Detection of Aleutian Disease Virus DNA in Tissues of Naturally Infected Mink

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SUMMARY

Organs of naturally infected mink were examined for the presence of Aleutian disease virus (ADV) DNA by in situ hybridization. Spleen, lymph nodes, thymus, bone marrow, kidney, liver, lung and small intestine were found to be positive for ADV to differing extents. Infected lymphoid organs showed a focal distribution of positive cells. Southern blot analysis of DNA extracted from infected organs revealed replicative forms of viral DNA in spleen and bone marrow samples only. These findings are consistent with a lymphotropism of ADV in vivo. Compared to the situation after experimental infection of mink these results indicate additional sites of virus replication and/or persistence of the naturally occurring disease.

Aleutian disease virus (ADV), an autonomous parvovirus (Bloom et al., 1980), causes a persistent infection in susceptible mink (Porter et al., 1980), which is characterized by perpetual viraemia, plasmacytosis and hypergammaglobulinaemia. The virus cannot be neutralized in vivo (Porter, 1986) and the deposition of circulating immune complexes in the kidney and small vessels during the course of the infection leads to chronic glomerulonephritis and arteritis, which determine the outcome of the disease.

Although Aleutian disease has been known for more than 30 years (Hartsough & Gorham, 1956), the replication site(s) of ADV in vivo is unknown. Due to the systemic deposition of immune complexes, detection of virus antigen in an organ does not necessarily imply virus replication at that site. However, as for all autonomous parvoviruses (Siegl et al., 1985), ADV replication is accompanied by the synthesis of replicative dsDNA forms (RFs), which are readily distinguished from the virion ssDNA by Southern blot hybridization (Bloom et al., 1983). The presence of these RFs of ADV DNA in certain organs is therefore a strong indication of virus replication at that site. Such RFs could be demonstrated in spleen and mesenteric lymph nodes of experimentally infected mink (Bloom et al., 1985). All published results of ADV DNA hybridization originate from tissues of experimentally infected mink. There are no data concerning viral DNA forms following natural infection, and this prompted us to screen organs of naturally infected mink for virus DNA by in situ hybridization. Tissues found to be virus DNA-positive were subsequently analysed for RFs by Southern blot hybridization.

Tissues were obtained from a commercial mink farm near Bremerhaven, F.R.G. which had a known record of Aleutian disease. The organ samples were immediately frozen and stored in liquid nitrogen until examination. Serum samples were tested by counter current immunoelectrophoresis (Cho & Ingram, 1972) and ELISA (Stolze & Kaaden, 1987) to prove the presence of anti-ADV antibodies (results not shown).

