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Pathogenesis of Aleutian Mink Disease Parvovirus Infection: Effects of Suppression of Antibody Response on Viral mRNA Levels and on Development of Acute Disease

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We suppressed the B-cell development and antibody response in mink by using treatment with polyclonal anti-immunoglobulin M (anti-IgM) to study the effects of antiviral antibodies on development of Aleutian mink disease parvovirus (ADV)-induced disease in more detail. Newborn mink kits were injected intraperitoneally with 1 mg of either anti-IgM or a control preparation three times a week for 30 to 34 days. At 21 days after birth, groups of mink kits were infected with the highly virulent United isolate of ADV. At selected time points, i.e., postinfection days 9, 13, 29, and 200, randomly chosen mink kits were sacrificed, and blood and tissues were collected for analyses. The efficacy of immunosuppressive treatment was monitored by electrophoretic techniques and flow cytometry. Effects of treatment on viral replication, on viral mRNA levels, and on development of acute or chronic disease were determined by histopathological, immunoelectrophoretic, and molecular hybridization techniques. Several interesting findings emerged from these studies. First, antiviral antibodies decreased ADV mRNA levels more than DNA replication. Second, suppression of B-cell development and antibody response in mink kits infected at 21 days of age resulted in production of viral inclusion bodies in alveolar type II cells. Some of these kits showed mild clinical signs of respiratory disease, and one kit died of respiratory distress; however, clinical signs were seen only after release of immunosuppression, suggesting that the production of antiviral antibodies, in combination with the massive amounts of free viral antigen present, somehow is involved in the induction of respiratory distress. It is suggested that the antiviral antibody response observed in mink older than approximately 14 days primarily, by a yet unknown mechanism, decreases ADV mRNA levels which, if severe enough, results in restricted levels of DNA replication and virion production. Furthermore, such a restricted ADV infection at low levels paves the way for a persistent infection leading to immunologically mediated disease. The potential mechanisms of antibody-mediated restriction of viral mRNA levels and mechanisms of disease induction are discussed.

We have been interested in the pathogenesis of disease caused by infection with the Aleutian mink disease parvovirus (ADV) for a number of years (3–5, 8–11, 14, 20, 21). ADV causes persistent infection in adult mink leading to a chronic, usually fatal, immune complex-mediated disease characterized by high anti-ADV antibody levels, hypergammaglobulinemia, glomerulonephritis, and arteritis (10, 39, 57–60). In contrast, mink kits infected with ADV as newborns produce only very low levels of anti-ADV antibodies and, moreover, develop an acute, lethal pneumonia characterized by a permissive, cytopathic infection of alveolar type II cells leading to hyaline membrane formation and respiratory distress (5, 7, 8, 11, 12). The development of acute or chronic disease in infected mink is probably determined by several factors. We have previously suggested that both the availability of specific, dividing target cells for viral replication and the host immune response may play major roles in determining the outcome of infection (6, 9–11, 14). Treatment of newborn kits with anti-ADV gamma globulin or mouse monoclonal antibodies against ADV structural proteins drastically reduces the acute mortality and the severity of clinical signs of respiratory disease. Interestingly, antibody-treated kits become persistently infected and develop chronic immune complex-mediated disease, but viral replication and transcription are restricted at the cellular level (14). This viral restriction in antibody-treated kits resembles that observed in persistently infected adult mink and suggests that antiviral antibodies play a role in protection from acute disease and in development of persistent infection and immune complex disease (10, 14).

To study the role of antibody in more detail, we wanted to suppress the B-cell development and antibody response in mink kits and then observe the effects of this immunosuppression on viral replication and viral mRNA levels, on distribution and levels of viral sequestration, and on development of acute versus chronic disease in ADV-infected mink. Mink kits are born without detectable levels of gamma globulin in their sera and acquire their neonatal gamma globulin (immunoglobulin G [IgG] only) via colostrum and milk. They are able to synthesize small amounts of IgG and IgM at birth, while IgA can first be synthesized at 2.5 months after birth (7, 56). These immunological features of mink kits together with results published on immunosuppression of mice (24–26) made treatment of newborn mink kits with anti-IgM antibodies a good candidate for selective suppression of an antibody response. On the basis of previous observations on the mortality of mink

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kits infected at different time points after birth (6) and the number of potential target cells (alveolar type II cells in division [42, 43]) available in the lung at a given time after birth, the age of 21 days of the mink kits was chosen for ADV inoculation. The rationale behind this was to infect kits at an age when they do not normally develop acute disease, i.e., interstitial pneumonia, but still have a reasonable number of alveolar type II cells in division and available as potential target cells for viral replication.

Results from these experiments indicated that the antibody response in anti-IgM-treated mink kits was severely repressed and that this lack of an antiviral antibody response correlated with high levels of ADV mRNA in the lung. This high level of ADV mRNA resulted, probably because of high levels of translation products, in acute cytopathology in the form of intranuclear inclusion bodies in alveolar type II cells; however, severe respiratory distress and hyaline membrane formation in affected lungs were not consistently observed. It is suggested that the antiviral antibody response observed in mink older than approximately 14 days restricts ADV mRNA levels which, if marked enough, results in restricted levels of DNA replication and virion production. Such a restricted ADV infection at low levels does not cause acute cytopathology but paves the way for a persistent infection leading to immunologically mediated disease. The potential mechanisms of antibody-mediated restriction of viral mRNA levels and mechanisms of disease induction are discussed.

MATERIALS AND METHODS

Virus. In vivo-passaged ADV-United (27), obtained from United Animal Division (Middleton, Wis.), was passaged once in newborn ADV antibody-negative mink kits. An inoculum was prepared from the lungs, spleens, and livers and adjusted to contain 10^7 adult mink 50% infective doses per ml. The preparation and use of this inoculum in mink kit studies have been described previously (8, 11, 14).

Antiserum production and characterization. Rabbit antibody to mink IgM was prepared as described previously (61). The antiserum was rendered mu chain specific by absorption with a mink IgG-bovine serum albumin copolymer (17) until no reaction with the light chains of mink IgG could be demonstrated by immunoelectrophoresis or double diffusion in agar (61). The IgG fraction of this rabbit antiserum was prepared by three precipitations at half saturation with $(NH_4)_2SO_4$, dialysis of the precipitate against 0.0175 M sodium phosphate buffer (pH 7.0), and application and elution of the protein on a column of DE-52 cellulose (Whatman), using the same buffer. Normal rabbit IgG was prepared in a similar fashion. The final products were shown to contain only rabbit IgG; the anti-IgM preparation was monospecific for mink IgM by immunoelectrophoresis and double diffusion in agar as described previously (61). The products were sterilized by passage through a 0.22-μm-pore-size filter (Millipore) and used at a concentration of 10 mg/ml.

Animals. The 25 bred female mink used in this study were standard dark mink (non-Aleutian, genotype A/A) and were housed in commercial mink cages and fed a standard mink diet. The mink were obtained from a farm known to be free of ADV infection for at least the last 3 years. Sera from these mink were negative for anti-ADV antibodies when tested by countercurrent immunoelectrophoresis (28) and rocket line immunoelectrophoresis (12). Kits born to these dams were kept as family groups with their dams until approximately 2 months old and then placed in pairs. Litters of kits were injected intraperitoneally within 16 h after birth with 1 mg of the anti-IgM preparation (anti-IgM group) or with 1 mg of normal rabbit IgG (normal Ig group). This treatment was repeated three times a week for 30 to 34 days. At 21 days after birth, kits were inoculated intraperitoneally with 0.1 ml of virus inoculum (virus-infected group) or with phosphate-buffered saline (uninfected control group). No distinction was made between female and male kits. Kits were observed clinically for signs of respiratory disease every second day. At 30, 34, 50, and 221 days of age (postinfection days [PID] 9, 13, 29, and 200, respectively), randomly selected groups of two to five mink kits were exsanguinated under pentobarbital anesthesia, and serum and tissue samples were collected immediately for analyses.

