Replication of Aleutian mink disease parvovirus in mink lymph node histocultures

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Aleutian mink disease parvovirus (ADV), causes an immune disorder with a persistent infection of lymphoid organs in adult mink. We studied replication of ADV in gel-supported histocultures prepared from adult mink mesenteric lymph node (MLN). Evidence of virus replication in the histocultures was first observed by indirect immunofluorescence 72 h after incubation with virus. Cells resembling lymphocytes and macrophages contained both ADV capsid (VP2) and non-structural (NS1 and NS2) proteins, and were present in a distribution suggestive of infected cells within germinal centres. ADV replicative form and encapsidated virion DNA were also detected in infected histocultures at time-points after 72 h. In addition, we were able to passage ADV-Utah to a new round of histocultures. These results suggested that the infected cells were actual target cells for ADV replication and that productive ADV-Utah replication, complete with the generation of virus, was occurring in the histocultures. The mink MLN histocultures provide a system to study the replication and molecular pathogenesis of ADV in target tissues.

Introduction

Aleutian mink disease parvovirus (ADV), a non-defective parvovirus (Bloom et al., 1980), causes classical Aleutian disease (AD), an immune disorder with a persistent infection of lymphoid organs, in adult mink (Porter, 1986; Bloom et al., 1994; Alexandersen, 1990; Aasted, 1985). The immune disorder is characterized by a relative increase in CD8+ lymphocytes (Aasted, 1989), plasmacytosis, hypergamma-globulinaemia and immune complex disease (Aasted, 1989; Porter, 1986; Alexandersen, 1990; Porter et al., 1983). Deposition of immune complexes leads to arteritis and glomerulonephritis (Porter et al., 1969, 1983; Porter, 1986), the usual cause of death.

The primary site of virus replication in adult mink is in lymphoid tissue. After infection with ADV-Utah, viral DNA is first detectable at day 5 (Alexandersen et al., 1988a) and specific proteins at day 8 (Porter et al., 1969); the highest concentrations of viral DNA and proteins and infectious virus are seen at day 10 (Porter et al., 1969; Bloom et al., 1985, 1987). Immunofluorescence studies on mesenteric lymph node (MLN) have shown that ADV capsid proteins are present in the cytoplasm of cells surrounding germinal centres (Bloom et al., 1985; Race et al., 1986). The distribution is thought to represent intact virus or virus proteins sequestered in phagocytic cells and not virus replication (Race et al., 1986). Large amounts of virion DNA co-localize with the capsid proteins further supporting the notion of virus sequestration (Alexandersen et al., 1988a; Mori et al., 1991a). A smaller number of cells actually supports virus replication as evidenced by the presence of viral mRNA (Alexandersen et al., 1988a). Some of these cells are located in the middle of germinal centres and others appear to be macrophages located in the subcapsular sinus of the lymph node. Consequently, the precise target cells for ADV replication remain uncertain.

When the findings from infected adult mink are contrasted with permissively infected cell cultures or newborn mink kits (Alexandersen, 1986), it is apparent that the infection in adults is restricted (Alexandersen et al., 1988a). The overall level of viral DNA and the relative ratio between the intracellular forms of viral DNA differs between permissive and restricted infections in that in the permissively infected cells the levels of replicative forms approximate those of virion DNA, whereas in the restricted infection, virion DNA is the more abundant form. This restriction of infection is confirmed by in situ hybridization studies (Alexandersen et al., 1988a). The genesis of the restriction is unknown, but the rapid antibody response
against ADV may play a major role in down-regulating levels of replicative intermediates (Alexandersen et al., 1988; 1994).

