Investigation of the Pathogenesis of Transplacental Transmission of Aleutian Mink Disease Parvovirus in Experimentally Infected Mink

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The transplacental transmission of Aleutian mink disease parvovirus (ADV) was studied in experimental infection of 1-year-old female non-Aleutian mink. The ADV-seronegative female mink were inoculated with ADV prior to mating or after the expected implantation of the embryos during pregnancy. A group of uninfected females served as a control group. Animals from each group were killed prior to or shortly after parturition. The in situ hybridization technique with radiolabeled strand-specific RNA probes was used to determine target cells of virus infection and virus replication. In both infected groups, ADV crossed the endotheliochorial placental barrier, although animals infected before mating already had high antibody titers against ADV at the time of implantation. The percentage of dead and resorbed fetuses was much higher in dams infected before mating. In the placentae of these mink, virus DNA and viral mRNA were detected in cells in the mesenchymal stroma of the placental labyrinth and hematoma but only occasionally in the cytotrophoblast of the placental hematoma. Placentae of animals infected during pregnancy showed in addition very high levels of virus and also viral replication in a large number of cytotrophoblast cells in the placental hematoma, which exhibited distinct inclusion bodies. In both groups, neither virus nor virus replication could be detected in maternal endothelial cells or fetal syncytiotrophoblast of the placental labyrinth. Fetuses were positive for virus and viral replication at high levels in a wide range of tissues. Possible routes of transplacental transmission of ADV and the role of trophoblast cells as targets for viral replication are discussed.

Aleutian mink disease parvovirus (ADV) in adult mink causes a persistent infection usually followed by a chronic, progressive disease associated with a disorder of the immune system (2, 19, 33, 34, 36, 39), which is characterized by the development of plasmacytosis, hypergammaglobulinemia, and immune complex-mediated glomerulonephritis and arteritis in the chronic stage of the disease. Viral replication was demonstrated in lymphatic organs, with a peak 10 days after infection, but then rapidly decreased, and by 60 days after infection, viral replication was below detectable levels (2).

In mink kits infected as newborns, an acute interstitial pneumonia with a high level of virus replication in alveolar type II cells occurs (1, 3, 42).

Observations on mink farms showed that farms positive for Aleutian mink disease (AD) have fewer pregnancies and a smaller number of kits per female than AD-free farms (20). Experimental studies revealed that ADV infection of pregnant female mink is also associated with decreased litter size, embryonic death, stillbirth, birth of weak kits, and abortion (1, 17, 20, 32). Haagsma (17) detected ADV in a stillborn mink kit after experimental infection of the female by the end of mating time. Padgett et al. (32) bred naturally ADV-infected females and collected the fetuses 5 days before the expected parturition. Of 53 examined fetuses, 32 (61%) contained virus. However, not much is known about the mechanism of transplacental transmission of ADV. Transplacental transmission is also known in other parvovirus infections, including porcine parvovirus (8, 29, 30), feline parvovirus (25), and human parvovirus B19 (10, 22).

Like that of other carnivores, the mink placenta has the features of a placenta endotheliochorialis (9, 13, 26) and is very similar to the cat placenta, which has been described in detail (28). It consists of three main areas: (i) the placental labyrinth, where the vascularized trophoblast forms syncytia surrounding maternal blood vessels, which is separated from the maternal bloodstream by the maternal endothelium and a periodic acid-Schiff-positive interstitial layer; (ii) the junctional zone or transition zone, where the fetal syncytiotrophoblast interdigitates with the epithelium of the maternal endometrium and in which, in areas where it contacts uterine glands, a cytotrophoblast which phagocytizes histiotroph is present; and (iii) the placental hematoma, where a direct contact between the cytotrophoblast and the maternal blood is present. The cytotrophoblast of the placental hematoma phagocytizes maternal erythrocytes.

The importance of the three parts of the placenta, which develop differently during gestation, in transplacental transmission of viruses is not known. Furthermore, differences between persistently infected females with chronic disease which become pregnant and acutely infected ADV-negative females during pregnancy have not been investigated. This study was designed to obtain more detailed information about the influence of different stages of infection on pregnancy and the pathogenesis of transplacental transmission. The in situ hybridization technique with strand-specific RNA probes is of particular value for the detection of target cells of viral sequestration and replication (2, 3).

MATERIALS AND METHODS

Virus. For experimental infections, the highly pathogenic virus strain ADV-United, obtained from United Animal Division (Middleton, Wis.), was used. The preparation of the inoculum has been described previously (4). The inoculum contained 10⁷ adult mink 50% infective doses per ml.

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TABLE 1. Experimental design and necropsy results

Group (day of infection, mo/day)	Mink no.	PID			No. of fetuses ^d		Fetus size ^b (CRL, cm)
		Mating 1	Matings 2/3	Necropsy	Normal	Resorbed or dead	
Persistently infected (2/15)	5	21		66	0	3	7
	1	22		69	3	3	
	4	20	30/33	69	NP, PC		
	8	19	29/32	69	NP, PC		
	2	27		70	8	2	3.5-5
	10	21	31/34	71	5 ^c	4	2
	9	22	32/35	72	2	3	6-7
	13	23	33/36	76	0	2	
	3	24	34	83	NP		
	Totals				18	17	
Acutely infected (4/13)	27	-34		9	3	1	4.5-5
	30	-37	-28	9	9	1	3-3.6
	31	-37	-28	12	8	0	1.5-2
	24	-35	-26	14	9	0	6
	23	-34	-26	14	5	4	6.5-7
	25	-36	-27	15	2	0	1.4
	28	-33	-25	15	8	0	5.5-6
	26	-33	-24	19	2n + 3f	1	7.5-8
	29	-33	-25	28	6n ^d		
	Totals				55	7	
Controls ^e (4/13)	14	-35	-27	8	9	0	4
	22	-37	-28	8	11	0	1.5-2
	15	-36	-27	13	8	1	5.5-6
	18	-32	-31	13	0	2	3.5
	16	-34	-25	13	8	2	4
	21	-32	-31	15	9	0	3.8-4
	17	-34	-26	16	9	0	7
	19	-35	-26		2n	1s	8
	20	-35	-26			1n ^f	
	Totals				56	7	