Electrophoresis. Countercurrent immunoelectrophoresis and rocket line immunoelectrophoresis for detection of antibodies to ADV were performed as described previously (12). Serum IgG and IgM were determined by using quantitative thin-layer rocket immunoelectrophoresis as described previously (7, 13). Serum gamma globulin was quantitated by densitometric scanning of electrophoresed sera on agarose gels stained with amido black as described previously (7).

Southern blot hybridization. Serum samples (200 μl) diluted 1:1 in TNE (0.15 M NaCl, 0.01 M Tris [pH 7.4], 0.001 M EDTA) were supplied with 1 μg of sheared salmon sperm DNA as carrier. DNA was then isolated from these samples and from tissue samples by the sodium dodecyl sulfate (SDS)-proteinase K digestion method described previously (8, 22, 23). One-microgram samples of tissue total DNA or samples corresponding to 100 μl of serum were analyzed by agarose gel electrophoresis and Southern blot hybridization as previously described (8, 11, 23). The number of copies of ADV DNA per cell or per milliliter of serum was estimated from known standards included in each blot as described previously (8, 11, 22, 23).

Northern (RNA) blot hybridization. Total RNA was extracted from lung and mesenteric lymph node by the guanidine thiocyanate method followed by centrifugation through a cesium chloride cushion as described previously (9) or by an acid phenol extraction procedure using a reagent kit from Promega (Madison, Wis.). Northern blot analysis after electrophoresis of total RNA samples in Formalin-containing agarose gels and blotting onto nylon filters was done as described previously (9).

Preparation of probes. Southern and Northern blot hybridization was performed by using strand-specific RNA probes radiolabeled with [^{32}P]UTP as previously detailed (23), with a reagent kit obtained from Promega. For in situ hybridization, the probes were radiolabeled with [^{35}S]UTP as previously described (10, 11, 14). The ^{35}S-labeled RNAs had a specific activity of approximately 5×10^8 cpm/μg, and the ^{32}P-labeled RNAs had a specific activity of approximately 2×10^8 cpm/μg. Control probes labeled with [^{35}S]UTP were prepared for the in situ hybridizations by using an irrelevant template included in the kit or a probe for the canine parvovirus as described previously (10, 63). When infected mink tissues were hybridized in situ with the control probes, no grain count over background was observed, indicating specificity of the ADV probes. Probes were kept at −80°C for up to 3 weeks before use.

Light microscopy and in situ hybridization. Transversely cut segments of the middle part of the cranial and caudal lung lobes and pieces of liver, spleen, kidney, heart, mesenteric lymph node, and gut (distal part of ileum) from sacrificed kits and from a single kit that died at day 44 (PID 23) were fixed in periodate-lysine-paraformaldehyde-glutaraldehyde (PLPG) fixative and processed for light microscopy and in situ hybridization as described previously (5, 10, 11, 14). Samples of the

same tissues were snap-frozen at −80°C and stored at this temperature until used for DNA and RNA analyses. This sampling technique has given highly reproducible morphological and quantitative results in several previous studies (5, 8, 10, 11, 14, 15). The in situ hybridizations were performed as previously described (10, 11, 14). ADV-infected Crandell feline kidney cells (72 h postinfection) with a known number of ADV genomes per positive cell were included in the hybridizations and served as an internal standard against which genome number could be calculated (10, 11, 14).

Flow cytometry. Lymphocytes were isolated from heparinized peripheral blood samples by the Ficoll-Paque (Nygaard, Oslo, Norway) density gradient centrifugation method as described previously (1, 2). Briefly, blood was diluted twofold with RPMI medium, carefully layered onto the Ficoll-Paque medium, and centrifuged for 45 min at 400 × *g*. The cells in the interphase were harvested and washed three times. These cells proved to be approximately 95% lymphocytes with a 5% monocyte contamination (1). Alternatively, total leukocytes were prepared from heparinized blood by the erythrocyte lysing procedure as described previously (1). Single-cell suspensions were obtained from spleens or mesenteric lymph nodes by passage through a stainless steel screen followed by dispersion in RPMI by pipetting. Cell debris was allowed to settle for 5 min before transfer of the cell suspension to a new tube.

Lymphocyte, monocyte, and granulocyte quantitation was done by using forward and side scatter diagrams prepared by a FACScan flow cytometer (Becton Dickinson) as described previously (1). Staining for surface Ig-positive cells (B lymphocytes) was done with rabbit antibodies to mink Igs, and staining for the CD8 surface marker was done with a mouse monoclonal antibody to human CD8 (T8 lymphocytes) as described previously (1). Calculation of percentage positive B lymphocytes or CD8-positive lymphocytes was done as described previously (1).

RESULTS

Clinical signs of respiratory disease. Newborn mink kits were injected three times a week with 1 mg of either normal rabbit IgG or anti-IgM and infected with ADV at 21 days of age. The group treated with anti-IgM antibodies and infected with ADV was the only group in which kits exhibited clinical signs of respiratory distress. In 5 of the 21 kits in this group, clinical signs were observed between days 34 (PID 13) and 44 (PID 23). Affected kits showed abdominal respiration and grunting in a period of 3 to 5 days and then recovered except for one kit that died at day 44 (PID 23).

Histopathology. No significant lesions were found in control uninfected mink. In ADV-infected mink kits sacrificed on PID 9 and 13, several viral inclusions (not shown) were observed in the lung of anti-IgM-treated kits; in contrast, no viral inclusions were observed in normal Ig-treated kits. Normal Ig-treated kits had many follicles in the lymph node cortex and in spleen; in contrast, no follicles were evident in anti-IgM-treated kits. The total lack of follicles in the cortex of mesenteric lymph nodes from the anti-IgM-treated kits can easily be observed in the in situ hybridization figures (see below). No significant lesions were found in any of the other organs examined. Severe interstitial pneumonia with hyaline membrane formation typical of ADV-induced pneumonia in newborn kits (5, 8) was observed only in the single ADV-infected, anti-IgM-treated mink kit that died at PID 23.

At PID 29, no signs of acute lesions were found in the kits examined. Infiltrations with mononuclear cells, including plasma cells, were found in most organs, including the lung. There was no apparent difference between normal Ig- and anti-IgM-treated kits.

TABLE 1. Determination of serum antibody titers against ADV in experimentally infected mink[a]

Treatment	Serum anti-ADV titer		
	Day 30 (PID 9)	Day 34 (PID 13)	Day 50 (PID 29)
Normal Ig	20	40	160
Anti-IgM	0	0	160
Dams[b]	0	0	20

[a] Serum anti-ADV antibody titers were determined by rocket line immunoelectrophoresis. In all cases, titers were 0 on day 21 (PID 0) and 640 (highest dilution tested) on day 221 (PID 200). Three mink kits were examined in each group at each of the indicated time points.

[b] The dams were not inoculated with ADV but became infected from their kits.

