JOURNAL OF VIROLOGY, Apr. 1994, p. 2720–2725
0022-538X/94/$04.00+0

Vol. 68, No. 4

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Role of Alveolar Type II Cells and of Surfactant-Associated Protein C mRNA Levels in the Pathogenesis of Respiratory Distress in Mink Kits Infected with Aleutian Mink Disease Parvovirus

BIRGITTE VIUFF,[1] BENT AASTED,[2] AND SOREN ALEXANDERSEN[1]*

Laboratory of Molecular Pathobiology, Department of Pharmacology and Pathobiology,[1] and Laboratory of Virology and Immunology, Department of Veterinary Microbiology,[2] The Royal Veterinary and Agricultural University of Copenhagen, DK-1870 Frederiksberg C, Denmark

Received 16 September 1993/Accepted 22 December 1993

Neonatal mink kits infected with Aleutian mink disease parvovirus (ADV) develop an acute interstitial pneumonia with clinical symptoms and pathological lesions that resemble those seen in preterm human infants with respiratory distress syndrome and in human adults with adult respiratory distress syndrome. We have previously suggested that ADV replicates in the alveolar type II epithelial cells of the lung. By using double in situ hybridization, with the simultaneous use of a probe to detect ADV replication and a probe to demonstrate alveolar type II cells, we now confirm this hypothesis. Furthermore, Northern (RNA) blot hybridization showed that the infection caused a significant decrease of surfactant-associated protein C mRNA produced by the alveolar type II cells. We therefore suggest that the severe clinical symptoms and pathological changes characterized by hyaline membrane formation observed in ADV-infected mink kits are caused by a dysfunction of alveolar surfactant similar to that observed in respiratory distress syndrome in preterm infants. However, in the infected mink kits the dysfunction is due to the replication of ADV in the lungs, whereas the dysfunction of surfactant in preterm infants is due to lung immaturity.

Infection with Aleutian mink disease parvovirus (ADV) causes a number of clinical and pathological syndromes in mink. Adult mink develop a persistent infection characterized by plasmacytosis, hypergammaglobulinemia, and immune complex-mediated glomerulonephritis and arteritis (1, 34). Furthermore, abortion and decreased fertility have been described (2). ADV infection causes an acute interstitial pneumonia in newborn, seronegative mink kits (2, 7, 29), whereas seropositive mink kits develop an immune complex-mediated disease similar to the disease seen in adult mink (3, 6, 8).

The acute interstitial pneumonia caused by ADV infection in newborn, seronegative mink kits is characterized by hypertrophy and hyperplasia of alveolar type II epithelial cells and the occurrence of intranuclear viral inclusions in such cells. Hyaline membranes cover the alveolar surface, and the infected mink kits develop a fatal respiratory distress syndrome (2, 4, 29). Respiratory distress syndrome with formation of hyaline membranes is also seen in preterm human infants and is in such cases due to lung immaturity (19, 24, 25). In human infants, a deficiency of pulmonary surfactant is known to be the cause of respiratory distress syndrome and treatment of preterm infants with exogenous surfactant has proven to be successful (24). A similar syndrome, adult respiratory distress syndrome (ARDS), is seen in human adults (9). This is an etiologically heterogeneous syndrome associated with sepsis, aspiration, toxins, circulatory collapse, and several other disorders (42). Although the mechanisms involved in ARDS are not well defined, the resulting lung abnormalities are similar to those in preterm human infants (9, 10) and in ADV-induced respiratory distress in mink kits.

The primary role of pulmonary surfactant is to reduce the surface tension at the air-liquid interface of the terminal airways, thus preventing collapse of alveoli (24, 46). Surfactant is synthesized in the alveolar type II cells where, prior to release, it is stored as lamellar bodies (24, 46). The contents of the lamellar bodies are secreted into the alveoli where it can form tubular myelin, which probably is a precursor of the surface-active monolayer of alveolar surfactant (46).