^a NP, not pregnant; PC, poor condition. f, fetuses; n, newborn mink kits; s, stillborn mink kits. Because implantation sites can still be detected when the fetus has been resorbed, the total number of normal fetuses plus the total number of resorbed and dead fetuses equals the number of implantation sites and newborns.

^b Only normal fetuses were measured.

^c Fetuses had subcutaneous, focal hemorrhages.

^d Two mink kits were killed on the day of birth; one was killed 5 days and two were killed 6 days after birth; and one was presumably eaten by the dam.

^e Controls received virus-free tissue suspension.

^f Died after 1 day.

Negative inoculum. As a negative control, a 10% tissue suspension, prepared from lungs, spleens, and livers from newborn ADV antibody-negative mink kits, was used (1).

Animals. Twenty-seven 1-year-old female black mink (non-Aleutian, genotype A/A) obtained from an AD-negative farm were used in this study. The animals were divided into three groups with nine animals in each group, referred to as persistently infected dams, acutely infected dams, and control dams. The mink were housed in commercial mink cages and fed a standard mink diet.

Experimental design. (i) Persistently infected dams. The females were moved to the experimental farm on February 9, 1994, and infected intraperitoneally on February 15, 1994, with 0.1 ml of virus inoculum. They were mated two or three times during March (Table 1). Blood samples were taken from each animal before inoculation, 10 days and 57 days after inoculation, and on the day it was killed. The dams were killed at a time near expected parturition, between April 22 and May 9, 1994, which is 66 to 83 days after infection (Table 1).

(ii) Acutely infected dams. The females were housed and mated twice during March on an AD-negative farm (Table 1). They were moved to the experimental farm and inoculated with 0.1 ml of virus inoculum on April 13, 1994, at a time when the implantation of the embryos should have already occurred. Blood samples were taken from each animal before inoculation, 9 days after inoculation, and on the day it was killed. The dams were killed 9, 12, 14, 15, 19, and 28 days after infection (Table 1), prior to or shortly after expected parturition, between April 22 and May 11. One female was allowed to give birth to two mink kits. The female and the mink kits were killed on the day of birth. Another female was allowed to give birth to six kits; two of these were killed on the day they were born, one was killed 5 days after birth, and two were killed 6 days after birth (Table 1).

(iii) Control dams. As a control group, nine dams were housed and mated twice during March (Table 1) on an AD-negative farm. They were moved to the experimental farm and inoculated with 0.1 ml of the virus-free tissue suspension

on the same day as the acutely infected group. They were kept in a separate, closed shed on the same farm. Blood samples were taken from each animal before inoculation, 9 days after inoculation, and on the day it was killed. The dams were killed 8, 13, 15, and 16 days after inoculation (Table 1), between April 21 and April 29. One female was allowed to give birth to three kits, and one female gave birth to one mink kit (Table 1).

Gestational stage. The gestational stage was estimated from the crown-rump length (CRL) of the fetuses, because the delay of implantation in mink makes it difficult to calculate the exact stage of pregnancy in the individual female from the mating data (for reviews on mink reproduction, see references 13, 21, 27, 37, and 41). After implantation (April 3 $\pm$ 1 week), the embryos develop to late-term fetuses in 30 $\pm$ 3 days, when they have a CRL of approximately 7 cm.

Fixation of tissues. On the selected days, the animals were exsanguinated under pentobarbital anesthesia. A necropsy was performed, the number of fetuses and implantation sites was counted, and the CRL of the fetuses was measured. Tissue samples of at least three placentae and fetuses were fixed in a freshly mixed solution of periodate-lysine-paraformaldehyde-glutaraldehyde (PLPG) (3). Placentae and small fetuses were fixed for 3 to 5 h at 4°C and then for 6 h at room temperature. The body cavities of big fetuses were opened, and the fetuses were fixed overnight at 4°C.

Samples of placentae and fetuses were also fixed overnight in 10% neutral buffered formalin. A piece of at least one placenta from each animal was fixed in 2.5% phosphate-buffered glutaraldehyde. Two different areas of the placenta, the placental hematoma and the placental labyrinth, were fixed in each fixative.

Samples of lung, liver, kidney, spleen, mesenteric lymph node, ovary, lymphocentrum ileosacrale, mammary gland (if present), and mammary lymph node of the adult females were fixed in PLPG in the same way as the placentae.

Samples of the same tissues were snap frozen in liquid nitrogen and stored at -80°C.

