Evidence of Restricted Viral Replication in Adult Mink Infected with Aleutian Disease of Mink Parvovirus

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Strand-specific hybridization probes were used in *in situ* molecular hybridization specifically to localize cells containing replicative intermediates of Aleutian disease of mink parvovirus (ADV). When adult mink of Aleutian genotype were infected with ADV Utah I, the largest number of cells positive for viral replication (i.e., containing replicative-form DNA and RNA) were found in the mesenteric lymph nodes and spleens at 10 days after infection. The localization of positive cells in the middle of germinal centers suggested that they were B lymphoblasts. Circulating leukocytes and bone marrow cells also contained viral RNA, but the levels of replicative-form DNA were below detectability. The levels of viral DNA and RNA in adult mink cells replicating ADV were decreased compared with those in permissively infected cell cultures or neonatal mink, suggesting that the replication of ADV in adult mink might be semipermissive or restricted at some early stage of viral gene expression. The low level of viral replication and transcription in lymphoid cells might provide a mechanism for the development of immune disorders and for the maintenance of persistent infection. Single-stranded virion DNA was found in other organs, but the strand-specific probes made it possible to show that this DNA represented virus sequestration. In addition, glomerular immune complexes containing virion DNA were detected, suggesting that ADV virions, or perhaps free DNA, may have a role in the development of ADV-induced glomerulonephritis.

We have analyzed two completely different patterns of disease caused by infection with the Aleutian disease of mink parvovirus (ADV). In seronegative newborn mink kits, ADV causes an acute interstitial pneumonia accompanied by the formation of hyaline membranes and a fulminant, fatal respiratory distress syndrome (3, 5, 6, 31). In contrast, adult mink do not become acutely ill when infected with ADV, but rather develop a chronic infection associated with a severe disorder of immune regulation. The disease in adult mink, known as classical Aleutian disease (AD), is characterized by marked hypergammaglobulinemia, plasmacytosis, and a fatal immune complex glomerulonephritis (26, 36-38). Although AD has been studied for more than 30 years, the mechanism by which ADV induces this severe disorder of immune function and establishes a persistent infection remains a conundrum.

One approach to studying ADV pathogenesis is to determine cellular sites of viral replication. If a particular cell population can be identified as a target for ADV replication *in vivo*, the function of that cell type may provide insight into how the virus infection produces a given clinical syndrome. For example, in infected mink kits, ADV acutely infects and kills the alveolar type II cells, thus causing a deficiency of pulmonary surfactant, the secretory product of these cells. This leads to an increase in alveolar surface tension and development of hyaline membranes and respiratory distress (3, 5, 6). In this instance, elucidation of the target cell population directly explains the clinical and pathological picture of the disease. In adult mink, on the other hand, the picture is less obvious, because the disease itself results from chronic immune complex formation and deposition.

The highest concentrations of infectious ADV, ADV-specific proteins, and DNA are found in organs of the lymphoreticular system 10 days after infection (14, 16, 23, 36, 41), but the precise identification of target sites for viral replication in adult mink has been hampered for several reasons. First, ADV is exceedingly stable (23) and might well be able to persist as particles in infected animals for a long time. Second, the virus is not effectively neutralized by the massive antiviral antibody response (35, 38), and therefore ADV can exist in infectious immune complexes which might be sequestered in phagocytic cells. Consequently, the demonstration of infectious virus, viral antigens, or viral DNA within an organ cannot be taken as proof that replication is occurring in that site.

We recently described a novel strategy involving strand-specific *in situ* hybridization (SSISH) to define target cells by identifying cells containing specific intermediates of viral replication (6). In the work described in this report we studied adult mink infected with the virulent ADV Utah I strain by using SSISH and extended the studies to include localization and quantitation of ADV-specific RNA. Potential target cells for ADV replication that contained viral replicative forms (RFs) of DNA and RNA were readily detected in mesenteric lymph nodes and spleens, and their distribution and morphology suggested that they were lymphocytes. Viral RNA was also found at a low level in bone marrow cells and circulating leukocytes. However, the level of viral replication and transcription in infected adult mink cells *in vivo* was decreased compared with that in permissive cell cultures, and this decrease might explain the persistent and semipermissive nature of ADV infection in adult mink. In addition, virion strands of DNA, presumably encapsidated within ADV virions, were localized to renal glomeruli and to elements of the phagocytic system, probably representing sequestered immune complexes containing complete virions.

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MATERIALS AND METHODS

Cells and viruses. Cell culture-adapted ADV G (15) was grown at 32°C in feline kidney (CRFK) (21) cells in RPMI 1640 supplemented with 10% fetal calf serum as previously described (15). Cells were infected at a multiplicity of infection of 2.5 and harvested 72 h postinoculation (p.i.). At the time of harvest, monolayers were trypsinized for 10 min at 37°C, made 10% in fetal calf serum, and washed once in phosphate-buffered saline.