Surfactant is a complex mixture of lipids and proteins containing approximately 90% lipids and 10% proteins. Four different surfactant-associated proteins have been described, designated SP-A, SP-B, SP-C, and SP-D (23, 35, 39). Surfactant-associated protein SP-A is a large glycoprotein which, depending on the species, is synthesized in alveolar type II cells only or in both alveolar type II cells and in nonciliated bronchiolar cells (31, 32). SP-A seems to participate in the formation of tubular myelin together with SP-B. Furthermore, SP-A may play a role in regulation of surfactant secretion and uptake and may be involved in host immunodefense mechanisms (23, 35). SP-B and SP-C are low-molecular-weight, hydrophobic proteins that accelerate the rate of surface-active monolayer formation at an air-liquid interface (23, 35). By using in situ hybridization, it has been found that SP-B mRNA in humans and rats is synthesized in alveolar type II cells and in nonciliated bronchiolar cells, whereas SP-C is synthesized in

* Corresponding author. Mailing address: Laboratory of Molecular Pathobiology, Department of Pharmacology and Pathobiology, Royal Veterinary and Agricultural University, 13 Bülowsvej, DK-1870 Frederiksberg C, Denmark. Phone: (45) 3528 2740. Fax: (45) 3528 2742.

alveolar type II cells only (18, 31, 32). SP-D is a glycoprotein and has been immunochemically localized to alveolar type II cells and nonciliated bronchiolar cells. It seems to be a host immunodefense protein acting in the lower respiratory tract (38, 39).

In the present study we have used double in situ hybridization, with the simultaneous use of a probe to detect SP-C mRNA and a probe to detect ADV replication, to confirm that alveolar type II cells are permissive for high-level ADV replication in infected mink kits. Furthermore, by comparing the levels of SP-C mRNA in infected and noninfected mink kits we were able to demonstrate that viral replication has a significant effect on the production of SP-C mRNA by alveolar type II cells. This finding substantiates the role of decreased surfactant levels in the pathogenesis of respiratory distress induced by ADV infection of newborn mink kits.

Mink used in this study were black mink (non-Aleutian, genotype *A/A*). Seronegative mink kits were inoculated intraperitoneally as newborns (within 16 h after birth) with 0.1 ml of a virus inoculum containing 10^7 50% adult mink infective doses per ml as previously described (7). Ten infected mink kits were exsanguinated under pentobarbital anesthesia at selected days (postinoculum days (PID) 8 to 13). Half the lung was quickly frozen in liquid nitrogen, and the other half was fixed in freshly prepared fixative containing periodate-lysine-paraformaldehyde-glutaraldehyde (PLPG) for in situ hybridization as previously described (7). Eight uninfected mink kits 8 to 14 days of age served as controls.

Total RNA was prepared from one-half of the lung tissue of five ADV-infected and five control mink kits by an acid phenol-chloroform extraction procedure as described previously (14). The total RNA was quantitated by measurement of A_{260}, and 5-μg samples were electrophoresed into 1% agarose gels containing 6% formaldehyde as previously described (5). Northern (RNA) blots were performed, using 6×10^5 cpm of ^{32}P-labeled RNA probe per ml. After exposure to film, bands were quantitated using a laser densitometer (LKB 2202 Ultroscan; LKB Products, Bromma, Sweden).

Both Northern blot and double in situ hybridizations were performed with strand-specific RNA probes. For Northern blot hybridization, the probes were radiolabeled with [^{32}P]UTP (New England Nuclear Corp., Boston, Mass.). For double in situ hybridization, radioactive probes were labeled with [^{35}S]UTP (New England Nuclear), whereas the biotin RNA probes were labeled with biotin-11-UTP (Sigma Chemical Co., St. Louis, Mo.). The probes were produced using a transcription kit obtained from Promega, Madison, Wis. The DNA template for the ADV probes used have previously been described in detail (12). The DNA template for the mink SP-C probe has previously been cloned in our laboratory (15) (EMBL accession number Z19516).