Light microscopy and in situ hybridization. The PLPG- and formalin-fixed

TABLE 2. Anti-ADV antibody titers and virion DNA in sera^a

Group	Mink no.	Exam 1		Exam 2		Exam 3		Necropsy findings	
		Titer	Viral DNA (pg)	Titer	Viral DNA (pg)	Titer	Viral DNA (pg)	Titer (PID)	Viral DNA (pg)
Persistently infected dams	5	0	NM	128	NM	5,120	ND	5,120 (66)	ND
	1	0	ND	128	NM	2,560	NM	2,560 (69)	NM
	4	0	NM	32	NM	2,560	ND	2,560 (69)	ND
	8	0	NM	128	10	640	ND	1,280 (69)	ND
	2	0	ND	32	NM	5,120	ND	5,120 (70)	NM
	10	0	ND	32	NM	2,560	NM	5,120 (71)	NM
	9	0	NM	64	NM	2,560	NM	2,560 (72)	NM
	13	0	NM	128	ND	2,560	ND	2,560 (76)	ND
Acutely infected dams	3	0	NM	64	5	2,560	ND	5,120 (83)	ND
	27	0	NM	64	20				
	30	0	NM	32	NM				
	31	0	NM	64	100			32 (12)	10
	23	0	NM	4	20			128 (14)	200
	24	0	ND	64	10			128 (14)	10
	25	0	NM	32	NM			128 (15)	100
	28	0	ND	64	NM			128 (15)	NM
	26	0	NM	128	10			256 (19)	5
	29	0	ND	64	100			256 (28)	NM

^a Data are reciprocal titers and total amount of virion DNA per 100 μ l of serum. NM, not measurable; ND, not done. Persistently infected dams were examined on PID 0 (Feb. 15), 10 (Feb. 25), and 57 (Apr. 13); acutely infected dams were examined on PID 0 (Apr. 13) and 9 (Apr. 22).

tissue samples were processed for light microscopy and in situ hybridization. Small fetuses were paraffin embedded in total; larger fetuses were either cut medially into halves or had cross sections made at different locations in the body. Histopathological examination was done on sections stained with hematoxylin and eosin. For further investigations, four persistently infected females in different stages of pregnancy, five acutely infected females at postinoculation day (PID) 9, 12, 14, 15, and 28, and four uninfected control females were selected for in situ hybridization with positive- and negative-sense ³⁵S-labeled RNA probes specific for the detection of ADV virion DNA (positive-sense probe) or RF DNA (positive- and negative-sense probes) and mRNA (negative-sense probe). As an additional negative control, selected tissues from infected animals were hybridized with a positive-sense ³⁵S-labeled RNA probe for the detection of the canine parvovirus (CPV) or mink enteritis virus genome (40). In situ hybridization was carried out with slight modifications of techniques described previously (3). For the in situ hybridization, the paraffin-embedded tissue was mounted on silane-coated slides because the placental tissue did not stick well on poly-D-lysine-coated slides. PLPG-fixed tissues were treated in proteinase K solution (5 μ g of proteinase K per ml, 10 mM Tris [pH 7.5], 2 mM CaCl₂) at 37°C for 10 min; formalin-fixed tissue was treated for 20 min. After proteinase K digestion, the slides were rinsed twice for 5 min each in 10 mM Tris (pH 7.5)-0.3 M NaCl and then refixed in phosphate-buffered 5% paraformaldehyde at 23°C for 30 min. Further treatment was carried out as previously described (2-4).

Southern blot hybridization. Serum samples (10 to 100 μ l) were diluted with phosphate-buffered saline (PBS) to 200 μ l, and 0.1 μ g of sheared salmon sperm DNA was added as a carrier. DNA extraction was performed with a commercially available kit (QIAamp Blood Kit; Qiagen, Kebo Lab, Albertslund, Denmark). The final elution had 2 μ g of glycogen added as a carrier and was ethanol precipitated. The pellet was resuspended in 20 μ l of TE buffer, and 10 μ l of each sample was analyzed by agarose gel electrophoresis and Southern blot hybridization (3). Tenfold dilutions of a DNA preparation containing 1 ng, 100 pg, 10 pg, or 1 pg of two 2-kb fragments of the ADV genome served as a positive control for the hybridization and estimation of the amount of DNA in the serum samples.

Preparation of probes. For the Southern blot hybridization, a radioactively labeled DNA probe of two approximately 2-kb fragments of the ADV genome was used. The DNA was labeled with [α -³²P]dCTP (Amersham, Birkerød, Denmark) by random primed labeling (14) and had a specific activity of approximately 5×10^8 dpm/ μ g.

The in situ hybridization was performed by using strand-specific RNA probes radiolabeled with [α -³⁵S]UTP (Amersham) with a commercially available kit (Promega, Bie & Berntsen, Rodovre, Denmark). The preparation of the probes has been described (3, 6, 40).

Serology. Serum samples were examined for specific antibodies against ADV antigen by countercurrent immunoelectrophoresis as described before (1).

RESULTS

Persistently infected dams. (i) Serology. All females were seronegative for anti-ADV antibodies at the beginning of the

study and seroconverted after inoculation (Table 2). They developed titers of up to 1:5,120 by the time of necropsy, between 2 and 3 months after infection. At the beginning of April, when implantation of the embryos should have occurred, these females had anti-ADV antibody titers of between 1:640 and 1:5,120.

(ii) Southern blot hybridization. Amounts of DNA down to approximately 5 pg were detectable by Southern hybridization. In the sera of two animals, virus DNA was detectable only at PID 10 (Table 2) before pregnancy, when the sera contained up to approximately 10 pg of virus DNA per 100 μ l, which equals about 4×10^6 copies of the viral genome. This calculation is based on the assumptions that the average molecular weight of one nucleotide is 322 and that the ADV genome consists of single-stranded DNA with about 5,000 nucleotides. During pregnancy and by the time of necropsy, virus DNA was not detectable in the sera of persistently infected dams.