A 10% suspension of ADV Utah I (18) was prepared from the spleens of adult sapphire mink and was adjusted to contain 10^7 adult mink 50% infective doses (ID_{50} s) per ml. Inocula prepared in this manner contain virus in immune complexes. To obviate any effects of such immune-complexed ADV in the inoculum, a portion of this inoculum was treated as described previously (1) to remove the antibody. This preparation was also adjusted to contain 10^7 ID_{50} s per ml.

Animals. All mink used in this study were maintained as described previously (3). Sixteen adult female sapphire (Aleutian, genotype a/a) mink were obtained from an ADV-free herd and were negative for circulating anti-ADV antibodies (7, 19).

The mink were divided into three groups as follows. Group 1 contained two mink, which were inoculated intraperitoneally with 1 ml of phosphate-buffered saline and killed 10 days later. Group 2 contained 10 mink, which were inoculated intraperitoneally with 10^7 ID_{50} s of the standard ADV Utah I inoculum. Two randomly selected mink were killed on days 0 (4 h after inoculation), 5, 10, 20, and 60 p.i.

Group 3 contained 4 mink, which were inoculated intraperitoneally with 10^7 ID_{50} s of the inoculum containing antibody-free ADV Utah I prepared from adult mink. Two mink were killed on days 5 and 10 p.i.

Mink were killed by exsanguination under pentobarbital anesthesia. Pieces of lung, liver, spleen, kidney, intestine (proximal jejunum), and mesenteric lymph node were fixed for in situ hybridization as previously described (6). Serum was collected and frozen at -70°C. Blood (1 ml) from each mink was stabilized with EDTA, and the leukocytes were purified from the buffy coat. Bone marrow cells were obtained from the femur by splitting the bone with a pair of forceps and scraping out the marrow with a sterile scalpel. The cells were suspended in sterile phosphate-buffered saline by vortexing.

The presence of typical progressive AD (38) was verified by histopathological examination of tissue sections and development of characteristic anti-ADV antibodies in counter-current electrophoresis (19).

Immunofluorescence. Cell suspensions of leukocytes and bone marrow cells were prepared for immunofluorescence (IFA) by cytocentrifugation of 10^5 cells onto acid-cleaned slides with a Shandon-Southern cytocentrifuge; they were then air dried and fixed in acetone. Frozen 5- to 10- μ m-thick sections of kidney were also placed onto acid-cleaned slides and fixed in acetone. Direct IFA with fluorescein isothiocyanate-conjugated immunoglobulin G from ADV-infected mink (14) was done as previously described (41). Direct IFA was done on kidney sections with fluorescein isothiocyanate-conjugated rabbit anti-mink immunoglobulin as described previously (6).

Quantitation of specific ADV DNA sequences. DNA was extracted from mink sera under nonannealing conditions as previously described (6). Agarose gel electrophoresis and

Southern blot analyses were then done as described previously (5, 16).

Preparation of probes. Southern blot hybridization was performed by using strand-specific RNA probes radiolabeled with [^{32} P]UTP as previously detailed (16), with a kit obtained from Promega Biotec, Madison, Wis. For in situ hybridization, the probes were radiolabeled with [^{35} S]UTP (New England Nuclear Corp., Boston, Mass.) as previously described (6). The ^{35} S-labeled RNAs had a specific activity of approximately 5×10^8 cpm/ μ g, and the ^{32} P-labeled RNAs had a specific activity of approximately 2×10^8 cpm/ μ g. Control probes labeled with [^{35} S]UTP were prepared for the in situ hybridizations by using an irrelevant template included in the kit and a 20- to 95-map-unit fragment of the canine parvovirus genome (kindly provided by Colin Parrish, Cornell University, Ithaca, N.Y.) cloned into the same transcription vector as the ADV probes. When infected cell cultures or infected mink tissues were hybridized in situ with these control probes, no grain count over background was observed, indicating specificity of the ADV probes. Probes could be kept at -70°C for up to 2 weeks before use.

In situ hybridization techniques. The in situ hybridizations were performed as previously described (6) with a single modification making it possible to detect RNA. This modification was simply to omit the RNase step before hybridization and to leave out the RNase T₁ from the posthybridization treatment. This modification made it possible to detect RNA plus DNA, while parallel slides treated with RNase were used to detect DNA alone. Infected CRFK cells (72 p.i.) with a known number of ADV genomes per positive cell were included in each hybridization and served as an internal standard on which the genome number could be calculated (6). The sensitivity of the assay was estimated to be 300 genomes per positive cell (6).