At PID 200, lesions of classical Aleutian disease, including glomerulonephritis and infiltrations in most organs with mononuclear cells, predominantly plasma cells, were found in all mink examined. There was no apparent difference between normal Ig- and anti-IgM-treated kits. No interstitial fibrosis or other residual lesions as evidence of previous severe acute pneumonia were found in any kit examined (data not shown).

Serology. Serum samples from mink kits and from their dams were analyzed for anti-ADV antibodies by using the highly sensitive and specific rocket line immunoelectrophoresis assay. The results are presented in Table 1. Anti-IgM-treated kits were negative for anti-ADV antibodies at PID 9 and 13, at which time normal Ig-treated kits had relatively high titers. At 29 days after infection with ADV, i.e., 16 to 20 days after the end of anti-IgM treatment, anti-IgM-treated kits and normal Ig-treated kits had equivalent, and high, titers.

Anti-IgM-treated kits had undetectable levels (i.e., less than 3 μg of IgM per ml of serum) of circulating free mink IgM when tested at 17, 30, and 34 days of age. Normal Ig-treated kits had circulating IgM levels of between 7 and 35 μg/ml at this age, ADV-infected kits having the highest levels. At 50 days after birth, mink kits had concentrations of 35 to 150 μg of IgM per ml of serum regardless of initial treatment. The levels of total gamma globulin were around 5 to 10% of total serum protein and were not significantly different among the groups until 50 days of age, when ADV-infected kits had up to 20% gamma globulin regardless of initial treatment. Levels of circulating mink IgG (2 to 5 mg/ml) were also not significantly different among groups except at 50 days of age, when ADV-infected kits had up to 20 mg of IgG per ml of serum. At 200 days after birth, all surviving ADV-infected mink had high anti-ADV antibody titers (titer higher than 640) and elevated levels of gamma globulin.

These results showed the efficacy of the immunosuppression due to the anti-IgM treatment. The treatment totally suppressed the production of detectable levels of anti-ADV antibodies or any detectable antibodies of IgM class. Moreover, the data also showed that the maternally transferred levels of IgG or total gamma globulin were not significantly altered and that the mink recovered after immunosuppression, i.e., had a normal antibody response to ADV at 16 to 20 days after the end of treatment.

Flow cytometry. The percentage of B cells and CD8-positive T cells were determined in mink tissues by fluorescence-based flow cytometry. The percentage of B cells was severely decreased in anti-IgM-treated kits at days 30 and 34 after birth, with very few surface Ig-positive B cells in blood and spleen

TABLE 2. Percentage of B cells and CD8-positive T cells in mink kits[a]

Treatment	% B cells			% CD8-positive T cells		
	Blood	MLN	Spleen	Blood	MLN	Spleen
Normal Ig	18.6 ± 7.3 (5)	25.8 ± 7.2 (4)	13.7 ± 0.6 (3)	7.0 ± 2.1 (5)	12.5 ± 2.4 (4)	2.7 ± 0.6 (3)
Anti-IgM	1.0 ± 0.8 (10)	6.0 ± 2.4 (5)	0.3 ± 0.5 (7)	10.7 ± 5.0 (10)	16.4 ± 1.9 (5)	11.7 ± 8.8 (6)

[a] B cells and CD8-positive T cells in blood, mesenteric lymph node (MLN), and spleen were determined by flow cytometry as described in Materials and Methods. The data for mink kits sacrificed at PID 9 and 13 were combined. Data are shown as mean value ± standard deviation; the number of individual samples is indicated in parentheses.

and a few B cells persisting in the mesenteric lymph node samples (Table 2). At 50 days after birth (16 to 20 days after the end of anti-IgM treatment), there was no significant difference in the percentage of B cells between the two groups (only two animals in each group; data not shown).

The percentage of CD8-positive cells was slightly higher in the anti-IgM-treated kits (Table 2). As described previously for ADV-infected adult mink (1), the number of CD8-positive cells increased slightly more in infected than in uninfected kits (data not shown).

In situ hybridization on infected mink tissues. Sections of lung, mesenteric lymph node, and liver from mink kits sacrificed at PID 9 and 13 were hybridized in situ, using strand-specific ADV RNA probes labeled with ^{35}S. Sections were not pretreated with RNase; therefore, grains over cells with the minus-sense probe represented viral replicative-form (RF) DNA and viral mRNA suggestive of viral replication in the cell, while grains with the plus-sense probe primarily represented the mere presence of virion DNA either being produced in the cell or sequestered at the site.

Sections from control mink consistently gave low levels of grains diffusely distributed over the whole slide and established the background level (not shown). Infected Crandell feline kidney cells (72 h postinfection) with a known amount of ADV genomes per positive cell were included in each hybridization and served as an internal standard against which copy numbers could be calculated (10, 11, 14).

Evidence of high levels of viral replication and sequestration was found in several tissues. The results for PID 9 and 13 samples were comparable and will be described together. Hybridization with the plus-sense probe on sections of mesenteric lymph node from normal Ig-treated mink kits sacrificed at 9 or 13 days after ADV infection showed the presence of high levels of ADV virion DNA concentrated to the follicles of the cortex and to the medullary sinuses (Fig. 1A). Similar hybridization on mesenteric lymph node sections from infected mink kits treated with antibodies to mink IgM showed the presence of virion DNA in a few scattered single cells of the cortex in which no lymph node follicles were evident. High levels of virion DNA were also found in the medullary sinuses of the lymph nodes in these kits (Fig. 1B).

Hybridization with the minus-sense probe showed low levels of ADV replication in cells in lymph node follicles of normal Ig-treated kits (Fig. 2A), similar to results described for adult ADV-infected mink (10). In contrast, only a few scattered positive single cells were found in the lymph node cortex of anti-IgM-treated kits; however, these few cells replicated ADV at high levels (Fig. 2B).

Lung sections from both normal Ig- and anti-IgM-treated kits at PID 9 or 13 probed with the plus-sense probe showed the presence of high levels of virion DNA or RF DNA in cells with a localization consistent with alveolar type II cells and alveolar macrophages (Fig. 3A and B). In contrast, a strong signal with the minus-sense probe was found only in alveolar type II cells in the anti-IgM-treated kits, indicating a high level of ADV mRNA and/or RF DNA in these cells (Fig. 3C and D and Fig. 4). The quantitative results from the in situ hybridization experiments on lung tissue are summarized together with quantitative Southern and Northern blot data (see below) in Table 3.

In the livers, of both normal Ig- and anti-IgM-treated kits, high levels of virion DNA were detected in cells resembling Kupffer cells (data not shown).

Southern blot hybridization. DNA was extracted from mink kit tissues and from serum by the SDS-proteinase K method. In both anti-IgM- and normal Ig-treated kits, the presence of ADV double-stranded RF and single-stranded ADV virion DNA was evident at high levels in lung tissue on days 30 and 34 (PID 9 and 13) (Fig. 5) and at low levels at day 50. RF DNA and virion DNA could also be detected in spleen and mesenteric lymph node at day 30 (PID 9). In these tissues, the levels of virion DNA were much higher than the levels of RF DNA (Fig. 6A); however, by using the minus-sense probe, the presence of RF DNA in the samples could be convincingly demonstrated (Fig. 6B). The mere presence of high levels of virion DNA could be demonstrated in the liver samples (Fig. 7). Liver was negative for RF DNA when the minus-sense probe was used (data not shown). Interestingly, there was no indication of significantly higher levels of RF or virion DNA in the anti-IgM-treated kits compared with the normal Ig-treated kits in any organ examined. The peak levels of ADV RF and virion DNA were found in the lung samples at PID 9 and 13 and corresponded to approximately 500 copies of RF DNA and 500 copies of virion DNA per total cell (Table 3).