(iii) Gross pathology. Two females were not pregnant at the time of necropsy. The uterus in both cases showed no signs of implantation. Both mink were in a poor general condition. One female (mink 3 [Table 1]) did not give birth to mink kits and was killed on May 9. Both uterine horns were thickened and contained small yellow masses. Implantation sites were not detected. The uteri of the other females in this group had implantation sites with dead or resorbed fetuses in addition to normally developed fetuses (Table 1). In total, of 35 fetuses or implantation sites, 18 fetuses looked normal, while 17 were dead or had been resorbed. Dead fetuses were discolored brown and were smaller than the normally developed fetuses. The implantation sites of resorbed fetuses consisted of brown, necrotic cell masses. The fetuses of one animal (mink 10 [Table 1]) showed focal subcutaneous hemorrhages.

(iv) Histopathology and in situ hybridization of the reproductive tract. The uteri, placentae, and fetuses of four females (mink 1, 2, 9, and 10) were examined by in situ hybridization. The size of the fetuses indicated a rather early stage of fetal development in one female (mink 10), the fetuses of one female were in the middle stage of gestation (mink 2), and two females had late-term, mature fetuses (mink 1 and 9). The

FIG. 1. (a) Detail from placental labyrinth after in situ hybridization with a ^{35}S -labeled negative-sense RNA probe on mink 2 (persistently infected). Positive cells are located adjacent to the syncytiotrophoblast (small arrow) and in the mesenchymal stroma of the fetal placenta (big arrow). f, fetal blood vessel; m, maternal endothelial cells; s, syncytiotrophoblast. Counterstained with hematoxylin. Bar, 30 μm . (b) Part of the placental hematoma of the same section. The positive cell is located in the stroma of the chorionic villi of the placental hematoma. e, maternal erythrocytes; c, cytotrophoblast; f, fetal blood vessel. Counterstained with hematoxylin. Bar, 30 μm .

results with the negative-sense probe for the detection of viral replication are presented in Table 3. In situ hybridization with the positive-sense probe revealed a similar distribution of positive cells, but the number of positive cells was often higher. In the uterine myometrium, a slight to moderate perivascular mononuclear infiltration was present. Only a very few cells near blood vessels in the uterine myometrium and occasionally in the stroma of the maternal epithelium (endometrium) were

very weakly positive for the positive- and negative-sense probes.

In the placentae of all normally developed fetuses, no pathological alterations were found, but cells positive at high levels for positive-sense RNA and also for negative-sense RNA probes were detected. Those cells were located in the mesenchymal stroma on the fetal side of the placental labyrinth (Fig. 1a) and of the chorionic villi in the placental hematoma (Fig.

FIG. 2. (a) Heart of a fetus from mink 1 (persistently infected) after in situ hybridization with the ^{35}S -labeled negative-sense RNA probe. Heart muscle cells show a positive signal. Counterstained with hematoxylin. Bar, 30 μm . (b) Thymus from the same fetus; in situ hybridization with ^{35}S -labeled negative-sense RNA probe, showing positive cells in the cortex of the thymus (arrows) and the interstitial connective tissue. Counterstained with hematoxylin. Bar, 60 μm .

1b). Often cells adjacent to the syncytiotrophoblast of the placental labyrinth (Fig. 1a) were found to be positive, but the syncytiotrophoblast itself was never positive with either the positive- or negative-sense probe. Positive cells appeared round and cytoplasm rich. Cytotrophoblast cells of the placental hematoma were occasionally positive, but only single cells at low levels. The number of positive cells in the placenta was higher late in gestation than early in gestation. Positive cells

were also detected in the wall of large fetal blood vessels in the placenta, sometimes so close to the lumen that they represent fetal endothelial cells. In the placentae of mink 10 (CRL of fetuses, 2 cm), intravascular hematopoiesis was present in fetal blood vessels of the placenta and a few hematopoietic cells showed a positive signal with both the positive- and negative-sense probes. Neither virus nor virus replication was present in the maternal endothelial cells of the placenta.

TABLE 3. Distribution of cells positive for in situ hybridization with radiolabeled negative-sense RNA probe (detection of RF DNA and mRNA of ADV) in placentae of persistently infected female mink

Mink no.	PID	Uterine wall ^b	No. of positive cells and condition ^a											
			1			2			3			4		
			Laby. mes./stroph	Hem. mes./ctroph	Fetus	Laby. mes./stroph	Hem. mes./ctroph	Fetus	Laby. mes./stroph	Hem. mes./ctroph	Fetus	Laby. mes./stroph	Hem. mes./ctroph	Fetus
10	71	(+)	(+)/-	-/-	+	(+)/-	-/-	(+)	-/-	-/(+)	(+)	(+)/-	Nec/-	Dead/-
2	70	-	(+)/-	(+)/-	++	+/-	(+)/(+)	+	ND	ND	ND	(+)/-	(+)/-	+
1	69	(+)	(+)/-	(+)/(+)	+	(+)/-	-/-	++	+/-	(+)/-	++	Nec/-	Nec/-	Res
9	72	(+)	(+)/-	+/-	+++	+/-	(+)/(+)	++	Nec/-	Nec/-	Dead/-	Nec/-	Nec/-	Res

^a No. of positive cells per section: (+), approximately 1 to 5; +, 6 to 50; ++, 51 to 100; +++, >100; -, none. Implantation sites: laby., placental labyrinth; hem., placental hematoma; mes., mesenchymal stroma; stroph, syncytiotrophoblast; ctroph, cytotrophoblast. Condition: Nec, necrotic; Res, resorbed. ND, not determined.