In situ hybridization was performed as described by Blum et al. (1983), with minor modifications. Briefly, frozen organ samples were cut with a cryostat and mounted on pretreated microscope slides. Bone marrow cells were prepared from femurs by flushing cells with RPMI 1640 medium and washing with phosphate buffered saline. For deposition on slides a Shandon Eliot cytocentrifuge (500 r.p.m. for 5 min) was used. After a few minutes of drying, specimens
were fixed for 15 min at room temperature in ethanol/acetic acid, 3:1. The slides were pretreated with 0.2 M-HCl, 2 × SSC and proteinase K as described (Brahic & Haase, 1978). After washing and dehydration in graded ethanol, RNA was digested with RNase A (100 μg/ml), RNase T1 (10 units/ml) in 2 × SSC, 1 mM-EDTA, 5% glycerol at 37 °C for 30 min in a humidified chamber. RNases were inactivated by two washings for 5 min each in 2 × SSC, containing 0.2% diethyl pyrocarbonate (Minnigan & Moyer, 1985). Post-fixation of the cells was done by treatment in freshly prepared 5% paraformaldehyde for 2 h in the dark (Haase et al., 1984). The slides were washed twice for 5 min each in 2 × SSC, then rinsed in distilled water and acetylated (Hayashi et al., 1978). After dehydration in a graded ethanol series, DNA was denatured by placing the slides in a solution containing 95% de-ionized formamide in 0.1 × SSC at 65 °C for 10 min. Samples were quenched by transfer into ice-cold 0.1 × SSC. After dehydration, the slides were hybridized for 48 to 72 h at 25 °C in the dark with 0.5 to 1 ng of probe in 5 to 10 μl of hybridization solution, containing 50% de-ionized formamide, 600 mM-NaCl, 0.02% each of Ficoll, polyvinylpyrrolidone and bovine serum albumin, 10 mM-Tris-HCl pH 7.5, 1 mM-EDTA, 10% dextran sulphate and 200 μg/ml calf thymus DNA. A cloned 73% central genome fragment (between map units 0.15 and 0.88) of ADV strain SL3 DNA (Löchelt & Kaaden, 1987), radiolabelled by nick translation (Rigby et al., 1977) with [35S]dCTP, was used as a probe. The specific activity was 1 × 10⁸ c.p.m./μg. The hybridization mixture was denatured at 100 °C for 5 min, quenched in ice water and applied to the slides. Following hybridization, the slides were washed extensively for 3 to 4 days in 50% formamide, 600 mM-NaCl, 10 mM-Tris-HCl pH 7.4 and 1 mM-EDTA at room temperature. After dehydration in a graded ethanol series containing 0.3 M-ammonium acetate, the slides were coated with Kodak NTB-3 emulsion, which had been melted at 43 °C and diluted 1:2 with 0.6 M-ammonium acetate. The slides were developed after exposure at 4 °C for 1 to 3 weeks and stained with Giemsa (Pardue & Gall, 1975). Positive hybridization results were visible as black grains by light microscopy, markedly differing from the non-specific background level of hybridization.

All sections were done at least in duplicate. The specificity was demonstrated in the following ways. (i) ADV-infected CCC Clone 81 cells were included (Fig. 1 a) as well as uninfected cells. (ii) No specific hybridization was detected in tissue sections of mink serologically negative for ADV or in heterologous mouse tissue. (iii) The specificity was further confirmed by pretreatment with DNase (Gowans et al., 1981), which reduced the signals to the background level.

Isolation of whole cell DNA from tissues and cells was done according to Bloom et al. (1985). Agarose gel electrophoresis and Southern blot hybridization (Southern, 1975) were performed as described recently (Löchelt & Kaaden, 1987), using the same probe as mentioned above. Table 1 shows the results obtained after in situ hybridization for the detection of ADV DNA. Only sections from mesenteric and non-gut-associated peripheral lymph nodes, spleen (Fig. 1 b, c), thymus, bone marrow (Fig. 1 d), kidney, liver, lung (Fig. 1 e) and small intestine were found to contain ADV-specific DNA sequences. No other organs examined gave (reproducible) positive results. In lymphoid organs (spleen, lymph nodes, thymus and bone marrow), very few cells displayed strong positive signals. Sometimes they were arranged as clusters of several cells (Fig. 1 c). This rare focal distribution explains the difficulty in assessing the state of virus DNA after extraction of whole cell DNA by Southern blot hybridization. Presumably the virus DNA is diluted out beyond the limit of detection by cellular DNA, a situation also known from other focal infections, e.g. with visna virus (Haase, 1986). RFs of ADV together with virus ssDNA could be demonstrated only in some spleen and bone marrow cells (Fig. 2), indicating a

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<tr>
<th>Organ</th>
<th>Spleen</th>
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<tr>
<td>Mesenteric lymph node</td>
<td>75</td>
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<tr>
<td>Peripheral lymph node</td>
<td>82</td>
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<tr>
<td>Thymus</td>
<td>55</td>
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<td>Bone marrow</td>
<td>33</td>
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<td>Kidney</td>
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<td>Liver</td>
<td>58</td>
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<td>Lung</td>
<td>50</td>
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<td>Small intestine</td>
<td>35</td>
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* Percentage positive (number of ADV DNA-positive organs/number of organs tested).
considerable amount of virus replication in these locations. Virus DNA forms could not be found in DNA extracts of other organs examined by Southern blot hybridization. The limit of detection was below 10 genome equivalents per cell. The existence of RFs was confirmed by Southern blot hybridization after alkaline gel electrophoresis (Maniatis et al., 1982) to discriminate true replicative forms from duplexes consisting of annealed virion ssDNAs of different polarity (data not shown). Taken together, these results confirm the superiority of in situ hybridization over blotting techniques for the detection of nucleic acids in virus infections, where only some cells would be expected to support virus replication. In the meantime we have generated single-stranded and strand-specific RNA transcripts, using an SP6 and T7 promoter-based dual transcription vector. These probes have already been used for Southern blot analysis (data not shown) and provide enhanced sensitivity. The planned use of strand-specific RNA
Short communication