Only a single mink kit died after having shown severe clinical signs of respiratory distress. This mink was treated with anti-IgM and infected with ADV and died 44 days after birth (PID 23). High levels of ADV RF and virion DNA were detected in the lung and virion DNA in the liver of this mink (data not shown).

Interestingly, on days 30 and 34 (PID 9 and 13), high levels of virion DNA could be detected in sera of anti-IgM-treated kits, but only relatively low levels were found in kits treated with normal Ig. The amount of virion DNA in sera from mink treated with anti-IgM was approximately 4×10^9 to 2×10^{10} viral genomes per ml. In contrast, the amount of virion DNA in sera from mink treated with normal Ig was about 20- to 500-fold lower (Table 4). On day 50 (PID 29), no serum viremia could be detected by this method (fewer than 4×10^6 genomes per ml).

Northern blot hybridization. Northern blot hybridization using the minus-sense probe showed the presence of large amounts of ADV mRNA in lung samples from three of four ADV-infected kits treated with anti-IgM and sacrificed at PID 9 or 13 (Fig. 8). In contrast, none of four ADV-infected kits treated with normal Ig had detectable levels of ADV mRNA. The levels of ADV mRNA in the anti-IgM-treated kits corresponded to approximately 2,000 copies per total cell (Table 3)

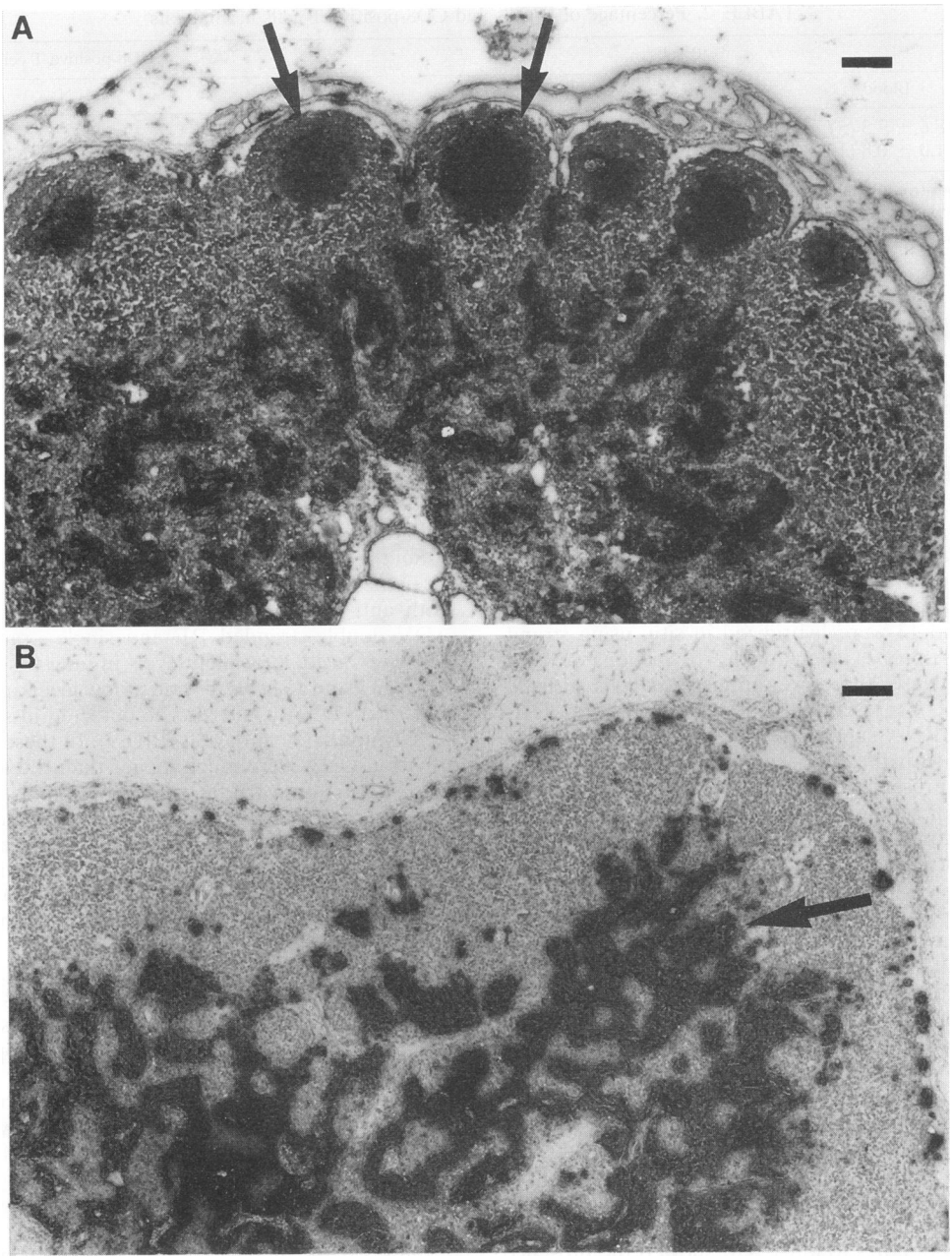

FIG. 1. In situ hybridization analysis using the plus-sense ^{35}S-labeled RNA probe for virion DNA on PLPG-fixed, paraffin-embedded sections of mesenteric lymph nodes from mink kits sacrificed 13 days after ADV infection. (A) Mesenteric lymph node from a normal Ig-treated mink kit. Heavy grain formation indicates the presence of high levels of ADV virion DNA concentrated to the follicles of the cortex (arrows) and in the medullary sinuses. (B) Hybridization similar to that in panel A but on a mesenteric lymph node section from a mink kit treated with anti-IgM. In this section, virion DNA is seen in scattered single cells of the cortex in which no follicles are seen and in the medullary sinuses (arrow). Bars, 150 μm.

and consisted of strong bands at 2.8 and 0.85 kb and a weak band at 4.3 kb as described previously for ADV mRNA from infected cell cultures (9).

DISCUSSION

We have previously shown that the kinetics of ADV DNA amplification and mRNA accumulation are slow compared with kinetics of the more prototypic parvovirus mink enteritis virus (10, 11, 14, 63), a mink parvovirus closely related to the canine and feline parvoviruses (49–54). Recent data from our laboratory suggested that the slow kinetics of ADV may be related to weak promoters for viral transcription (29, 62). ADV replication and virus sequestration reach maximal levels at 9 to 14 days after infection of both neonatal and adult mink (10, 11, 14). However, the levels of ADV replication and levels of ADV mRNA are much lower in adult mink than in newborn mink kits, and we have previously hypothesized that the decreased