^b Positive cells in the uterine wall of persistently infected dams showed a much lower signal (more than 10 times less) than in the placenta.

In all living fetuses examined, virus and viral replication were detectable, but there were no pathological findings. Fetuses early in gestation as well as late-term fetuses were infected at rather high levels. A strongly positive signal for the positive- and negative-sense RNA probes was present in a wide range of tissues. In particular, a large number of cells in the liver, spleen, connective tissue of the skin, interstitial connective tissue in other organs, heart muscle cells (Fig. 2a), cells in the cortex and stroma of the thymus (Fig. 2b), and a few bone marrow cells showed a strong signal for both probes. In the liver, a positive signal was often found over immature hematopoietic cells, but single hepatocytes also showed a positive signal. In the interstitium of the kidney and in the brain, only occasional single cells appeared positive. In the lung, only a rather small number of cells were positive compared with other organs. In young fetuses, positive cells in the lung were located in the interstitium; in late-term fetuses, accumulations of big cytoplasm-rich cells in the alveoli were also strongly positive for the positive-sense probe but only very weakly positive for the negative-sense probe.

The placentae of resorbed fetuses consisted of necrotic cell masses. The endometrium in those cases showed a slight to moderate infiltration with neutrophils and lymphocytes. The placentae of dead fetuses were partly necrotic, especially in the area of the placental hematoma, whereas the placental labyrinth in three cases appeared normal. These placentae were negative for virus and viral replication except for two (mink 10 [Table 3]), for which single cells in the stroma of the placental labyrinth were positive. Histopathologically, dead fetuses consisted of accumulations of cells with an intact nucleus but without distinct differentiated organs. Three of four dead fetuses contained few cells positive with the positive-sense probe, and one was also positive with the negative-sense probe.

As an additional negative control, one placenta and fetus of mink 10 were also hybridized with a positive-sense RNA probe for detection of the CPV genome and were negative.

Acutely infected dams. (i) Serology. All dams in this group developed low antibody titers against ADV antigen, up to 1:256 between 9 and 28 days after infection (Table 2). The newborn mink kits had detectable antibody levels of up to 1:320 except for one, which was killed on the day of birth and did not receive colostrum from the mother (Table 4). The body fluid of one late-term fetus was examined for anti-ADV antibodies and was negative.

(ii) Southern blot hybridization. Sera from the dams showed detectable virus DNA levels on PID 9, 12, 14, 15, and 19 (Table 2). Most of the dams developed viremia during pregnancy, with up to 200 pg (8×10^7 virion copies) per 100 μ l of serum. One

fetus and one newborn kit of female 26 killed at the day of birth had levels of up to approximately 2,000 pg (8×10^8 virion copies) per 100 μ l of body fluid or serum. The newborn kits, which had already received maternal antibodies through the milk, had virus DNA levels of between 10 pg (4×10^6 virion copies) and 400 pg (1.6×10^8 virion copies) per 100 μ l of serum (Table 4), or 5 to 200 times less.

(iii) Gross pathology. Seven animals in this group were pregnant in different stages at the time of necropsy (Table 1). The animals were in good condition. One female gave birth to six kits. One kit was presumably eaten by the mother; the other five developed normally up to the day they were killed. Another female (mink 26) gave birth to two kits, which were killed the same day. One mink kit had milk in its stomach already, and the other one did not. The mother was killed the same day. Three fetuses were still in the uterus. In total, of 62 fetuses, newborns, or implantation sites, 7 fetuses were dead or resorbed (Table 1).

(iv) Histopathology and in situ hybridization of the reproductive tract. The uteri, placentae, fetuses, and newborns of five females at PID 9, 12, 14, 15, and 28 were selected for further investigation. The females were in different stages of pregnancy. One female had rather small fetuses (1.5 to 2 cm; mink 31 on PID 12), two females were in a middle to late stage of pregnancy (4.5 to 5 cm for mink 27 on PID 9 and 5.5 to 6 cm for mink 28 on PID 15), one was near term (6.5 to 7 cm; mink 23 on PID 14), and one gave birth to six mink kits (mink 29 on PID 28). The results of the in situ hybridization with the negative-sense probe showing viral replication are presented in Table 5.

In the female at PID 9, two placentae had only a very few weakly positive cells for both probes in the cytotrophoblast of the placental hematoma; the corresponding fetuses were negative. One placenta of the same female showed a high number of positive cells in the mesenchymal stroma of the labyrinth and hematoma but not in the cytotrophoblast of the hematoma. The fetus showed positive cells only in the umbilical cord and the liver, which was negative with the negative-sense probe.

The placentae of two females (minks 31 and 28) on PID 12 and 15 showed a high percentage of cytotrophoblast cells in the placental hematoma with intranuclear, mainly basophilic, or amphophilic inclusion bodies (Fig. 3a). These cytotrophoblast cells were detaching from the trophoblast into the hematoma to a much higher extent than in uninfected placentae and showed a very strong positive signal for both probes over the nucleus but also over vacuoles in the cytoplasm (Fig. 3b). There was no inflammatory reaction present in uterine myo-

