developed antibodies 7 to 9 days postinfection. A total of 24 virus-inoculated fetuses and 20 control fetuses were studied. Fetuses infected at 35, 48, and 55 days of gestation died between about 5 and 22 days after infection. Virus was isolated from their organs and fetal blood. Virus spread to control fetuses but did not cause death and mummification or stimulate antibody production. Fetuses from sows infected at 72, 99, and 105 days of gestation survived. They developed high antibody titer in utero. Control piglets remained antibody free.

After initial isolation in Europe (1, 2, 4), porcine parvoviruses (PPV) have been reported to occur worldwide (6, 11, 19-21). All known strains were shown to be antigenically related or identical. The incidence of this virus infection in pigs is very high. The percentage of antibody-positive animals range from 33% (4) up to over 50% in herds (1, 2, 11, 19, 21, 24) kept under different husbandry conditions.

The pathogenic effects induced by PPV in pigs have not been clearly established. Cartwright and Huck (4) made several PPV isolations from stillborn, aborted, and mummified fetuses. They associated the infection with reproductive failures in pig herds. However, they made isolations from apparently healthy, normal piglets as well. More recent studies also indicate frequent isolations of PPV from aborted or mummified fetuses (20, 21, 24). Isolations were also made from pigs exhibiting clinical signs of respiratory tract infection (6, 19). Darbyshire and Roberts (6) reported an induced pneumonitis after infection with their isolate of PPV.

Since parvoviruses have a preference for rapidly growing cells to support their replication (16), embryonic and fast-developing tissues

offer optimal conditions for parvovirus growth. A logical consequence was to look for damage to the fetus or the newborn piglet after PPV infection. Cartwright et al. (5) and Johnson and Collings (12, 13) studied pregnant sows at various stages of gestation infected with PPV orally or intravenously. Transplacental crossing of PPV to the fetus was reported. Fetuses from sows infected at later stages of pregnancy developed antibody against the virus. Infection of the sows did not result in abortion, abnormal numbers of stillbirths, or small litters in any of the sows infected.

Since precise time of infection and concentration of virus reaching the fetus could not be determined from previous studies, experiments were planned whereby piglets were infected in utero at different times of development by direct inoculation of PPV into the amniotic fluid.

MATERIAL AND METHODS

Virus. PPV strain G10/1 (1, 18) in the 12th serial passage in swine kidney (SK) cell cultures (14) was used in this study. The inoculum had an SK cell infectivity titer of $10^{4.9}$ tissue culture infective doses/0.2 ml and a hemagglutination (HA) titer of 1:256, using guinea pig erythrocytes.

Virus isolation. Virus isolations were performed by inoculating 0.3 ml of 10% organ suspensions into the SK cell cultures 16 h after cell seeding. Four tubes each were inoculated with all specimens from dead or clinically abnormal fetuses studied. Inoculated cul-

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tures were observed for 7 days postinfection (dpi). After repeated freezing and thawing, an HA test was carried out with the culture suspension. Negative specimens were serially passaged six times under the same conditions. To assure specificity of the isolates, all HA-positive specimens were tested in the hemagglutination inhibition (HI) reaction with a monospecific rabbit anti-PPV hyperimmune serum.

From apparently normal fetuses, virus isolations were attempted from explant tissue cultures of lung, kidney, and testis (male piglets). Cultures were prepared by standard techniques. After cells grew to monolayers, at least two to three subcultures of each tissue were made. The supernatant as well as a portion of frozen and thawed cells were checked for HA activity. Cell culture medium used in all studies was Earle balanced salt solution with 0.5% lactalbumin hydrolysate and 10% calf serum.

HA and HI. HA and HI reaction techniques described previously (1, 18) were adapted to microtiter plates for this study. Guinea pig erythrocyte concentration was increased to 1%. Reaction temperature was 4 C. Readings were recorded after 4 h of incubation. The monospecific hyperimmune serum was prepared in rabbits by using density gradient-purified PPV G10/1, 10th passage. The HI antibody titer was 1:1,024, using 4 antigen (HA) units.