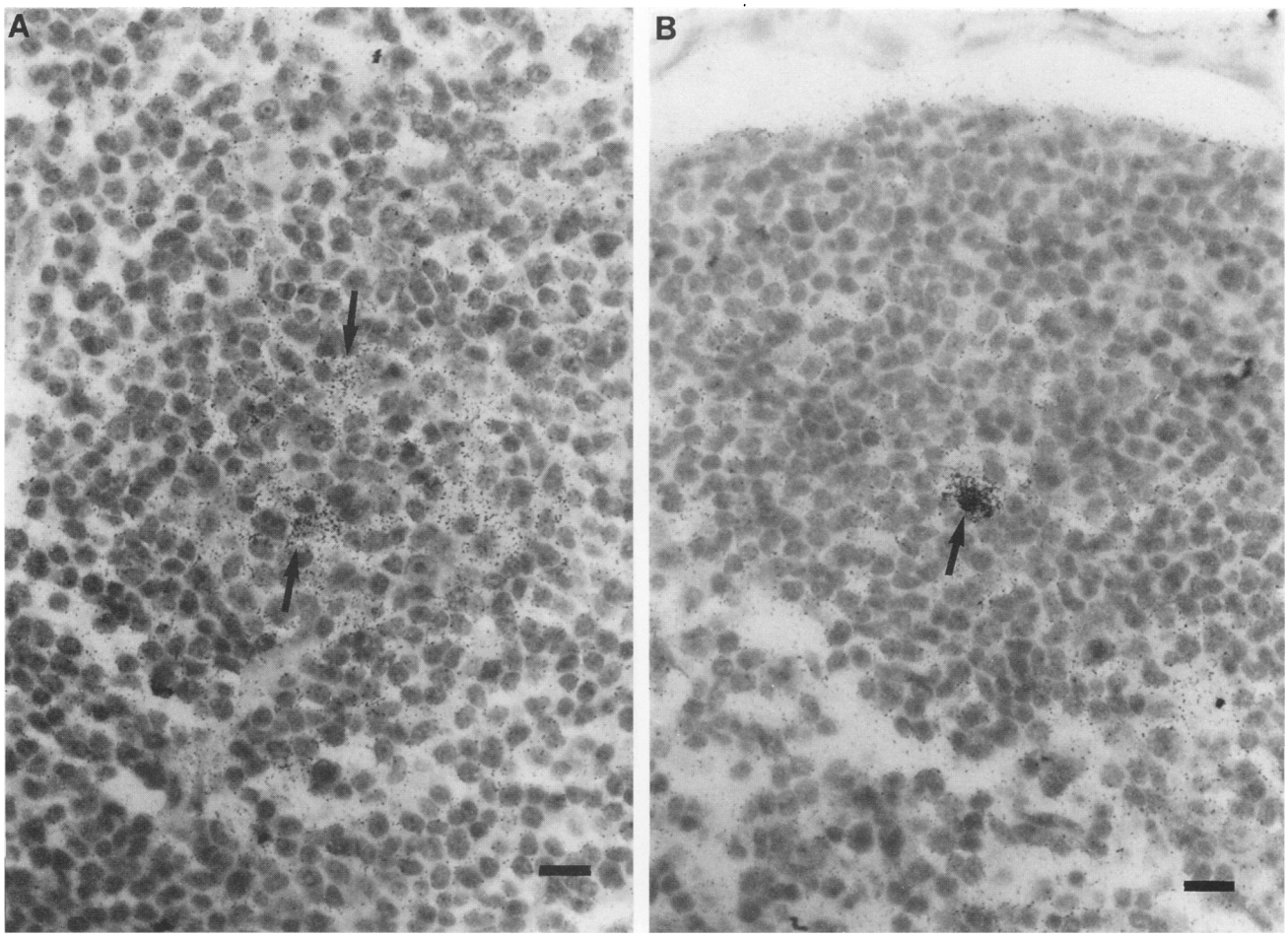

FIG. 2. In situ hybridization analysis using the minus-sense ^{35}S-labeled RNA probe for ADV mRNA and RF DNA on PLPG-fixed, paraffin-embedded sections of mesenteric lymph nodes from mink kits sacrificed 13 days after ADV infection. (A) Mesenteric lymph node from a normal Ig-treated mink kit. Light grain formation is seen over cells in the middle of a germinal center (arrows) and indicates a low level of ADV replication in these cells. (B) Hybridization similar to that in panel A but on a mesenteric lymph node section from a mink kit treated with anti-IgM. High-level ADV replication is seen in a single cell in the cortex (arrow). Bars, 15 μm.

level of ADV replication seen in adult mink is due to a fast antibody response (10, 14).

In this study, the level and cellular localization of virus replication and sequestration during experimental ADV infection of immunosuppressed mink were examined by nucleic acid hybridization techniques. The data were correlated to the findings obtained by using conventional serological and histopathological methods in order to obtain a detailed picture of the pathogenesis of ADV-related disease. Mink kits were treated with antibodies against mink IgM, and the efficacy of the anti-IgM treatment was verified by the severe suppression of B-cell development observed by flow cytometric analysis and by the lack of antiviral antibodies. This is in itself a promising result for further studies because before this study, very little was known about the immunosuppressive effect of anti-IgM antibodies in animals other than mice (24–26).

Several interesting findings emerged from the studies. First, it appears that antiviral antibodies decrease ADV mRNA levels more than DNA replication. Second, suppression of B-cell development and antibody response resulted in high levels of ADV replication in mink kits infected at 21 days of age; moreover, the ADV infection in such kits caused production of viral inclusion bodies in alveolar type II cells. Some of these kits showed mild clinical signs of respiratory disease, and one kit died of respiratory distress; however, clinical signs were seen only after release of immunosuppression, suggesting that the production of antiviral antibodies, in combination with the massive amounts of free viral antigen present, somehow is involved in the induction of respiratory distress.

In our previous work using in situ hybridization analysis of tissue sections from mink infected with ADV (10, 11, 14), calculations were made of the amount of specific virion DNA, RF DNA, and mRNA present in infected cells. The amount of ADV RF DNA and mRNA in mesenteric lymph node cells at the peak of infection of adult mink (PID 10) with ADV reached less than 10,000 copies per infected cell, while alveolar type II cells from mink kits infected as newborns with ADV contained up to 80,000 copies of RF DNA and about 400,000 copies of mRNA per infected cell (6, 10, 11). The results presented here suggested that for ADV infection in young (3 weeks old) immunosuppressed mink kits, each infected alveolar type II cell contains approximately 50,000 copies of ADV RF DNA and 200,000 copies of mRNA, i.e., a level resembling the amount present in the permissive ADV-infected newborn