The double in situ hybridization was performed by simultaneous hybridization with a biotin-labeled probe and a radioactive probe. Hybridizations were performed as previously described (6–8). The hybridization solution contained 10^5 cpm (0.4 ng/μl) of ^{35}S-labeled RNA probe and 1 to 5 ng of biotin-labeled RNA probe per μl. Hybridizations were done at 42°C for 12 to 16 h. After being washed, the slides were immersed in Tris HCl-buffered saline (TBS) (0.05 M Tris HCl [pH 7.5], 0.15 M NaCl) for 5 min. The sections were then incubated for 30 min at room temperature in streptavidin (DAKO, Glostrup, Denmark) in a moist chamber and washed two times for 5 min each in TBS. Sections were incubated for 30 min at room temperature in biotinylated alkaline phosphatase (DAKO) in a moist chamber followed by two 5-min washes in TBS. The slides were then immersed in veronal acetate buffer (30 mM sodium acetate, 30 mM sodium barbitone [5.5 diethylbarbituric acid sodium salt; E. Merck AG, Darmstadt, Germany], 100 mM NaCl, 50 mM $MgCl_2$, pH adjusted to 9.2) for 30 min, and alkaline phosphatase activity was demonstrated by incubating the slides in a filtered solution of 0.05% (wt/vol) Fast Red TR Salt (Sigma), 0.05% Naphthol AS-BI Phosphate (Sigma) (predissolved in dimethylformamide to 25% [wt/vol] immediately before addition to buffer solution), and 0.024% Levamisole (Sigma) in veronal acetate buffer (pH 9.2). The slides were kept in total darkness during development. Slides hybridized with the biotin-labeled plus-sense ADV probe were incubated for 45 min, whereas slides with the biotin-labeled minus-sense ADV probe and the SP-C probe were incubated overnight. The slides were then rinsed several times in 0.3 M ammonium acetate and immersed in 70% ethanol containing 0.3 M ammonium acetate and air dried. Slides were dipped in Kodak nuclear track emulsion NTB-2 as previously described (7), and exposure was done in a dry and totally dark environment at 4°C. Sections hybridized with the ^{35}S-labeled ADV plus-sense probe were exposed for 1 to 2 days, whereas sections hybridized with the ^{35}S-labeled ADV minus-sense probe and the SP-C probe were exposed for 7 days. The slides were developed as previously described (7). After development the slides were counterstained with Gills hematoxylin no. 3 (Polysciences, Inc., Warrington, Pa.) for two min, washed in distilled water for 5 min, air dried, and mounted in a water-based mounting medium (Glycergel; DAKO).

As a control for hybridization to mRNA, some sections were treated with RNase before hybridization, after which the hybridization signal from mRNA disappears; this RNase step was performed as described previously (7).

In situ hybridization using ADV probes. Sections from the kits were hybridized in situ using ^{35}S-labeled or biotin-labeled RNA probes. By using the strand-specific RNA probes, we could detect cells in which ADV was replicating. The minus-sense probe localized sites of viral replication by demonstrating mRNA and replicative-form (RF) DNA. In contrast, the plus-sense ADV probe mainly localized sites of viral sequestration by demonstrating virion DNA and RF DNA as previously detailed (4, 6, 7, 12).

In all infected mink kits, positive cells could clearly be detected with both ADV probes. Some individual differences were seen, but as previously described about 2% of the total lung cells allowed the replication of ADV to high levels as detected by the minus-sense ADV probe (7). The positive cells were widely distributed throughout the lung tissue and were not confined to areas with visible lesions (Fig. 1). As previously described, most of the positive cells had a morphology and distribution resembling that of alveolar type II cells (7, 8). The bronchiolar epithelium was always negative for ADV, and endothelial cells were only very rarely positive for ADV replication. The signal detected by the biotin-labeled ADV probes resembled what was seen with the ^{35}S-labeled ADV probes, although the ^{35}S-labeled probes seemed to be more sensitive.