TABLE 3—Continued

No. of positive cells and condition ^a															No. of sites examined/ total
5			6			7			8			9			
Laby. mes./ stroph	Hem. mes./ ctroph	Fetus	Laby. mes./ stroph	Hem. mes./ ctroph	Fetus	Laby. mes./ stroph	Hem. mes./ ctroph	Fetus	Laby. mes./ stroph	Hem. mes./ ctroph	Fetus	Laby. mes./ stroph	Hem. mes./ ctroph	Fetus	
Nec/−	Nec/−	Res	ND	ND	ND	ND	ND	ND	−/−	Nec/−	Res	(+)/−	Nec/−	Dead/(+)	7/9
Nec/−	Nec/−	Res	ND	ND	ND	+/−	(+)/−	++	Nec/−	Nec/−	Dead/−	+/−	(+)/−	++	7/10
Nec/−	Nec/−	Res	Nec/−	Nec/−	Res										6/6
Nec/−	Nec/−	Res													5/5

metrium, endometrium, or placenta. In the same animals, various numbers of cytotrophoblast cells in the junctional zone were strongly positive for both probes. In situ hybridization of one placenta of mink 31 and two placentae and one fetus of mink 28 with a positive-sense RNA probe for the detection of CPV gave a negative result.

The placentae of the animal killed at PID 14 had many fewer positive cytotrophoblast cells than those of the other two females, and inclusion bodies were only observed occasionally.

In the placental labyrinth, the positive signal was confined to cells in the mesenchymal stroma and adjacent to the syncytiotrophoblast. There was no significant difference in the strength of signal and number of positive cells in the placental labyrinth between acutely infected dams and persistently infected dams. On the maternal side, a few spindle-shaped cells in the uterine myometrium, cells near blood vessels, and cells in the interstitial endometrial stroma (Fig. 4) were positive. Maternal endothelial cells of the placenta were negative.

There were no pathological alterations in normally developed fetuses, although they had a very high signal for virus and virus replication at the same locations as the fetuses from persistently infected dams. Fetuses of mink 31 (CRL, 1.5 to 2 cm) also showed a positive signal with probes of both senses in hematopoietic cells of the yolk sac (Fig. 5).

Necrotic placentae and dead fetuses were negative with both probes.

In the newborn mink kits, a slight accumulation of lymphocytes in the portal areas of the liver was present. All newborn kits examined were positive for both probes, especially cells in the liver, spleen, and heart muscle, and in four kits, epithelial cells in the stratum basale of the esophagus showed a high signal with both probes (Table 5). In the lung, only single cells in the interstitium of the interalveolar septae were positive.

In persistently and acutely infected dams, cells in the interstitium and sometimes epithelial cells of the mammary gland showed a positive signal for both probes. In the ovary, in most cases only single cells of the ovarian stroma were positive with the positive-sense probe but not with the negative-sense probe.

Control dams. (i) Serology. All sera from the animals in this group and their newborn mink kits remained negative for antibodies against ADV.

(ii) Southern blot hybridization. Virus DNA was not detectable in the sera of either the adult mink or the newborn mink kits.

(iii) Gross pathology. Seven females in this group were pregnant at the time of necropsy and in good condition except for one female that died on PID 13 because of stress (weight loss, stomach ulcers, and diarrhea). One control female had a malformed fetus with an ectopia of the liver and one empty implantation site. One female gave birth to two normal kits and one stillborn kit. Another female gave birth to one kit, which

died after 1 day. In total, of 63 fetuses, newborns, and implantation sites, 7 fetuses or newborns were dead or resorbed (Table 1).

(iv) Histopathology and in situ hybridization of the reproductive tract. The placental hematoma and placental labyrinth of the normally developed fetuses in this group showed no pathology. The placentae of dead fetuses showed no differences from those of animals in the infected groups. The newborn mink kits had a slight accumulation of lymphocytes in the portal areas of the liver. No significant pathological findings were observed in ovaries and mammary glands. Two placentae and fetuses from minks 14, 22, 15 (also one dead fetus and the corresponding placenta), and 17 (Table 1) and organs from one newborn mink kit were selected for in situ hybridization. All tissues examined were negative with both probes.

DISCUSSION

ADV crossed the placental barrier in mink infected before or after the initiation of pregnancy, although animals infected before breeding already had high antibody levels against ADV at the time of pregnancy.

The percentage of transplacentally infected fetuses from AD-positive females is of practical interest. In our study, 34 of 36 living fetuses or newborn mink kits from both groups examined were infected with ADV. Only two fetuses of one acutely infected animal examined at PID 9 were negative. However, the design of our study and the relatively small number of animals do not allow a representative calculation. The females in this study were inoculated intraperitoneally with a highly virulent virus strain at a rather high dose, which does not resemble a natural infection. However, the high rate of transplacental transmission in animals from both groups in

TABLE 4. Anti-ADV antibody titers and virion DNA in sera from newborn mink kits from acutely infected dams 26 and 29^a

Dam	Kit no.	Age (days)	Titer	Viral DNA (pg)
26	1 ^b	0	0	2,000
	2	0	4	400
	3 ^c	f ^d	0	2,000
29	1	1	10	100
	2	1	40	200
	3	5	320	10
	4	6	320	100
	5	6	320	20

^a Data are reciprocal titers and total amount of virion DNA per 100 µl of serum.

^b This mink kit did not receive colostrum from the mother.

^c Fluid from the body cavities was examined in this case.

^d f, fetus.

FIG. 3. (a) Detail of placental hematoma from mink 31 (acutely infected), showing cytotrophoblast cells with intranuclear inclusion bodies and hyperchromatic nuclear membrane (big arrows); note also the phagocytosis of maternal erythrocytes by the cytotrophoblast (small arrows). Hematoxylin and eosin staining. Bar, 30 μ m. (b) Parallel section, same location as in panel a; in situ hybridization with ³⁵S-labeled negative-sense RNA probe, showing replicative form of virus DNA and viral mRNA, strongly positive signal over the nucleus and also cytoplasm of the cytotrophoblast, corresponding to the cytotrophoblast with intranuclear inclusion bodies seen in panel a. Counterstained with hematoxylin. Bar, 30 μ m.

our study suggests the potential for placental crossing and an affinity of ADV for fetal tissues with high mitotic activity.