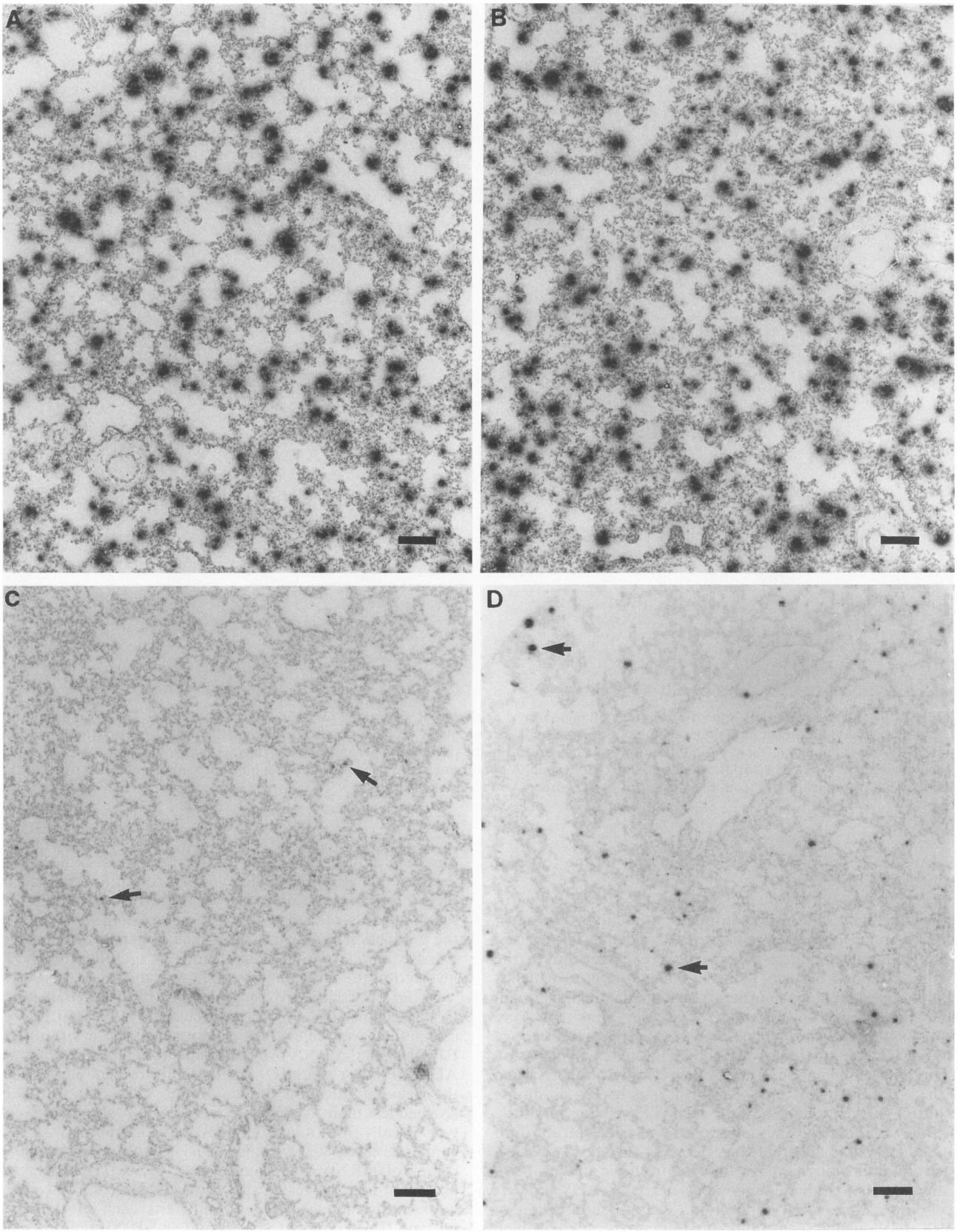

FIG. 3. In situ hybridization analysis using the strand-specific ^{35}S-labeled RNA probes on PLPG-fixed, paraffin-embedded lung sections from mink kits sacrificed 13 days after ADV infection. (A) Lung section from a normal Ig-treated mink kit probed with the plus-sense probe. Heavy

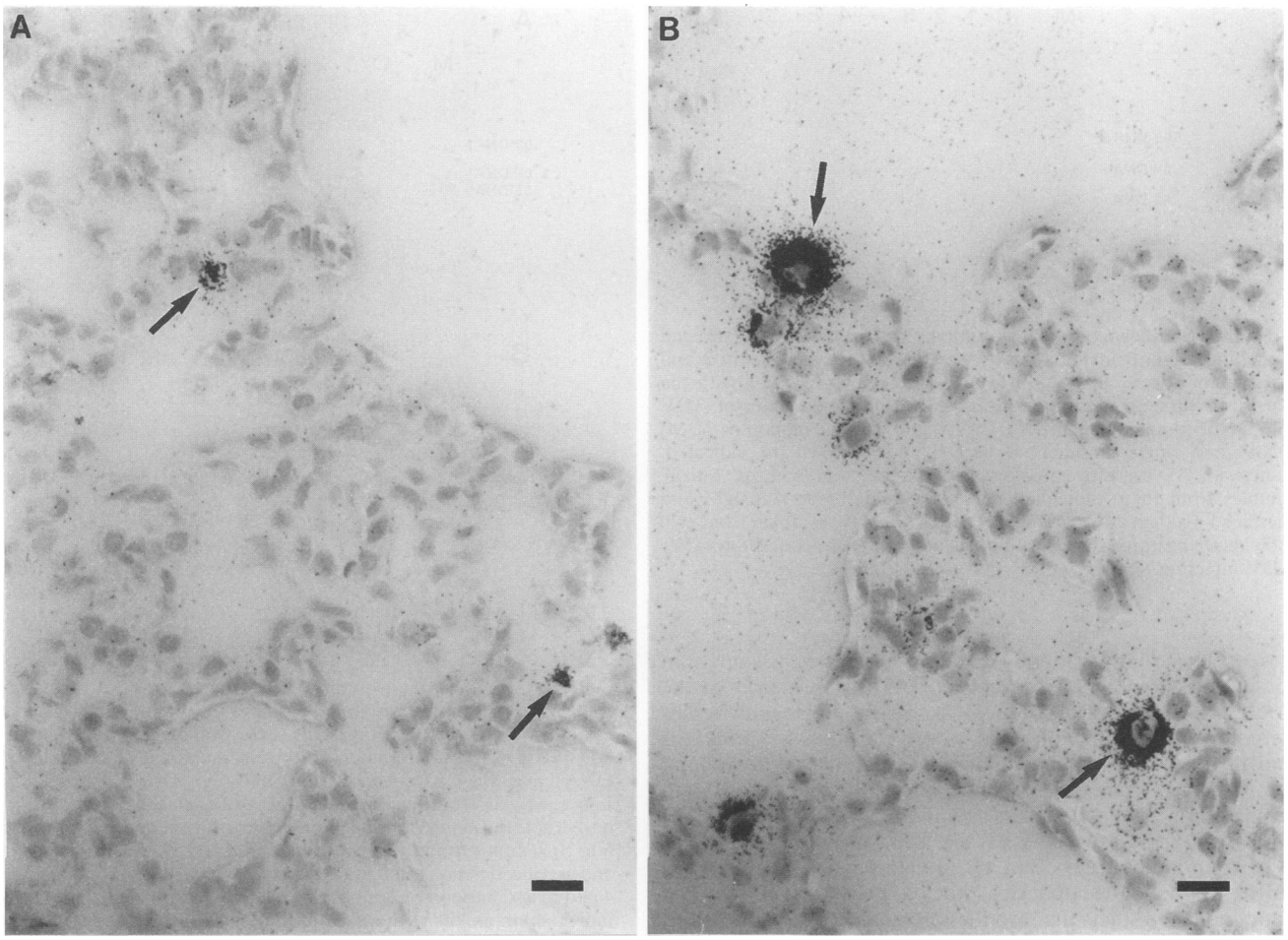

FIG. 4. In situ hybridization analysis on the same sections as in Fig. 3 but at a higher magnification. (A) Lung section from a normal Ig-treated mink kit probed with the minus-sense probe. Only light grain formation is seen over positive cells (arrows), indicating a low level of ADV mRNA and/or RF DNA in cells with a localization consistent with alveolar type II cells. (B) Lung section from a mink kit treated with anti-IgM and probed with the minus-sense probe. Heavy grain formation is seen especially over the cytoplasm of cells (arrows), indicating high levels of ADV mRNA and/or RF DNA in cells with a localization consistent with alveolar type II cells. Bars, 15 μm.

mink lung or in mink enteritis virus-infected adult mink (63). Although the anti-IgM-treated kits infected with ADV at 21 days of age had almost as high levels of viral DNA replication and mRNA levels as did kits infected as newborns and also contained nuclear inclusion bodies in the alveolar type II cells, they did not develop clinical signs of respiratory distress as severe as those found in kits infected as newborns. There could be several explanations for this. Older kits may not be as susceptible as newborn kits to a reduction or change in alveolar surfactant; alternatively, older kits may have a more efficient production and regulation of surfactant synthesis.