In sections prepared from control kits, there were no positive cells when hybridized with either of the ADV probes. A low background level of grains was consistently seen diffusely distributed over the whole slide when ^{35}S-labeled probes were used. When biotin-labeled probes were used, no background was seen over lung tissue, whereas a diffusely distributed red color sometimes was seen over liver tissue, probably representing endogenous biotin.

In situ hybridization using the SP-C minus-sense probe. When lung sections from mink kits were probed with a

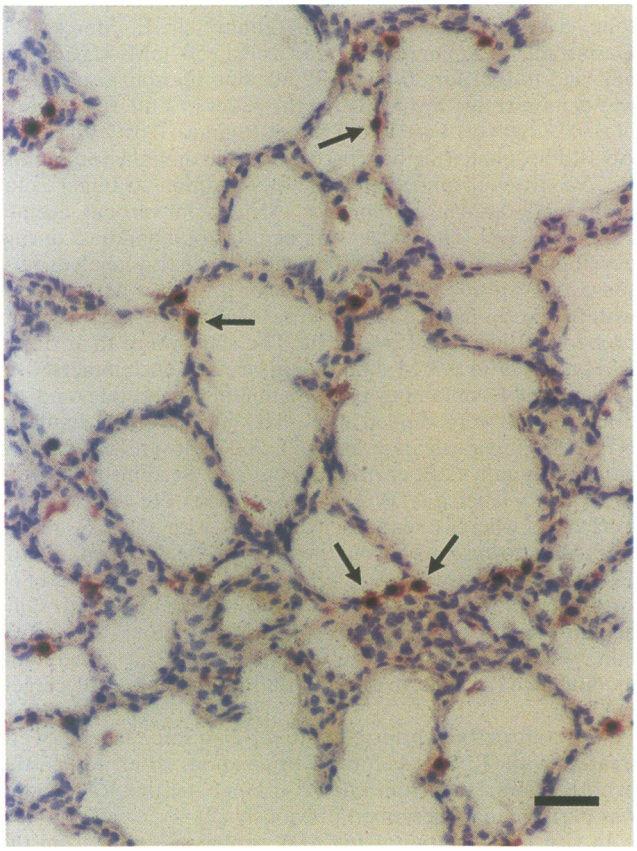

FIG. 1. In situ hybridization analysis using a biotin-labeled ADV minus-sense probe for detection of ADV mRNA and RF DNA on a PLPG-fixed, paraffin-embedded section of the lung from a mink kit sacrificed 8 days after ADV inoculation. Cells with ADV replication are seen as red cells. They are distributed throughout the lung (arrows). Bar, 40 μm.