RESULTS

Analysis of ADV RNA in infected-cell cultures. Our previous experiments indicated that RFs of DNA can serve as markers for viral replication within cells and tissues. Another specific intermediate for viral replication would be the presence of viral RNA molecules. All RNAs encoded by the parvoviruses, including ADV (S. Alexandersen and M. E. Bloom, manuscript in preparation), are plus in sense (17) and thus should be detected with the minus-sense probe. Consequently, we investigated the presence and quantity of ADV RNA in permissively infected cell cultures to provide a framework on which to study ADV transcription and replication in the organs of infected adult mink.

When CRFK cells infected with ADV G for 72 h were studied by SSISH for both RNA and RF DNA by using the minus-sense probe, most of the grains detected were found over the cytoplasm of the cells (Fig. 1b), indicating that the majority of the RNA was cytoplasmic. The number of grains was approximately five times that observed in parallel slides treated with RNase (i.e., only the RF signal would be detected) (Fig. 1a) and corresponded to approximately 250,000 RNA copies per infected cell. Furthermore, the number of cells positive for RF DNA and RNA were the same, strongly suggesting that all cells containing RFs of DNA also contained RNA. The number of grains over cells found with the plus-sense probe was not affected by RNase treatment (not shown), indicating that all detectable RNA is plus in sense. These results indicated that SSISH for RNA and RF DNA should be significantly more sensitive than for RF DNA alone.

FIG. 1. In situ hybridization analysis of ADV G-infected CRFK cells. (a) In situ hybridization on periodate-lysine-paraformaldehyde-glutaraldehyde (PLPG)-fixed CRFK cells infected with ADV G for 72 h. The cells were pretreated with RNase and hybridized with the minus-sense probe. At short exposure, only a few grains over the nucleus of an infected cell can be observed (arrow). These grains represent hybridization to RF DNA localized in the nucleus of the infected cell (6). (b) In situ hybridization on cells infected, hybridized with the minus-sense probe, and exposed as in panel a but not pretreated with RNase. Heavy grain production is seen over the cytoplasm of an infected cell (arrow) and represents hybridization to cytoplasmic ADV mRNA. A few grains can be seen over the nucleus and represent hybridization to RF DNA or a small amount of nuclear ADV RNA. Bar, 10 μ m.

FIG. 2. Estimation of the percentage of positive cells and genomes per total cell in mesenteric lymph node by in situ hybridization. In situ hybridization was performed on PLPG-fixed paraffin-embedded tissue as described in Materials and Methods. Cells containing virion DNA were detected by using the plus-sense probe, RF DNA was detected on RNase-treated slides by using the minus-sense probe, and RF DNA plus RNA was detected by using the minus-sense probe on slides not treated with RNase. (a) Percentage of cells positive in mesenteric lymph nodes. (b) The number of genomes per total cell was calculated by multiplying the content of genomes per positive single cells by the percentage of positive cells. The ID₅₀ in the organs was estimated by assuming a virion-to-ID₅₀ ratio of 100 (16) and assuming that 500 mg of tissue corresponds to approximately 5×10^8 cells (14).

In situ hybridization on infected mink tissues. The previous sets of experiments showed that it was possible to identify ADV RNA by SSISH and that probing for RNA might significantly enhance the ability to detect cells replicating ADV. Therefore, we performed SSISH on tissue sections from adult mink infected with ADV Utah I by using the ³⁵S-labeled RNA probes. Sections were probed either with the plus-sense probe to localize probable sites of viral sequestration or with the minus-sense probe to detect RF DNA and RNA. Sections prepared from control mink consistently gave low levels of grains diffusely distributed over the whole slide and established the background level (see Fig. 3a). When tissues from mink killed on day 0 p.i. were examined, no grain counts over background were found, implying that no cells had accumulated enough genomes to be detected by in situ hybridization (i.e., they contained less than 300 genomes).

From day 5 p.i., grains in excess of background were formed over cells in mesenteric lymph nodes and spleens when either of the probes was used. Both the number of grains per cell and the number of positive cells were maxi-

mum at day 10 p.i., had decreased by day 20, and decreased further until day 60 (Fig. 2). The numbers of genomes calculated by this in situ hybridization technique with internal standards are close to earlier published estimates obtained by Southern blot hybridizations (16). Assuming a genome-to-ID₅₀ ratio of 100 (16), the levels of ID₅₀s could be estimated, and the results are shown in Fig. 2b. The percentage of cells positive for ADV-specific DNA or RNA in the different tissues at the various time points is shown in Table 1.