No acute lesions were observed in the normal Ig-treated kits infected with ADV at 21 days of age. The only differences in viral parameters between these kits and the kits treated with anti-IgM were the decreased levels of ADV mRNA in the alveolar type II cells and a decrease in viremia. Apparently the levels of ADV DNA amplification were the same in both normal Ig- and anti-IgM-treated kits (Table 3). Thus, it appears that the antiviral antibody response in the normal Ig-treated kits selectively decreases ADV mRNA levels but not DNA replication and furthermore that the decreased levels of mRNA together with the marked decrease in viremia brings the ADV infection below a certain threshold for induction of acute cytopathology and clinical disease. Our previous experiments with passive antibody treatment of ADV-infected newborn mink indicated that this treatment decreased intracellular levels of both ADV DNA replication and mRNA levels (14) similarly to what is observed in adult ADV-infected mink with

grain formation indicates the presence of high levels of virion DNA in cells with a localization consistent with alveolar type II cells and alveolar macrophages. (B) Lung section from a mink kit treated with anti-IgM. The section was probed with the plus-sense probe and shows the presence of high levels of virion DNA in cells with a localization consistent with alveolar type II cells and alveolar macrophages. (C) Lung section as in panel A but probed with the minus-sense probe. Only light grain formation is seen over cells (arrows), indicating a low level of ADV mRNA and/or RF DNA. (D) Lung section as in panel B but probed with the minus-sense probe. Heavy grain formation is seen over many cells (arrows), indicating high levels of ADV mRNA and/or RF DNA. Bars, 150 μm.

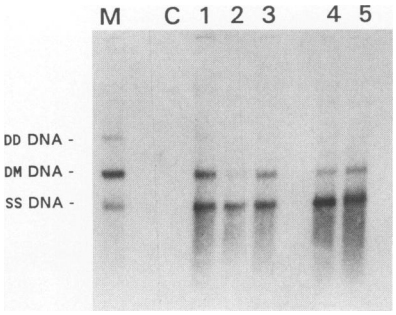

FIG. 5. Southern blot hybridization. Total DNA was extracted from lung tissues from mink kits by the SDS-proteinase K method, and 1-μg samples were analyzed by Southern blot hybridization with the plus-sense probe as described in the text. Relative positions of ADV RF DNA (duplex dimer [DD] DNA and duplex monomer [DM] DNA) and single-stranded virion DNA (SS DNA) are indicated. Lanes: M, ADV-positive marker; C, ADV-negative control; 1 to 3, samples from three individual mink kits treated with anti-IgM and sacrificed 13 days after ADV infection; 4 and 5, samples from two individual mink kits treated with normal Ig and sacrificed 13 days after ADV infection.

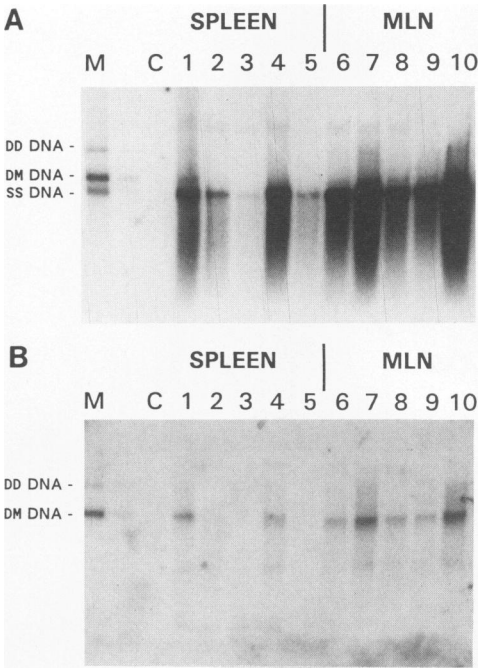

FIG. 6. Southern blot hybridization of total DNA extracted from spleen and mesenteric lymph node tissues from mink kits. (A) Southern blot hybridization with the plus-sense probe. Relative positions of ADV RF DNA (duplex dimer [DD] DNA and duplex monomer [DM] DNA) and single-stranded virion DNA (SS DNA) are indicated. Lanes: M, ADV-positive marker; C, ADV-negative control; 1 to 3 and 6 to 8, spleen (lanes 1 to 3) and mesenteric lymph node (MLN) (lanes 6 to 8) samples from three individual mink kits treated with anti-IgM and sacrificed 9 days after ADV infection; 4, 5, 9, and 10, spleen (lanes 4 and 5) and mesenteric lymph node (lanes 9 and 10) samples from two individual mink kits treated with normal Ig and sacrificed 9 days after ADV infection. (B) The probe was eluted from the filter by alkaline treatment, and the filter was hybridized with the minus-sense probe.

a vigorous antiviral antibody response (10). If we assume that the antibody-mediated restriction of ADV is primarily at the mRNA level, we can propose a model for acute versus persistent ADV infection. In newborn seronegative mink kits and in older kits immunosuppressed by anti-IgM treatment, the virus will have time, before an antibody response, to replicate for 9 to 14 days, slowly building up high levels of RF and virion DNA as well as mRNA levels in the infected cells. When antibodies are present, the situation is entirely different. In the normal Ig-treated kits infected at 21 days of age, the data indicated that the production of antiviral antibodies, albeit at relatively low levels, suppressed ADV mRNA levels, while DNA replication and single-strand formation were unaffected. When the suppression of ADV mRNA is even more marked, as observed in kits treated passively with antibodies or in adult mink with a fast antibody response, our previous results indicate that DNA replication is also decreased (6, 10, 14), most likely because of a lack of ADV translation products such as the large nonstructural protein shown to be absolutely required for parvovirus DNA replication (19, 30).

The exact mechanism by which the antiviral antibodies restrict ADV mRNA levels at the single-cell level is currently not well understood. On the basis of the finding that the efficient transcription and replication of the minute virus of mice parvovirus requires a specific amino acid motif on the incoming viral capsid (18, 34), we previously suggested that the antibodies may mask or change an important motif in the incoming viral capsid (14). That antibodies can bind to virions and induce conformational changes that ultimately, without affecting viral attachment and penetration, result in restricted viral replication and transcription has been shown for other viruses (31, 37, 46), and antibody-mediated restriction of viral transcription has been implicated in persistent measles virus infection (33, 38, 44). If we assume that multiple rounds of DNA amplification, virion production, and reinfection lead to the final high levels of DNA replication and transcription seen in antibody-free young mink, a restrictive function of antibodies is easily envisioned. In this scenario, infected cells in antibody-free animals at PID 9 to 14 could be infected at extremely high input doses from the second or third round of virus replication, while in contrast, older animals or kits treated with antiviral antibodies have a markedly decreased viremia (10, 14) due to antibodies already present after the first round of replication. Thus, incoming virions in the second or third round of replication will be reduced in number and furthermore are likely to be bound to antiviral antibodies found in

TABLE 3. Hybridization analyses of lung tissue from mink kits[a]

Treatment	No. of genomes/ positive cell[b]	No. of genomes/ total no. of cells[b]
Normal Ig	50,000 RF	500 RF
	50,000 virion	500 virion
	<10,000 mRNA	<100 mRNA
Anti-IgM	50,000 RF	500 RF
	50,000 virion	500 virion
	200,000 mRNA	2,000 mRNA

[a] Mink were inoculated with an ADV inoculum, and two mink kits from each group were sacrificed at PID 13. Sections of lung were processed for in situ hybridization analysis, and frozen lung tissue was processed for Southern and Northern blot analysis as described in Materials and Methods. The proportion of positive cells (1% in each case) was determined by in situ hybridization. The number of positive cells and number of grains over each positive cell were recorded for an area containing 20 positive cells or at least 10,000 cells in total.