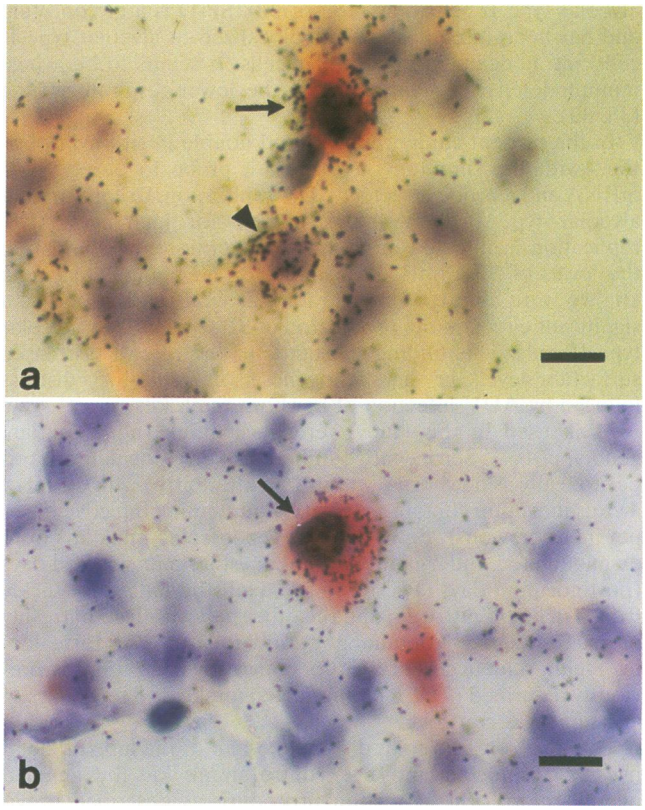

FIG. 2. Double in situ hybridization using a ^{35}S-labeled SP-C minus-sense probe to detect SP-C mRNA and a biotin-labeled ADV minus-sense probe to detect ADV replication. (a) Lung tissue from an ADV-infected mink kit (PID 8). Grain production is seen over an alveolar type II epithelial cell containing SP-C mRNA (arrowhead). An alveolar type II cell with replicating ADV is seen as a red cell covered with grain (arrow). Bar, 8 μm. (b) Lung tissue from an ADV-infected mink kit (PID 10). An alveolar type II cell with replicating ADV is clearly seen (arrow). Bar, 8 μm.

^{35}S-labeled minus-sense probe for surfactant-associated protein C (SP-C), mRNA for SP-C could easily be detected. A distinct hybridization signal was found to occur throughout the alveolar type II cells of the lung tissues in both infected and control kits (data not shown). No cells of the bronchiolar epithelium in the lungs were found positive. Also, no grain in excess of background level was seen in liver, mesenteric lymph node, spleen, thymus, or bone marrow cells. Therefore, as in human and rat tissues (32), SP-C in mink is only synthesized in the alveolar type II cells in the lungs. When sections were incubated with RNase prior to hybridization with the SP-C minus-sense probe, no positive cells were found in any tissue (data not shown). These results showed that the minus-sense SP-C probe hybridized specifically with mRNA for SP-C located in alveolar type II cells, which means that the SP-C minus-sense probe is a good marker for the alveolar type II cells.

There were some local differences in the level of SP-C mRNA in different areas of the lobe. The difference seen was not created by poor distribution of the probe, since double in situ hybridization demonstrated strongly ADV-positive cells in the same area. Neither was the difference caused by the ADV infection, since regional variation in the level of SP-C mRNA was also found in noninfected mink kits. Regional differences in the expression of SP-C mRNA has previously been described by using RNA slot blot analysis to compare different lobes of the lung (20). In our studies, regional differences in the same lobe were demonstrated. It has been shown that a large part of surfactant is recycled (37, 46) and that the synthesis, secretion, and catabolism of surfactant are tightly regulated, maybe through a feedback mechanism (46). The local differences we observed in the SP-C mRNA levels could be caused by such a feedback mechanism.

Double in situ hybridization. We previously suggested that the alveolar type II cell most likely was the permissive cell type for ADV replication in lung tissue of infected mink kits (2, 4, 7). To confirm this hypothesis we developed a double in situ hybridization method using an SP-C probe to detect alveolar type II cells and a strand-specific ADV probe to detect ADV replication.

When lung tissue from infected mink kits was hybridized simultaneously with a ^{35}S-labeled minus-sense SP-C probe and a biotin-labeled minus-sense ADV probe, signals from both probes were detected in cells with a morphology consistent with alveolar type II cells (Fig. 2). Because the ADV minus-sense probe hybridizes with ADV mRNA and ADV RF DNA and the SP-C minus-sense probe hybridizes with SP-C mRNA, this shows that ADV replication takes place in cells producing SP-C, which thus are alveolar type II cells. Some cells were