Animal experiments. Six bred large white specific-pathogen-free sows of varying ages kept under isolation were studied. Before infection with PPV, the sows had low antibody titers against porcine enterovirus types 1 through 6. They were free from antibodies to porcine enterovirus types 7 and 8, parvovirus, hog cholera virus, pseudorabies virus, and transmissible gastroenteritis virus. The antibody titers against the enteroviruses did not change during the experiment. In utero infection of their fetuses was done at 35, 48, 55, 72, 99, and 105 days of gestation. The sows were tranquilized with chlorpromazine (thorazine injectable, 25 mg/ml; Smith, Kline & French Laboratories, Philadelphia, Pa.) (0.44 mg/kg of body weight) administered by ear vein. Lumbar epidural analgesic was induced by injection of approximately 20 ml of 2% lidocaine at the lumbosacral space according to R. Getty (Proc. Annu. Meet. Am. Vet. Med. Assoc., 1966). A light plane of surgical anesthesia was induced by cone administration of halothane, 5% concentration, thereafter reduced to 1 to 2% concentration for maintenance. Inhalation anesthesia was selected for its relatively low toxicity to the fetus and rapid recovery rate. The primary demand for sensory anesthesia was borne by the lumbar epidural. This combination anesthesia provided the degree of relaxation and analgesia necessary for aseptic laparotomy and atraumatic manipulation of the gravid uterus with minimal risk to the fetuses and for the intra-amniotic injections of the virus. The gravid uterine horns were exteriorized through a 20- to 25-cm incision in the linea alba. After identification of the embryos and injection of the virus, the uterine horns were returned to the abdominal cavity and the abdomen was closed with four layers of suture (peritoneum, linea alba, superficial fascia, and skin). Procaine penicillin and dihydrostreptomycin were administered by intramuscular injection at 12-h intervals for 3 days thereafter as prophylactic medication.

Fetal pigs of one horn were infected by inoculation of 0.25 ml of fluids containing $10^{6.5}$ mean tissue culture infective doses of PPV G10/1 into the amniotic sac. Control fetal pigs in the opposite horn received 0.25 ml of cell culture fluids without virus. Blood samples were taken from the sows at 7-day intervals. The sows were sacrificed 28 days after infection of fetuses. The uterus was saved for virological examination. Sows SW 2343 (inoculated at 99 days of gestation) and SW 2344 (inoculated at 105 days gestation) were allowed to deliver the piglets normally. Fetal pigs were designated "L" from the left horn and "R" from the right horn. Fetuses in each horn were numbered separately, starting at the bifurcation.

Precolostrum serum samples were obtained from piglets for antibody tests at birth. A total of 44 fetuses, 24 virus inoculated and 20 controls, was studied.

RESULTS

None of the sows developed clinical signs of infection after PPV inoculation. However, all sows developed HA-inhibiting antibody titers 7 days after infection of the fetal amniotic fluid. Fetuses that were infected with PPV G10/1 via inoculation of the amniotic cavity between the 35th and 55th days of gestation died (Table 1). Crown-rump measurements of the partly mummified fetuses enabled estimation of approximate time of death. It was concluded that death occurred 5 to 22 dpi. In sow SW 2345 (infected on day 35), two fetuses died approximately 5 dpi, two died 14 dpi, and one died 20 dpi. In sow SW 2332 (infected on day 48), one fetus died 10 dpi, three died 12 dpi, and one died 22 dpi. In sow SW 2363 (infected on day 55), one piglet died 9 dpi and two died approximately 14 dpi.

Piglets inoculated with PPV between the 72nd and 105th days of gestation all survived and developed normally. There were no differences between virus-inoculated and control piglets (Table 1).

Two sows (SW 2332 and SW 2378) apparently had dead fetuses in the uterus at the time of infection. These were not inoculated and could easily be recognized after hysterectomy on the basis of size. Crown-rump measurement indicated death before or at the time of inoculation.

Surviving virus-inoculated and control piglets were bled from the umbilical cord after removal of the placenta. Normally delivered piglets were sampled before ingestion of colostrum. All sera from apparently normal control piglets were negative for antibody, whereas the normally developed virus-inoculated piglets of sows SW 2378, inoculated at day 72 of gestation, SW 2343, inoculated at day 99 of gestation, and SW 2344, inoculated at day 105 of gestation, all developed high serum antibody titers (Table 2). The sera from all fetuses and naturally delivered piglets (before colostrum) did not have