These results suggested that some individual cells in the mesenteric lymph nodes and spleens were clearly positive for viral replication, as indicated by the presence of RF DNA and RNA. Such positive cells were most often found in the middle of germinal centers (Fig. 3b, c, and d), but their cell type could not be identified with certainty. The location is characteristic for stimulated B lymphoblasts, although dendritic cells and macrophages may also be found in this location (42, 50). It should be pointed out that repeated studies with IFA have failed to identify cells displaying ADV

TABLE 1. Percentage of cells positive for ADV-specific RF DNA and/or RNA by SSISH^a

Cell type	% Positive on following day p.i. ^b :				
	0	5	10	20	60
Mesenteric lymph node cells	0, 0 ^c	0.2, 0.3	1.8, 2.1	0.4, 0.5	0, 0
Spleen cells	0, 0	0.04, 0.06	0.15, 0.2	0.1, 0.09	0, 0
Liver, kidney, gut, and lung cells	0, 0	0, 0	0, 0	0, 0	0, 0
Bone marrow cells ^d	0, 0	0.8, 1	2.5, 3	1.3, 1.5	0.09, 0.1
Leukocytes ^d	0, 0	0, 0	1.15, 1.25	0, 0	0, 0

^a Adult sapphire mink were inoculated with ADV Utah I, and two mink were killed at each of the indicated time points. Tissue and isolated bone marrow cells and peripheral blood leukocytes were processed for in situ hybridization as described in Materials and Methods. Cells were scored positive for viral RF DNA or RNA when the numbers of grains were more than three times background when probed with the minus-sense probe.

^b Each number represents the individual value for each of the two mink examined at the various time points.

^c The number of positive cells was recorded for an area containing approximately 100,000 cells. Therefore, 0% positive means no positive cells among 100,000 cells examined (<0.001% positive).

^d Positive cells in bone marrow and leukocytes could be found only when scoring for RF DNA and RNA. No cells were positive when scoring for RF DNA alone.

FIG. 3. In situ hybridization analysis with the minus-sense probe on sections of mesenteric lymph node from noninfected (control) and infected mink. (a) In situ hybridization on PLPG-fixed paraffin section of mesenteric lymph node from a normal control mink with the minus-sense probe. The section was not pretreated with RNase. Only background levels of grains can be detected. The germinal center seen is small and unstimulated, as normally seen in healthy adult mink. Bar, 24 μ m. (b) In situ hybridization on PLPG-fixed paraffin section of mesenteric lymph node from an infected mink killed 10 days after infection. The minus-sense probe was used, and the section was not pretreated with RNase. Light grain production is seen over cells in the middle of a germinal center (arrow). The germinal center is hyperplastic, which is characteristic for early ADV infection in mink. Bar, 24 μ m. (c) Higher magnification and longer exposure of parallel section to section shown in panel b. The minus-sense probe was used, and the section was not pretreated with RNase. Heavy grain production is seen over cells in the middle of a germinal center (arrows). Bar, 10 μ m. (d) Similar section as in panel c, hybridized with the minus-sense probe, but pretreated with RNase. Most of the grains have disappeared, indicating that most of the grains seen in panel c are due to hybridization to RNA. Bar, 10 μ m.

FIG. 4. In situ hybridization with the plus-sense probe on sections of mesenteric lymph node from infected mink. (a) In situ hybridization on PLPG-fixed section of mesenteric lymph node from a mink killed 10 days after infection. Heavy grain production is seen over cells at the periphery of germinal centers (arrow). Bar, 100 μ m. (b) Parallel section to section shown in panel a, but at a higher magnification and with a shorter exposure time. Grain production can be seen diffusely distributed over cells in the periphery of a germinal center (arrow). Bar, 15 μ m. (c) In situ hybridization on the same section as in panel a. Heavy grain production is seen over cells in the reticular network of the lymph node sinuses. Bar, 100 μ m.

antigens in this particular location (14, 41; S. Alexandersen and M. E. Bloom, unpublished observations).

The distribution of grain production was totally different when the plus-sense or virion probe was used on spleens and mesenteric lymph nodes. Most grains were formed over cells in the periphery or germinal centers (Fig. 4a and b) and in the reticular network of the sinuses (Fig. 4c). This localization of virion DNA correlated well with the finding of viral antigen around germinal centers in the mesenteric lymph nodes and spleens by IFA (14, 41) and indicated viral sequestration. As expected, grains were also found over cells in the middle of germinal centers, thus indicating that the cells in this location that were replicating ADV also contained virion DNA and were most probably producing virions.

Circulating leukocytes from infected mink were also examined; no cells positive for viral DNA could be demonstrated, but at day 10 p.i. a few cells (ca. 1%) contained viral RNA at low levels (approximately 2,000 copies per positive cell). Bone marrow cells contained no RF DNA, but they did contain low levels of single-stranded virion DNA and RNA (Fig. 5). The morphological preservation was not sufficient to allow characterization of the cells, but the size and shape of the cells and their nuclei suggested that they were lymphoblasts, monocytes, or promyelocytes. Other cell types which could be recognized by morphology, such as small lymphocytes and polymorphonuclear leukocytes, were consistently negative for ADV sequences. Further characterization of positive cells for viral replication in the mesenteric lymph nodes, bone marrow, and among circulating leukocytes must await the development of specific markers for mink cell subpopulations.