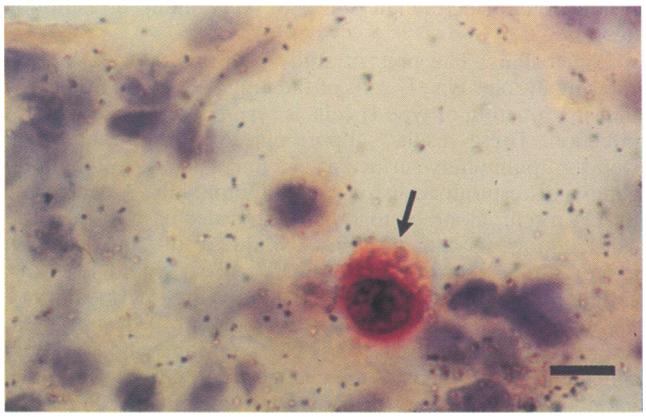

FIG. 3. Lung tissue from an ADV-infected mink kit (PID 8). Double in situ hybridization with a ^{35}S-labeled SP-C minus-sense probe and a biotin-labeled ADV minus-sense probe. A cell with replicating ADV but without demonstrable SP-C mRNA is seen (arrow). Bar, 8 μm.

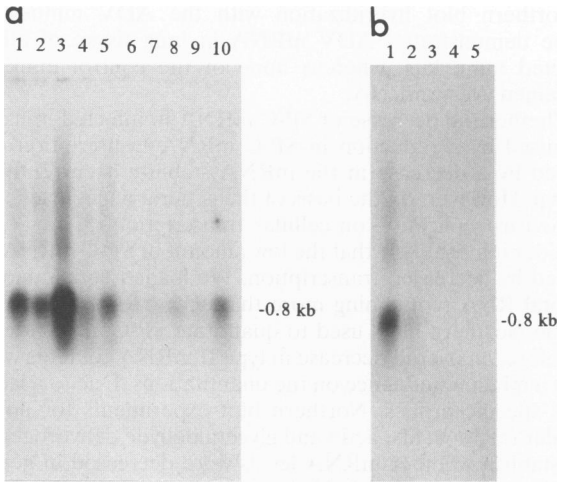

FIG. 4. Northern blot analysis of total RNA. Equal amounts of total RNA were loaded on the gel. The filters were hybridized with a ^{32}P-labeled SP-C minus-sense probe. (a) Lanes: 1 through 5, total RNA from the lungs of noninfected mink kits; 6 through 10, total RNA from the lungs of ADV-infected mink kits. A band at around 800 bases is detected for all animals, but the group of ADV-infected mink kits shows a significant decrease in the levels of SP-C mRNA. (b) Lanes: 1, total RNA from the lung of a noninfected mink kit; 2, total RNA from the lung of an ADV-infected mink kit; 3, total RNA from the spleen of an ADV-infected mink kit; 4, total RNA from the thymus of an ADV-infected mink kit; 5, total RNA from the mesenteric lymph node of an ADV-infected mink kit. SP-C mRNA was only detected in the lungs of the mink kits.

only positive for the SP-C probe and represented alveolar type II cells not actively allowing ADV replication (Fig. 2a). Especially in early infection many cells were positive for transcription of SP-C mRNA and not ADV mRNA, whereas later in infection fewer cells were positive for SP-C mRNA only. No more double-positive cells were seen later in infection, thus, the decrease in the number of cells positive for SP-C mRNA only was not caused by a higher number of double-positive cells. Both in early and late infection some cells were positive for the ADV minus-sense probe only (Fig. 3). These cells are considered alveolar type II cells which have stopped their SP-C mRNA production due to the ADV infection or, alternatively, another cell type permissive for ADV replication.

ADV replication was not observed in bronchiolar cells and was only very rarely seen in endothelial cells. Generally, parvovirus replicates in dividing cells (40, 41). This is in agreement with the pronounced division of alveolar type II cells just after birth (26). However, endothelial cells and other cell types are also dividing just after birth (13, 26). Therefore, our data indicate that there exists either a specific receptor on the alveolar type II cells which is necessary for ADV infection or that some factors in the alveolar type II cells are especially favorable for ADV replication.