The total number of implantation sites in persistently infected dams was much lower than expected. This group had 35 implantation sites, only about half of the total number of implantation sites in the acutely infected and control groups. In

addition, the rate of dead and resorbed fetuses was also much higher in the persistently infected group (~50%) than in the acutely infected and control groups (12%). This is in agreement with earlier observations on AD-positive mink farms (20), which have a poorer breeding result, and experimental studies, in which 22% of the fetuses in AD-seropositive non-

FIG. 4. Uterine wall of mink 27 (acutely infected) after in situ hybridization with ^{35}S -labeled positive-sense RNA probe. Positive cells are located in interstitial connective tissue of myo- and endometrium (arrows) of the pregnant uterus. m, myometrium; e, endometrium; u, uterine gland. Counterstained with hematoxylin. Bar, 60 μm .

Aleutian mink and 49% of the fetuses in AD-positive Aleutian mink were dead (32).

In contrast to earlier observations (1, 20), abortions were not observed in our study. In earlier studies, abortion was observed when the females were infected on April 6 (1a) or by the end of mating time (20), that is, before implantation of the blastocysts. The induction of abortion might be dependent on the time of infection or the virus isolate used.

The infection of fetuses early in gestation in one persistently infected mink and the finding of infected late-term fetuses in two other minks in the same group indicate that the fetuses are able to develop normally to a certain extent despite being infected with ADV at high levels. Therefore, ADV obviously does not cause tissue damage to an extent that would lead to fetal death. On the other hand, the rate of fetal death was much higher in the persistently infected group, although virus was either not detected or detected only at very low levels in dead fetuses. The placentae of normally developed fetuses showed no morphological changes, and the slight perivascular mononuclear infiltrations in the uterine myometrium cannot by themselves explain the high rate of fetal death in this group. The infection with ADV might therefore not be the only cause of fetal death, as some fetuses also died during gestation in the uninfected group. In early stages of pregnancy, up to 11 fetuses were found in the uterus. The average number of newborn mink kits per female in black mink on AD-free farms in Denmark in 1994 was approximately five (11a), indicating that a certain percentage of implanted embryos die throughout gestation in normal pregnancy, which is in accordance with earlier studies (21).

In the acutely infected group, all infected fetuses survived the infection to the time that they were killed. The earliest time that transplacental transmission of ADV was detected was 9 days after inoculation, when one out of four fetuses and three out of four placentae were positive. It seems likely that the

infection in this group was too short in duration to cause fetal death.

In both infected groups, virus and virus replication were detected in cells of the fetal placenta and the fetus. The virus also seems to replicate in these positive areas, as indicated by a signal with probes of both senses.

A remarkable difference was observed between the persistently and acutely infected groups in the placental hematoma, where the fetal cytotrophoblast is in direct contact with the maternal blood. The cytotrophoblast of the placental hematoma in persistently infected females might be protected from virus infection and replication by anti-ADV antibodies present in the maternal blood. In contrast, the cytotrophoblast of the hematoma and the junctional zone in acutely infected dams is exposed to the viremic phase of the infection. It not only takes up free virions or parts of the virus as a component of immune complexes from the maternal blood or from the histiotroph of the uterine glands but also replicates the virus as mitotic active cells, which supports parvoviral replication (11). The transplacental route of ADV through cytotrophoblast cells of the hematoma and junctional zone seems most likely.

In the placental labyrinth of both infected groups, virus-replicating cells were located adjacent to the syncytiotrophoblast and represent cytotrophoblast cells, which persist and are connected as a second cell layer to the syncytiotrophoblast (5, 28). The positive cells in the mesenchymal stroma of the placental labyrinth could resemble macrophage-like cells, which in our study were detected in the same locations in light and electron microscopic sections (data not shown). Although placental macrophages have been described for human and mouse placenta (15, 38) but not for carnivore placenta, their presence in carnivore placenta should be considered, and a further characterization of those cells and their role as possible targets for parvovirus infection and replication for transplacental transmission could be a subject for further studies. Al-

TABLE 5. Distribution of cells positive for in situ hybridization with radiolabeled negative-sense RNA probe (detection of RF DNA and mRNA of ADV) in placentae of acutely infected female mink^a

Mink no.	PID	No. of positive cells and condition												
		Ute- rine wall ^b	1			2			3			4		
			Laby. mes./ stroph	Hem. mes./ ctroph	Fetus	Laby. mes./ stroph	Hem. mes./ ctroph	Fetus	Laby. mes./ stroph	Hem. mes./ ctroph	Fetus	Laby. mes./ stroph	Hem. mes./ ctroph	Fetus
27	9	(+)	+/-	+/(+)	(+) ^c	-/-	-/-	-	Nec/-	Nec/-	Dead/-	-/-	-(+)	-
31	12	++	+/-	+/(+++)	+++	(+)/-	+/(++)	+++	+/-	+/(+++)	++	+/-	+/(+++)	ND
23	14	+	++/-	+/(+++)	++	++/-	+/(++)	+++	Nec/-	Nec/-	Dead/-	Nec/-	Nec/-	Dead/-
28	15	+	++/-	+/(+++)	+++	+/-	(+)/+	ND	+/-	(+)/++	+++	+/-	(+)/+++	+++

^a See Table 3, footnote *a*. Five newborn kits from mink 29 were tested on PID 28. One showed positive cells especially in the liver, spleen, and heart; the other four showed positive cells especially in the liver, spleen, heart, and esophageal epithelium.