In the liver, only cells positive with the plus-sense or virion probe were detected (Fig. 6). No cells positive for viral replication were found (Fig. 7a), although overall kinetics resembled those for mesenteric lymph nodes (Fig. 6). The localization of grains over a population of cells reacting only with the virion probe (Fig. 7b) suggested that this was due to viral sequestration, probably in phagocytic Kupffer cells. Endothelial cells in larger vessels were also frequently positive for viral sequestration.

In the kidneys, no cells positive for ADV replication were found. Cells in glomeruli were consistently negative for virus replication (Fig. 8a), but when the virion probe was used, glomeruli were positive (Fig. 8b and c) on days 10 and 60 p.i., suggesting that at these time points, virus particles were being sequestered in these structures. Various kinds of tubular epithelial cells and a few interstitial and endothelial cells in larger vessels were also positive for viral sequestration.

The intestines had no positive cells for viral replication. The cells positive for ADV sequestration were always in the lamina propria of villi or in cells around blood or lymph vessels in the intestinal wall (data not shown). The epithelial cells were always negative with either probe.

The lungs had no cells positive for viral replication and had the smallest number of cells positive for viral sequestration among the tissues examined (data not shown). Positive cells were demonstrable only on day 10 p.i. and consisted of a few (ca. 0.05%) positive cells located around larger blood vessels and bronchi. These cells had a morphology consistent with their being macrophages. This finding is remarkable when the massive levels of ADV replication in lungs of newborn mink kits is considered (5, 6). Clearly, the lungs are not targets for ADV replication in adult mink.

Demonstration of ADV antigen and mink immunoglobulins in infected mink. IFA was done on frozen sections of kidneys

FIG. 5. In situ hybridization analysis of bone marrow cells from infected mink. PLPG-fixed cytopspins of bone marrow cells from mink killed 10 days after infection were subjected to SSISH analysis. (a and b) In situ hybridization on bone marrow cells not pretreated with RNase and with the minus-sense probe. Grain formation is seen primarily over the cytoplasm of a few cells (arrows). Since no grains were detected over control cells or cells treated with RNase, most of the signal must be due to ADV-specific RNA. Bar, 15 μ m. (c and d) In situ hybridization on bone marrow cells treated with RNase and with the plus-sense probe. Grains are developed over the cytoplasm of a few cells (arrows) and represent hybridization to virion DNA. Bar, 15 μ m.

from mink killed at the different time points. No ADV-specific fluorescence was seen in either control or infected mink kidney sections. However, mink immunoglobulins were found in a granular "lumpy bumpy" pattern in the glomeruli (Fig. 9b) of infected mink on days 10 and 60 p.i. Control kidney sections (Fig. 9a) and sections from mink killed at days 5 and 20 p.i. were negative for mink immunoglobulins. No ADV-specific fluorescence was detected in bone marrow cells or in leukocytes from infected mink.

Demonstration of specific ADV DNA in serum samples from infected mink. To examine whether distribution of virions via the bloodstream had occurred, we examined DNA extracted from serum samples by Southern blot hybridization. In the serum samples from infected mink, no DNA could be

detected on day 0 p.i., indicating that input virion DNA was below the detection limit ($<2 \times 10^6$ genomes per ml). By day 5 a concentration of about 4×10^8 single-stranded ADV genomes per ml of serum was detected. At day 10 the content in serum rose to a maximal concentration of about 4×10^9 genomes per ml: a level about 10 times lower than that reported for infected mink kits (6). After day 10 the content fell, to about 4×10^7 genomes per ml of serum on day 20 and to about 4×10^6 genomes per ml on day 60. This DNA in serum has previously been shown to be contained within virions (6, 14, 16).

Differences between groups. Group 1 consisted of normal control mink, and no ADV-specific antibodies, DNA, or RNA were detected. No differences could be detected

FIG. 6. Estimation of the percentage of positive cells and genomes per total cell in liver by in situ hybridization. In situ hybridization was performed on PLPG-fixed paraffin-embedded tissue as described in Materials and Methods. Cells containing virion DNA were detected by using the plus-sense probe, RF DNA was detected on RNase-treated slides by using the minus-sense probe, and RF DNA plus RNA was detected by using the minus-sense probe on slides not treated with RNase. (a) Percentage of cells positive in liver. (b) The number of genomes per total cell was calculated by multiplying the content of genomes per positive single cells by the percentage of positive cells. The ID₅₀ in the organs was estimated by assuming a virion-to-ID₅₀ ratio of 100 (16) and assuming that 500 mg of tissue corresponds to approximately 5×10^8 cells (14).

between group 2, inoculated with immunocomplexed virus prepared from adult mink, and group 3, inoculated with the same inoculum stripped of antibodies (data not shown).