[b] Estimated from in situ hybridization and Southern and Northern blot hybridization analyses by comparison with standards of ADV-infected Crandell feline kidney cells as described previously (8, 10, 11, 14, 22, 23).

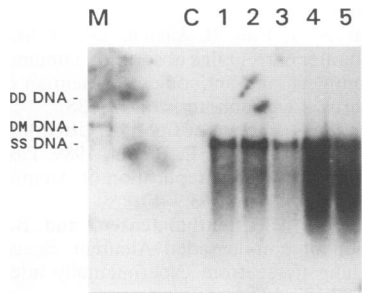

FIG. 7. Southern blot hybridization on total DNA extracted from liver samples. Total DNA samples (1 μg) were analyzed by Southern blot hybridization with the plus-sense probe. Relative positions of ADV RF DNA (duplex dimer [DD] DNA and duplex monomer [DM] DNA) and single-stranded virion DNA (SS DNA) are indicated. Lanes: M, ADV-positive marker; C, ADV-negative control; 1 to 3, samples from three individual mink kits treated with anti-IgM and sacrificed 13 days after ADV infection; 4 and 5, samples from two individual mink kits treated with normal Ig and sacrificed 13 days after ADV infection.

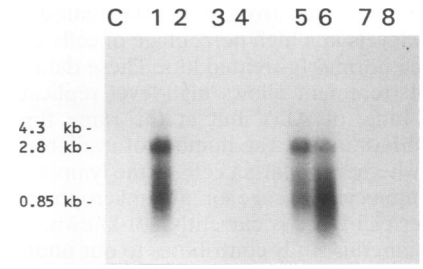

FIG. 8. Northern blot analysis of RNA extracted from lung samples. Total RNA was extracted, and 25-μg samples were analyzed by Northern blot hybridization with the ADV minus-sense probe as described in the text. The relative sizes of the ADV RNAs are indicated at the left. Lanes: C, ADV-negative control; 1 and 2, samples from two individual mink kits treated with anti-IgM and sacrificed 9 days after ADV infection; 3 and 4, samples from two individual mink kits treated with normal Ig and sacrificed 9 days after ADV infection; 5 and 6, samples from two individual mink kits treated with anti-IgM and sacrificed 13 days after ADV infection; 7 and 8, samples from two individual mink kits treated with normal Ig and sacrificed 13 days after ADV infection.

excess in serum (10, 14). Such virions may initiate DNA replication but may be inefficient in initiating transcription. Finally, an indirect effect of the antiviral antibodies in down-regulation of viral mRNA levels is an attractive possibility. The presence of large amounts of virus-antibody complexes in antibody-positive animals may induce production of certain cytokines, such as tumor necrosis factor alpha (TNF-α) and interferons, that theoretically could restrict ADV transcription or, alternatively, could induce rapid degradation of the mRNA. That cytokine signals can regulate cellular and viral mRNA levels has been shown in several instances (32, 35, 41, 45, 48, 55, 66). Moreover, the involvement of cytokines would also explain the fact that the most severe clinical symptoms are observed after the peak of viral expression when low levels of antibodies are produced. In this setting, it is worth mentioning that the cytokine TNF-α has been implicated in the pathogenesis of respiratory distress in humans (47, 64), in experimental immune complex alveolitis, and in adenovirus pneumonia (36, 65). Moreover, TNF-α has been shown to down-regulate mRNAs for surfactant-associated proteins (66). Also, the function of macrophages, known to synthesize TNF-α and interferons, has specifically been shown to be altered by immune complexes in respiratory disease caused by influenza virus (16, 40).

However, how can the scenario described above explain the development of persistent infection and immunologically mediated disease? The low initial replication of ADV results in no or very few cells killed, and the prolonged release of virions coupled to the extreme stability and trypsin resistance of ADV may play a role. Perhaps the lack of acute cell killing and the fact that the majority of virions from the first rounds of replication are released into an excess of antibodies somehow change immune system recognition or initiation (cytotoxic T lymphocytes, large granular lymphocytes, B-cell epitope response, levels of specific cytokines produced, etc.). Preliminary experiments do in fact indicate that the initial immune recognition may play a role in the development of antibodies against conformational or linear epitopes and that excessive levels of antibodies against linear epitopes somehow are coupled to development of chronic immune complex-mediated disease (7a). It is tempting to speculate that antibodies against ADV conformational epitopes play a role in restricting levels of viral replication and spread in early infection. Later the aberrant, excessive production of antibodies against linear epitopes interferes with this function and may be directly involved in immune complex formation and deposition, perhaps by binding to ADV breakdown products. Germane to this notion is the finding that early-infection immune complexes apparently are different from late-infection complexes (10).

TABLE 4. Determination of serum viremia in experimentally infected mink[a]

Treatment	No. of viral genomes/ml of serum		
	Day 30 (PID 9)	Day 34 (PID 13)	Day 50 (PID 29)
Normal Ig	4×10^7–2×10^8	$<4 \times 10^6$	$<4 \times 10^6$
Anti-IgM	4×10^9	2×10^{10}	$<4 \times 10^6$

[a] Serum viremia was determined by Southern blot hybridization as described in Materials and Methods. Three mink kits were examined in each group at each of the indicated time points.

The results on replication of ADV in alveolar type II cells of the lung in anti-IgM- and normal Ig-treated mink kits were rather clear-cut. In contrast, the definition and characterization of target cells in lymph node and spleen was more difficult. Theoretically, the anti-IgM treatment should have knocked out all B cells from the animals and thus, if B cells are the target for ADV replication in lymphoid organs, have abolished ADV replication at these sites. However, although no circulating IgM or antiviral antibodies could be detected in treated mink kits, a few residual B cells could be detected in lymph node samples. No follicles could be observed in such lymph nodes and no circulating antiviral antibodies could be detected, suggesting that residual B cells did not survive long enough to go through class switching and clonal expansion. However, the finding of residual B cells in the lymph nodes of treated kits makes the interpretation of the data more difficult. As determined by Southern blotting, the total levels of DNA amplification and sequestration were similar in anti-IgM- and normal Ig-treated kits. However, determination of ADV mRNA levels in such lymph nodes was rather inconsistent but did not seem to indicate a significant difference in mRNA levels between the two groups (data not shown). Interestingly, in situ hybridization showed high-level replication in a few single cells in the

cortex of lymph nodes from anti-IgM-treated kits and, in contrast, low levels in a high percentage of cells in lymph node follicles of the normal Ig-treated kits. These data indicate that the anti-IgM treatment allows high-level replication, as also seen in the lung, of ADV but at the same time limits, or prevents proliferation of, the number of available target cells. In any case, whether the target cells in the lymphoid tissues are of the B-lymphocyte lineage or are macrophages, dendritic cells, or other cell types is currently not known.

In conclusion, this study contributes to our understanding of the pathogenesis of disease caused by ADV infection in mink, although several lines of possibilities still have to be pursued. Further studies using modulation of the immune response of infected mink paired with careful quantitative analysis of data obtained by using sensitive molecular and conventional techniques are needed.

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