TABLE 1. *Data of sows and piglets used for in utero infection with PPV G10/1*

Sow no.	Infection day of pregnancy	Infected uterus horn/no. of piglets	Control uterus horn/no. of piglets	Piglets 4 weeks after infection	
				Infected horn	Control horn
SW 2345, 5th litter	35	Left/5	Right/6	5 dead and mummified piglets, length 5-9 cm	6 normal piglets, length 15.5 cm
SW 2332, 3rd litter	48	Left/6 (position 4 was dead, 5 infected)	Right/2	6 dead and mostly mummified piglets, length 11-17 cm (position 4 only 4 cm)	2 normal piglets, length 20 cm
SW 2363, 1st litter	55	Left/4	Right/4	4 dead and mummified piglets, length 13-15 cm	4 normal piglets, length 22 cm
SW 2378, 3rd litter	72	Left/7 (position 5-7 were dead, 4 infected)	Right/5 (position 6 was dead)	4 normal piglets, length 26 cm; 3 mummified, length 12 cm	4 normal piglets, length 26 cm 1 mummified, length 12 cm
SW 2343, 1st litter	99	Left/3	Right/1	4 piglets naturally delivered, bled before colostrum	
SW 2344, 1st litter	105	Left/3	Right/2	5 piglets naturally delivered, bled before colostrum	

TABLE 2. *Blood HI antibodies in sows and fetal pigs (virus inoculated and control) after inoculation with PPV G10/1 at different times of gestation^a*

Sow no.	Samples days after infection					Fetal pigs	
	Pre	7	14	21	28	Virus inoculated (L)	Control (R)
SW 2345	<4	512	2,048	1,024	1,024	L NT ^b	R-1 to R-6 negative
SW 2332	<4	512	1,024	1,024	1,024	L NT	R-1, R-2 negative
SW 2363	<4	256	1,024	1,024	2,048	L NT	R-1 to R-4 negative
SW 2378	<4	512	1,024	1,024	1,024	L-1 256 L-2 512 L-3 256 L-4 256	R-1 to R-4 negative
SW 2343 ^c	<4	2,048	2,048	2,048	2,048	L-1 256 L-2 512 L-3 1024	R-1 negative
SW 2344 ^c	<4	1,024	2,048	2,048	2,048	L-1 256 L-2 256 L-3 256	R-1 to R-3 negative

^a Antibody titers are given in reziproke dilution values.

^b NT, Not tested.

^c Piglets of sows SW 2343 and SW 2344 were bled before ingestion of colostrum.

antibodies to any of the known eight serotypes of porcine enteroviruses. Virus isolations were successful from all organs of virus-inoculated piglets that died after infection. This was true with sows SW 2345 (five dead), SW 2332 (five dead), and SW 2363 (four dead). Explant tissue cultures from organs of piglets from sows SW 2378, SW 2343, and SW 2344 were consistently positive. Only one virus-inoculated piglet was negative (SW 2344, L-3). For details, see Table 3.

Explant cultures from organs of many con-

trol piglets were also positive for virus isolation. Virus was isolated from at least one organ sample in 13 of 20 piglets tested. The organ material most consistently positive was lung. Virus was not isolated from control piglets of sow SW 2332 (Table 3).

DISCUSSION

Porcine fetuses did not survive intrauterine infection with a PPV when inoculated on the 35th, 48th, and 55th days of gestation. Death and subsequent mummification occurred be-

TABLE 3. *Virus isolation from fetal pigs inoculated with PPV G10/1 and control pigs 4 weeks after inoculation*

Sow no.	Inoculated at days of gestation	Virus isolations from:	
		Virus-inoculated fetuses	Control fetuses
SW 2345	35	L-1, L-2, L-3, L-4, L-5 positive from all organs	R-1 lung, testis positive R-2 lung positive R-3 negative R-4 lung, kidney positive R-5 lung positive R-6 testis positive
SW 2332	48	L-1, L-2, L-3, L-5, L-6 positive from all organs	R-1 negative R-2 negative
SW 2363	55	L-1, L-2, L-3, L-4 positive from all organs	R-1 negative R-2 lung positive R-3 lung, kidney positive R-4 negative
SW 2378	72	L-1 lung, testis positive L-2, L-3, L-4 saved for pathology Mummies negative	R-1 negative R-2 lung, kidney positive R-3 lung, kidney positive R-4 lung positive Mummies negative
SW 2343	99	L-1 testis positive L-2 kidney positive L-3 kidney, testis positive	R-1 kidney, testis positive
SW 2344	105	L-1 kidney positive L-2 lung, kidney positive L-3 negative	R-1 kidney, lung positive R-2 negative R-3 kidney, testis positive