DISCUSSION

The identification of target cells for ADV replication in infected adult mink is a major puzzle. Because the clinical picture is one of severe chronic immune complex disease and immune dysregulation (13, 38), interest has focused on cells of the immune system. In the experiments described in this paper, we used SSISH to probe for two specific intermediates of ADV replication, i.e., RF DNA and RNA.

The in situ hybridization technique gave direct evidence for viral replication in certain cells of lymphatic organs. In the mesenteric lymph nodes and spleens, up to 2% of the cells replicated ADV on day 10 p.i., but subsequently the number rapidly decreased, and by day 60 p.i. the apparent number of target cells was below detectable levels (<0.001%). The peak at day 10 and the calculated levels of ID₅₀s in the organs correlate well with earlier studies in which infectious virus was used as a marker (23, 36). The cells positive for replication could not be characterized with certainty by morphological criteria; however, they were located in the middle of germinal centers, which is a characteristic location for stimulated B lymphoblasts (42). These cells rapidly divide in stimulated germinal centers (42, 51), and replication of ADV in such cells would correlate well with the requirement for cells in early S phase for the replication of parvoviruses (48).

Two other cell populations showed some characteristics of target cells, but differed from those found in the mesenteric lymph nodes and spleens. Circulating leukocytes from infected mink were examined, and although no cells were positive for virion DNA or RF DNA, a few cells contained low levels of ADV-specific RNA. In addition, up to 3% of the cells in bone marrow were positive for virion DNA and RNA, but negative for RF DNA. Unfortunately, the exact identity of these cells cannot be established before functional assays or specific markers for mink cell subpopulations are developed.

Our results suggested that the nature of ADV replication in adult mink may be somewhat heterogeneous and might differ significantly from that observed in permissively infected CRFK cells or mink kits. To illustrate this point, we have summarized the characteristics for target cells from the three ADV models we have studied (Table 2). Permissive infection of CRFK cells and the type II alveolar cells of mink kits is characterized by high levels of RFs of DNA and virion DNA (50,000 to 100,000 copies per infected cell) and amplified levels of virus-specific RNA (250,000 to 400,000 copies per infected cell). Furthermore, intranuclear ADV antigens are readily demonstrable in infected CRFK and type II cells (3, 5, 6). The apparent differences between these two permissive systems and the findings for infected adult mink were striking. The levels of all three types of intracellular ADV nucleic acids (mRNA, RF DNA, and single-stranded DNA) were damped by at least a factor of 10. Furthermore, the relative ratios between the three species of nucleic acid were altered. Finally, repeated examination of tissue sections and cytocentrifuge preparations from infected adult mink (14, 41; data not shown) has failed to reveal any cells displaying ADV nuclear antigen, whereas such cells were readily identified in cell cultures and lungs of infected mink kits. These findings have led us to speculate that ADV replication within individual target cells in adult mink is not fully permissive, but is somehow restricted.

The concept of restricted or semipermissive infections has been discussed for other parvoviruses (45) and also implied in the pathogenesis of other slow viral diseases (43), including visna of sheep and the acquired immune deficiency syndrome of humans (25). Our results suggested that restricted infection may have a role in ADV, an important animal model of immune dysfunction. This state of semipermissiveness could be mediated either through the viral particles themselves or through host factors found in adult mink. Recent studies (P. Tattersall, R. Moir, and E. Gardiner, Second Parvovirus Workshop, MM5, p. 16, 1987) showed that fully productive replication of minute virus of mice depends upon a particular protein sequence motif contained within the incoming capsid proteins. The capsid

FIG. 7. In situ hybridization analysis of liver from infected mink. Sections of PLPG-fixed paraffin-embedded liver from mink killed 10 days after infection were subjected to SSISH analysis. (a) Liver section not pretreated with RNase and hybridized with the minus-sense probe. Only background levels of grains can be detected, indicating the presence of fewer than 300 copies of RF DNA or RNA in any single cell and therefore probably no replication of ADV. (b) In situ hybridization on parallel liver section hybridized with the plus-sense probe. Grains are seen over cells lining the hepatic sinuses, indicating the presence of viral sequestration in phagocytic Kupfer cells (arrows). Bar, 24 μ m.