When the ADV plus-sense probe was used together with the SP-C minus-sense probe, a few double-positive cells could be demonstrated, representing alveolar type II cells containing virion DNA and/or RF DNA. Moreover, cells positive with only one of the probes could be demonstrated. Some of the cells positive only for the ADV plus-sense probe could be alveolar type II cells not transcribing SP-C mRNA. Alternatively, they could be alveolar macrophages containing sequestered virion DNA.

As a control, double in situ hybridization was performed on infected mink kit tissues with a ^{35}S-labeled plus-sense SP-C probe combined with one of the two biotin-labeled ADV probes. This resulted in a positive ADV signal with no cells having grain counts above background level (data not shown). When noninfected mink kits were hybridized with the ^{35}S-labeled minus-sense SP-C probe and either of the biotin-labeled ADV probes, the SP-C probe hybridized to SP-C mRNA in the type II cells, but no positive cells were found with the ADV probes (data not shown). These data showed the specificity of the double in situ hybridization. The sensitivity of the probes were the same in double in situ hybridization as they were in single in situ hybridization. Thus, the signal from the biotin-labeled probe does not reduce the signal from the ^{35}S-labeled probe or the other way around.

Northern blot hybridization. Results from the in situ hybridization experiments on the infected mink kits at different times after infection suggested that the ADV infection caused a decrease of SP-C mRNA levels. However, the regional differences in SP-C mRNA levels made it difficult to determine whether this decrease was significant. Therefore, total RNA was extracted from five infected mink kits and from five noninfected kits of approximately the same ages and analyzed by Northern blot technique to obtain an average of lung SP-C mRNA content.

The concentration of total RNA extracted from the lung tissues was quantitated by densitometry, and an equal amount of total RNA from the different animals was used for Northern blot analysis. The 28S ribosomal bands from the different animals were compared by densitometric quantitation to control the quantity and quality of the RNA. The Northern blots were hybridized to a ^{32}P-labeled SP-C minus-sense RNA probe. In both infected and control mink kits a single band at around 800 bases was detected (Fig. 4a). Some variation was seen among individual animals in both the infected group and the control group. However, it was obvious that there was a difference in lung SP-C mRNA content between the two groups. The hybridization was repeated on several different Northern blots with the same result. The autoradiographs of the blots probed with the SP-C probe were quantitated with a densitometer, and this analysis showed a fourfold lower content of SP-C mRNA in lung tissue from the infected group than in the control group ($P < 0.02$, using the t-test). When total RNA from other tissues was hybridized to the same probe, no band was detected (Fig. 4b).

Northern blot hybridization with the ADV minus-sense probe demonstrated ADV mRNA in lung tissue of all the infected mink kits, whereas none of the control mink kits contained ADV mRNA.

Whether the decrease of SP-C mRNA in infected mink kits is caused by a reduction in SP-C mRNA transcription or is caused by a decrease in the mRNA stability is currently not known. However, on the basis of the general negative effect of parvovirus replication on cellular transcription (5, 16, 36) we consider it most likely that the low amount of SP-C mRNA was caused by decreased transcription. We loaded equal amounts of total RNA (containing more than 95% rRNA and tRNA) on the Northern blots used to quantitate SP-C mRNA levels; therefore, an overall decrease in type II mRNA contents would have a minimal influence on the quantitations. Due to a lack of mink-specific probes, Northern blot experiments for normal cellular mRNAs, like actin and glyceraldehyde dehydrogenase, to establish whether mRNA levels were decreased in general have been unsuccessful. If the overall transcription rate of ADV-infected alveolar type II cells is severely decreased, this would give a reduction of other type II cell-specific proteins, including SP-A and SP-B as well, and thus add to the lack of surfactant function which ultimately leads to hyaline membrane formation and respiratory distress in ADV-infected mink kits. Further studies are needed to establish whether mRNAs for other type II cell-specific proteins are decreased during ADV infection.