Fig. 2. Southern blot hybridization of whole cell DNA from bone marrow of naturally infected mink. Single-stranded (virion) DNA (ssDNA) as well as 4.8 kb duplex monomeric (dmDNA) and 9.6 kb duplex dimeric (ddDNA) replicative forms are demonstrated.

probes for in situ hybridization will furthermore distinguish the real replication site(s) from sequestered virus.

The data obtained by in situ hybridization, in particular the high percentage of ADV DNA-positive lymphoid organs, support the concept of a lymphotropism of ADV in vivo, a feature shared with other parvoviruses like feline panleukopenia virus and canine parvovirus (Siegl, 1984). The finding of virus DNA in spleen and lymph nodes parallels the data obtained after experimental infection (Bloom et al., 1985). Furthermore, the focal distribution of ADV DNA in lymphoid organs suggests that ADV replicates in certain lymphocyte subpopulations only. This is consistent with previous in vitro infection studies, carried out with purified peripheral blood lymphocytes of serologically ADV-negative mink (Kaaden et al., 1986), in which a tropism of ADV for the B cell-enriched fraction was found. The focal in situ hybridization pattern seen in our study may reflect the fact that in addition to a proliferative activity of the target cell, intracellular, probably differentiation-dependent factors are also of paramount importance for productive replication of parvoviruses (Cotmore & Tattersall, 1987). Of particular interest is the so far undescribed existence of replicative ADV DNA forms in bone marrow cells. These findings supplement the detection of ADV-specific antigen and infectious virus in bone marrow cells of naturally infected mink (Roth et al., 1984). It is tempting to speculate that this site is of importance for the persistent course of the disease, e.g. as a virus reservoir.

Likewise, the presence of virus DNA in adult lung tissue has so far not been described. A primary lung injury after a naturally occurring outbreak of ADV has been documented (Larsen et al., 1984), in which only mink kits of ADV-negative dams suffered from severe pneumonia with high mortality. This disease was reproduced experimentally (Alexandersen & Bloom, 1987) and revealed the presence of a great amount of ADV RF DNAs in lung tissue of the infected mink kits. The tissues used in our study originate from a mink farm where Aleutian disease is endemic, thus rendering an analogous situation unlikely. The presence of virus DNA in the lung may be caused by natural infection via the respiratory tract or by secondary infiltration of infected lymphocytes.

Interestingly, ADV DNA was only occasionally detected in sections of the small intestine. Our results from the in situ hybridization may reflect that the sensitivity of the probe is too low to detect single virus DNA forms originating from oral transmission. ADV DNA in liver tissue suggests phagocytosis of cell debris and immune complexes rather than virus replication, whereas the presence of virus DNA in kidney is most likely caused by the glomerular deposition of circulating immune complexes.

Taken together, our data indicate a lymphotropism of ADV in vivo. The detection of ADV DNA forms in naturally infected mink complements the results from experimental infection. In
the natural infection, however, additional findings have been made, i.e. the presence of virus DNA in lung tissue and of virus RF DNA in bone marrow cells. These differences may be related to the experimental approach. The experimental infection (Bloom et al., 1985) was carried out using a known (highly virulent) virus strain of defined titre by intraperitoneal application. Furthermore, the animals were destroyed 10 to 60 days after infection. In contrast, the material that we examined originated from a heterologous mink population after natural infection (by intra-uterine, oral and/or respiratory transmission). The time of infection as well as the virus strain(s) involved are unknown. Furthermore, the animals were killed late compared to the experimental approach. More investigations are necessary to reveal the importance of our findings with respect to the natural course of the disease.

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REFERENCES


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