^b Signal comparable with that of cells in the placenta.

^c Umbilical cord only.

though the maternal endothelium and the syncytiotrophoblast of the placental labyrinth were never positive for virus or virus replication, it is possible that virions or virus as a component of immune complexes can pass the placental barrier by pinocytosis or endocytosis. But since the highly modified maternal endothelium and the differentiated syncytiotrophoblast, in contrast to the cytotrophoblast, are not permissive for virus infection and replication, virus DNA levels in these cells are too low to be detected by in situ hybridization. This hypothesis is supported by the observation that two fetuses of mink 10 were infected at high levels, although the corresponding placentae did not have positive cells in the cytotrophoblast of the hematoma or the junctional zone but only in the placental labyrinth. Therefore, transplacental transmission seem to occur also in the placental labyrinth. The placenta also might have different transport systems early in gestation than late in gestation, and thus the mechanism of transplacental transmission of viruses could be different depending on the gestational stage.

The findings in both infected groups suggest that infectious virus is transported through the trophoblast despite the presence of antibodies, replicates in the cytotrophoblast and cells of the mesenchymal stroma of the fetal placenta, and enters the fetal vascular system, where it replicates in hematopoietic

cells and other tissues. That indicates that the placenta not only plays a role in transporting the virus but is also a site of viral replication, which cannot be protected by maternal antibodies. In the placental labyrinth, where the fetal trophoblast is not in direct contact with the maternal blood, and in the fetus throughout gestation, there is no evidence of restricted viral replication, as is observed in adult mink (2).

A tropism of ADV for lymphocytes in adult mink has been demonstrated and discussed earlier (2, 18, 23). In this study, fetal cytotrophoblast cells, cells of the mesenchymal stroma of the placenta, which could include placental macrophages, cells in the wall of fetal blood vessels, fetal endothelial cells, and fetal hematopoietic cells besides other fetal tissues were identified as targets for ADV infection and replication. Macrophages have also been considered as targets for ADV in adult mink, and their possible role in the persistence of the infection is discussed in other studies (7, 24, 31). Studies on human placentae and placentae of laboratory animals indicate that trophoblast cells share many characteristics with macrophages (16). Cytotrophoblasts and macrophages might have some common features, which support ADV infection.

Although newborn mink kits were infected transplacentally, they were able to develop normally up to 6 days after birth. An

FIG. 5. Detail of the yolk sac of a fetus from mink 31 (acutely infected) after in situ hybridization with ³⁵S-labeled negative-sense probe, showing a positive signal over hematopoietic cells. Note also the mitotic figure (arrow). Counterstained with hematoxylin. Bar, 30 μ m.

TABLE 5—Continued

No. of positive cells and condition															No. of sites examined/total
5			6			7			8			9			
Laby. mes./stroph	Hem. mes./ctroph	Fetus	Laby. mes./stroph	Hem. mes./ctroph	Fetus	Laby. mes./stroph	Hem. mes./ctroph	Fetus	Laby. mes./stroph	Hem. mes./ctroph	Fetus	Laby. mes./stroph	Hem. mes./ctroph	Fetus	
+/-	+/++	ND	+/-	+/++	ND	+/-	+/+	+	+/-	ND	+				4/4
Nec/-	Nec/-	Dead/-	++/-	++/++	+++	++/-	+/+	+++	+/-	-(+)	+++	Nec/-	Nec/-	Dead/-	8/8
(+)/-	+/+++	ND	ND	ND	ND	+/-	+/+++	+++	+/-	+/+++	+++				9/9
															7/8

inflammatory reaction in infected organs was not present at this time. A maternal antibody titer was detected in the serum, and the amount of virus genome in the serum was much lower than in the mink fetuses. As soon as the mink kits obtain milk from the mother, maternal antibodies are absorbed and the amount of virus in the serum decreases rapidly, but large amounts of virus and virus replication were still detectable in several organs. Lung lesions with a high level of viral replication in alveolar type II cells, as occurs in mink kits infected as newborns (1, 3, 42), were not observed in transplacentally infected fetuses or newborn mink kits up to 6 days after birth.

Transplacental infection might lead to a partial immunotolerance for ADV. In an earlier study, a reduced severity of lesions and a marked reduction in anti-ADV antibody levels in mink born from females that had been infected with ADV during pregnancy were observed (35). A reduced immune response of those mink to ADV was suggested. However, immunotolerance in transplacentally infected mink has not been shown. Further investigations are necessary to study the possibility of immunotolerance and persistence of the infection in transplacentally infected mink.

Virus was also present in the mammary glands of adult females in both infected groups, so that transmission of virus to newborn mink kits from AD-positive females through the milk is also possible.

This study demonstrates the possible transplacental transmission of ADV, in which the cytotrophoblast plays an important role as a target for viral infection and replication in the placenta. Transplacental infection was shown for a high percentage of fetuses and newborn mink kits, even in the presence of circulating anti-ADV antibodies in the mother. Infection prior to the breeding season had a higher impact on reproduction capacity than infection during gestation, which might be due to the shorter exposure to the virus.

In veterinary practice, breeding of AD-positive females, although they might not have clinical signs, should be avoided in order to eliminate the virus and the disease.

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