FIG. 8. In situ hybridization analysis of kidney from infected mink. (a) In situ hybridization on PLPG-fixed paraffin-embedded section from a mink killed 10 days after infection. The section was not treated with RNase and was hybridized with the minus-sense probe. Only background levels of grains can be detected, indicating that there is no replication of ADV. (b) Parallel section of kidney hybridized with the plus-sense probe. Low levels of grains are developed over the capillary loops of a glomerulus (arrow). (c) Kidney section from a mink killed 60 days after infection, hybridized with the plus-sense probe. Low levels of grains are developed over the cells in a glomerulus, indicating sequestration of virus in this structure. Bar, 24 μ m.

proteins of ADV particles are proteolytically degraded (2), and an effect of this degradation might be to interrupt this motif and thus reduce the ability of such particles to initiate sequential rounds of productive infection. Germane to this notion might be the finding that the capsid proteins in

FIG. 9. Immunofluorescence on kidney sections of uninfected (control) and ADV Utah I-infected mink. (a) Immunofluorescence on acetone-fixed cryostat section of kidney from a normal control mink with FITC-conjugated rabbit anti-mink immunoglobulin. Only background fluorescence can be observed. (b) Immunofluorescence with FITC-conjugated rabbit anti-mink immunoglobulin on section of kidney from a mink killed 60 days after infection. Positive fluorescence is seen in a "lumpy bumpy" pattern in a glomerulus. The pattern of fluorescence suggests that the immunoglobulins are deposited along the glomerular capillaries and not in the mesangium. Bar, 24 μ m.

infected mink kit lungs are not degraded to a similar extent (8) and that the infection in this site is fully permissive.

Alternatively, the target cells for viral replication might be inherently semipermissive and therefore able to suppress productive replication. As a consequence, the replication and expression of viral genes might be tightly regulated by transcriptional or translational factors. Such a regulation of viral expression has been demonstrated in vitro for minute virus of mice and can be a consequence of the state of host cell differentiation (44, 45). If the target cells for ADV replication are of the lymphocytic series, these requisite factors might be expressed only during a narrow window of differentiation. The nature of the limiting viral cellular factors would be of great interest. Comparative studies on the transcriptional programs of ADV in cell culture, infected kits, and adult mink are currently in progress and may shed some light on which of the viral gene products are affected.

Finally, another mechanism of down regulating infection might relate to the rapid host response found in adult mink compared with mink kits (3-5, 36). Anti-ADV antibodies, which can be demonstrated as early as day 8 p.i. (Alexan-

TABLE 2. Characteristics of cells replicating ADV^a

Cell type	No. of copies of:			Presence of nuclear ADV proteins ^e
	RF DNA ^b	mRNA ^c	Virion DNA ^d	
CRFK cells	50,000	250,000	50,000	+
Type II alveolar cells	82,000	400,000	100,000	+
Mesenteric lymph node cells	1,500	8,000	12,500	—
Spleen cells	1,000	9,000	7,500	—
Bone marrow cells	ND ^f	6,000	3,000	—
Peripheral blood leukocytes	ND	2,000	ND	—

^a Adult sapphire mink were inoculated with ADV-Utah I and killed at 10 days after infection. Tissue and isolated bone marrow cells and peripheral blood leukocytes were processed for in situ hybridization as described in Materials and Methods. The number of grains per positive cell was correlated to the number of genomes by comparison with an included standard of infected CRFK cells containing a known number of ADV genomes. The numbers of genomes tabulated for CRFK cells and mink kit alveolar type II cells are derived from reference 6, except for the content of RNA, which was evaluated on the same samples by using the method described in this report.

^b The content of RF DNA was based on in situ hybridization with the minus-sense probe and RNase treatment of the cells.

^c The content of mRNA was calculated by subtracting the value of RF DNA obtained from the value of RF DNA plus RNA obtained by using the minus-sense probe and no RNase treatment.

^d The content of virion DNA was calculated by subtracting the number of RF DNA genomes from the number of genomes obtained by using RNase treatment and the plus-sense probe.

^e The presence of nuclear ADV proteins in the samples was examined by using immunofluorescence with anti-ADV sera as described previously (6, 14, 41).

^f ND, Not detectable (below detection limit of the assay, i.e., 300 genomes).

dersen and Bloom, unpublished observations), might suppress virus growth either by reducing the number of input particles or by somehow interfering directly with intracellular viral replication. The level of viral replication within individual type II alveolar cells is reduced in seropositive compared with seronegative mink kits (Alexandersen and Bloom, in preparation), and this finding might support the idea that antibody is somehow able to suppress ADV replication at a cellular level. In the adult mink in these experiments, however, the presence or absence of antibody on the viral inoculum did not alter the rate or level of viral replication to a detectable degree, suggesting that antibody may not be a significant factor in adult mink.

Another major finding to emerge from these studies related to the evidence obtained about viral sequestration in lymphoreticular organs of ADV-infected adult mink. In spleens and mesenteric lymph nodes, the regions positive for viral sequestration were situated around germinal centers and in the reticular network of the mesenteric lymph node sinuses, consistent with IFA results published for adult mink (14, 41). These findings suggested that virus was sequestered in phagocytic, perhaps antigen-presenting, cells often found in the periphery of germinal centers (33, 34, 42, 50). Another possibility is that the virus around the germinal centers is bound to receptors on the surfaces of B lymphocytes also found in this location (42, 50). Both possibilities would provide an explanation for the huge antibody response observed in AD and would correlate with the localization of other antigens during an immune response (10, 28, 34).