tween 5 and 22 dpi based on measurements of crown-rump lengths (7). Sows of the infected fetuses did not show any clinical symptoms. Since virus was readily reisolated from all organs of these fetuses, it was supposed that death was due to damage induced by the virus. These results agree with observations of Redman et al. (23) in fetuses of one sow infected at 62 days of gestation. Infection of these fetuses occurred at a time when they lacked immunological competence. The majority of reports dealing with immunological responses in fetal pigs established onset of immunological competence at 60 to 74 days of gestation (7, 10, 25, 27). Cartwright et al. (5) and Johnson and Collings (12, 13) did not report fetal death after natural PPV infection although transplacental crossing of the virus occurred.

Intrauterine infections at later stages of gestation, at 72, 99, and 105 days, did not result in death of fetal pigs, and antibodies were produced readily.

PPV invariably spread from virus-inoculated fetuses in one horn to the control fetuses in the other horn. This occurred regardless of the period of gestation that fetal pigs were inoculated. However, no fetal deaths were observed in the control fetuses to which virus had spread.

Similar findings were reported by Cartwright et al. (5) and Johnson and Collings (12, 13), who observed transplacental infection of piglets

after oral and intravenous infection of sows at various periods of gestation. No deaths directly attributable to PPV infection were reported in these two papers, and no clinical or pathological changes in infected piglets were found. However, fetuses of sows infected at late stages of pregnancy developed antibodies to PPV.

In sows SW 2332 and SW 2378, some of the fetuses were dead before inoculation and SW 2343 only had four fetuses. Although it cannot be excluded that a virus infection may be responsible for these mummified fetuses (8), negative serological and virological tests do not support this hypothesis.

Similar discrepancies between direct in utero inoculation of fetuses and infection by transplacental crossing of virus to fetuses were described by Margolis and Kilham (16, 17). They studied infections of reovirus type 3 in pregnant rats and found fetuses infected but generally free from disease at birth when inoculated via the maternal blood circulation. However, when fetuses were infected by direct intra-amniotic inoculation, a high incidence of death and resorption was recorded. Margolis and Kilham (16) postulate a resistance factor in placental tissue and/or in the fetus which limits the infection. A maturation process may be responsible for fetal recovery from reovirus infections. Further experimental evidence for this hypothesis has been presented by Watson (26) with

mumps virus-infected chicken embryos and Osburn et al. (22) with blue-tongue virus in sheep embryos.

It is possible that other factors may be involved if only small quantities of virus pass transplacentally to infect fetal pigs, resulting in a latently infected state. The techniques required for isolation of virus from these piglets (13) suggest such a possibility. In these studies, recovery of PPV from fetuses infected by virus spreading from fetuses infected directly were made only by growing explant cultures from infected organs of these fetuses and not by conventional methods. Lack of antibody production in the piglets of the control uterus horn that were infected by spreading virus further supports the presence of a low concentration of transplacentally transferred virus. It has been shown that low levels of antigen fail to induce antibody production in fetal pigs at the 88th day of gestation when infected in utero with pseudorabies virus (11).

An additional factor may be involved, namely, the body temperature of the sow. The normal body temperature of pigs ranges from 38 to 40 C, with a mean of 39 C. Pigs are sensitive to changes of environmental temperatures and normal body temperatures may vary considerably. In vitro studies showed that PPV replicated in cell cultures at 40 C only when cultures were inoculated with large amounts of virus (3). Low quantities of PPV induced carrier states in cells that were activated by lowering the incubation temperature to 37 C. Similar conditions may occur in vivo.

Antibody production in immunologically competent piglets can occur within 9 days after direct inoculation with virus. The fact that these piglets did not develop antibodies to PPV within 4 weeks after inoculation suggests that in addition to quantity of antigen, the time involved in the spread of the virus from fetuses in the virus-inoculated horn to the control horn may also be a factor. Many control piglets were in a gestation period that rendered them immunologically competent when infected. Since the mechanisms of the spread are not fully known (15), it may well be that 28 days, the time between inoculation of the pigs in one horn and the time of hysterectomy of the sow, may allow the virus to spread to fetuses in the other horn, but may be too short for induction of measurable amounts of antibodies in fetal pigs.

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