The decrease in the amount of SP-C mRNA is marked considering the number of infected cells in the lungs. Assuming that mink, in addition to rats (22), have around 14% alveolar type II cells, then only about 10 to 15% of the type II cells are infected with ADV according to our in situ hybridization data. We have previously shown that the sensitivity of the in situ hybridization is approximately 300 genomes per infected cell (7). Thus, theoretically there could be more ADV-infected alveolar type II cells than those detected by in situ hybridization and such cells could also be deficient in SP-C mRNA. Furthermore, hyperplasia and hypertrophy of alveolar type II cells are characteristically observed in ADV-induced interstitial pneumonia in mink kits (2). This increased proliferation of type II cells is a general response of these cells to many kinds of alveolar injury (26). Experiments with O_2-induced lung injury have shown that during hyperplasia the alveolar type II cells do not produce surfactant of normal composition (27). Thus, although ADV apparently only infects around 10 to 15% of the alveolar type II cells, it is possible that the proliferation of uninfected type II cells, which then temporally may lack surfactant-synthesizing capability, amplifies the direct effect of ADV on infected type II cells. The result would be a severe reduction in the overall surfactant production.

In addition, a decrease in SP-C mRNA levels during ADV infection could be influenced by other factors. Several in vitro and in vivo studies have shown that surfactant-associated proteins are regulated by hormones, cytokines, and growth factors (11, 17, 28, 30, 44, 46, 47). The regulation appears to be a complex process, and the surfactant protein genes seem in part to be individually regulated (30, 44). Interestingly, an in vitro study demonstrated that tumor necrosis factor alpha (TNF-α) decreased the cellular content of SP-A mRNA and SP-B mRNA (SP-C mRNA was not included in the study) (45). Furthermore, a recent study in humans concluded that TNF-α production in alveolar macrophages obtained by bronchoalveolar lavage from patients with ARDS was significantly increased (43). Therefore, one might consider whether secretion of TNF-α or other cytokines from activated macrophages could play a role in decreasing alveolar type II cell surfactant production during the late stages of ADV-induced respiratory distress.

Conclusions. The present study confirms that ADV replicates in alveolar type II cells of the lung. We also demonstrate that the function of type II cells is severely disturbed by ADV infection. These results support our hypothesis that a deficiency of pulmonary surfactant is a major factor in the pathogenesis of respiratory distress seen in mink kits. A decrease in SP-C protein, alone or together with a decrease in the other surfactant-associated proteins, would result in decreased and/or altered surfactant production. This would lead to a collapse of pulmonary alveoli and an increased permeability of the alveolar lining followed by development of hyaline membranes and respiratory distress. The symptoms and pathological changes are similar to the changes seen in preterm human infants with respiratory distress syndrome. In these children, the immaturity of the lung tissue is the cause of the low level of surfactant. The same symptoms and pathological changes can be demonstrated in human adults with ARDS, following a variety of different insults. In patients with ARDS, it has been shown that there is a decrease in the amount of SP-A and SP-B (the level of SP-C was not investigated) (21, 33). Thus, there are several causes for the development of respiratory distress syndrome, but the common link is the development of a dysfunction of surfactant, which then results in the characteristic symptoms and pathological findings.

A canine SP-C probe used in our cloning of the mink SP-C probe was kindly provided by Tyler White, California Biotechnology Inc., Mountain View, Calif.

This work was supported in part by grants from the Danish Agricultural and Veterinary Research Council, the Biotechnology Center for Livestock and Fish Production, the Danish Fur Breeders Association Research Foundation, and the Novo Nordisk Foundation. S.A. is a research professor of the Danish Ministry of Science and Education.

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