The level of viral sequestration was low at day 5 p.i., but reached peak values at day 10. The relatively low level of replication compared with the high level of virus sequestered at day 10 indicated that virus from several successive replication cycles must be accumulated to produce the large amount of virus observed. This might have come from cells being destroyed after infection, or perhaps the cells replicating ADV are able to produce and release virions without being killed. This might explain why IFA studies have failed to detect intranuclear ADV antigens in infected adult mink (14, 36, 41). This sequestered DNA in some organs probably is from cells releasing ADV there, but the high level of ADV DNA in serum suggested that viremia may also be important as a factor in virus sequestration.

In the liver, large amounts of virion DNA were found in Kupffer cells, similar to the sequestration of ADV found by

IFA for adult mink (36). No RF DNA or RNA could be detected in the liver by in situ hybridization, indicating that no cells in this organ supported viral replication.

The kidney glomeruli were positive for sequestered virion DNA and were also positive for mink immunoglobulins on day 10 and 60 p.i. This is the first report giving direct evidence for the presence of ADV DNA in glomeruli of infected adult mink. Moreover, the coexistence of virion DNA and mink immunoglobulins suggested the presence of immune-complex glomerulonephritis, in which intact virions or virion DNA was one of the components. The fact that virion DNA and low levels of mink immunoglobulins were cleared between days 10 and 20 p.i. suggested that the renal mesangial cells were capable of removing deposited immune complexes early in ADV infection. However, by day 60 p.i., both virion DNA and high levels of mink immunoglobulins were again detectable in a small number of glomeruli. This suggested that in late infection the phagocytic potential of the mesangial cells and infiltrating macrophages and polymorphonuclear leukocytes had been compromised. The apparent lack of immune complex removal in late infection might have several explanations. First, the mononuclear phagocytic systems of ADV-affected sapphire mink (Aleutian genotype) are greatly impaired in late infection (D. L. Lodmell, R. K. Bergman, W. J. Hadlow, L. C. Ewalt, and R. E. Race, manuscript in preparation). This decrease in phagocytic activity observed in infected mink could be induced by the circulating immune complexes, as observed for other types of immune complexes (9, 24, 32, 47); alternatively, Aleutian genotype mink may be especially predisposed, since these mink have the Chediak-Higashi syndrome, which includes decreased clearance of exogenous protein by the reticuloendothelial system, including the mesangial cells (39, 40). Another explanation could be that late-infection immune complexes are different from early-infection immune complexes and that these differences affect the clearance of the complexes as described for serum sickness glomerulonephritis (20, 22, 30). Early circulating immune complexes probably consisted of a relatively large number of virus particles (heavy viremia and relatively large amounts of virion DNA deposited) complexed to a relatively small number of antibody molecules (low antibody titer and little immunoglobulin deposited). In contrast, late immune complexes might consist of a small amount of virus complexed to a large number of antibody molecules (heavy

immunoglobulin deposition). The heavy deposition of immunoglobulins and low levels of virion DNA in late infection suggested that a substantial fraction of the deposited complexes consist of other than intact virions. Perhaps viral proteins, DNA, autoimmune complexes (11, 27), or rheumatoid factors (49) also are constituents. This is further suggested because a considerable number of the immune complexes are too small to contain intact viral particles (35).

As a final point of discussion, the concepts of viral restriction, limited permissivity, and viral sequestration can be integrated into a hypothetical scheme that accounts for the disorder of the immune system, the persistent infection, and the inability to detect cells replicating ADV late in infection. ADV might infect regulatory cells of the immune system in a semipermissive fashion. This restricted infection might not inevitably kill the infected cells, but in some cases might merely suppress cellular transcription, as described for adeno-associated viruses (12, 46), and decrease the ability of these cells to exert their regulatory functions. As is evident from studies on the acquired immune deficiency syndrome, only very small numbers of cells need be infected to wreak havoc on normal immune function (29). If the number of infected cells in late ADV infection were exceedingly small, or if the restriction of viral expression were very severe, the infected cells might escape detection by the techniques we have used. At the same time, these cells might release small amounts of virus. This hypothesis would explain both the persistent infection and the development of immune disorders by a mechanism of semipermissive infection of cells of the immune system. Another possibility would be that the massive sequestration of virus in areas of the lymph nodes and spleens known to be responsible for antigen presentation and immune stimulation (33, 34, 42, 50) directly provokes the immense humoral immune response and that this heavy antigen load induces the characteristic disorders of immune regulation either owing to difficulties in processing or via immune complexes.

Further studies by SSISH and Northern blot analysis are in progress to further characterize the subpopulations of lymphocytes responsible for ADV replication in adult mink and to delineate the possible suppression or block of viral replication and transcription observed in lymphocytes, circulating leukocytes, and bone marrow cells of ADV-infected adult mink.

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