The antibody response complicates the analysis of ADV infection in other ways, too. Antiviral antibody is present as soon as virus is detected and virus can persist in infectious immune complexes (Porter & Larsen, 1967). Furthermore, antibody can mediate ADV infection of phagocytic cells (Mori et al., 1991; Kanno et al., 1992; 1993; Dworak et al., 1997) and has also been shown to neutralize infectivity in cell culture (Kanno et al., 1993). Consequently, antibody may interfere with identification of target cells, and the study of ADV infections in the absence of a systemic immune response might facilitate identification of primary target cells.

In order to identify target cells for virus infection and to extend the analysis of the complex pathogenesis of AD, an ex vivo system would be an advantage. Recent studies describe the use of gel-supported histocultures for examining HIV pathogenesis (Margolis et al., 1995a; Glushakova et al., 1997). The advantage of the histoculture system compared to conventional culturing systems is a preservation of the microscopic three-dimensional cytoarchitecture in the cultured organ, even in very dynamic tissues such as secondary lymphoid organs (Nilsson, 1971). These ex vivo cultures can partially compensate for a lack of inbred animals because replicate cultures can be established from each animal. In addition, histocultures allow for precise kinetic studies because they can be synchronously infected after explantation. Finally, problems attendant to a systemic immune response are largely avoided. During culture of secondary lymphoid organs, critical structures and at least some normal physiological functions, such as lymphocyte migration and antibody production, are maintained (Margolis et al., 1995a, b; Glushakova et al., 1997; Nilsson, 1971; Tew et al., 1978; Tew & Stavitsky, 1974).

In this work we developed a histoculture system of MLN tissue from adult mink. We were able to infect these cultures with the pathogenic isolate ADV-Utah and demonstrate production of replicative intermediates and encapsidated viral DNA. We were as well able to passage this virus to another round of histocultures.

**Methods**

**Virus.** The ADV-Utah isolate was propagated and isolated from mink kits essentially as previously described (Alexandersen, 1986; Alexandersen et al., 1987). The titre was 1 × 10⁶ ID₅₀/ml. Before use the virus was diethylether extracted, dialysed 2 × 12 h, sonicated, centrifuged at 9000 g for 12 min at room temperature (RT) and filtered through 0.45 and 0.22 μm filters. The virus was diluted in RPMI 1640 supplemented with 200 μl/mL penicillin, 100 μg/mL streptomycin sulfate and 15% foetal calf serum (FCS) (complete media) to a final concentration of 1 × 10⁴ ID₅₀/ml.

**Histoculture system.** The mink histoculture system was modelled on previously described examples (Margolis et al., 1995a; Glushakova et al., 1997; Li et al., 1991; Freeman & Hoffman, 1986; Nilsson, 1971; Sorour et al., 1975). ADV-negative mink were euthanised by cardiac puncture and the MLN was aseptically removed. Fat was trimmed off and the organ was subsequently rinsed in complete media. The lymph node was cut into pieces, ca. 1 mm thick, 2 mm wide and 2 mm long, each fragment containing both cortex and medulla. The organ fragments were kept at room temperature in complete medium for no longer than 30 min before initiation of culturing.

Pieces of sterile gel matrix (Gelfoam, Upjohn), 12 × 10 × 7 mm, were pre-soaked in two changes of complete media for 2 h. Lymph node fragments were placed on the floating pieces of gel matrix in six-well plates in 5 ml of complete medium at 37°C. By this method, the tissue fragments were covered by a thin film of media and kept at the liquid–air interface. Complete medium was changed every day. In more than 20 experiments no bacterial or fungal contamination was noted.

For infection, the gel matrix was hydrated for 2 h in complete medium containing 1 × 10⁵ ID₅₀ ADV-Utah/ml prior to addition of lymph node fragments. The tissue fragments were then placed on the gel matrix in 5 ml fresh complete medium containing 1 × 10⁵ ID₅₀ ADV-Utah/ml. After 24 h the fragments were rinsed in complete medium, transferred to fresh pieces of pre-hydrated gel matrix and then incubated at 37°C for various times.

**Histoculture viability.** To assess tissue viability and changes in cytoarchitecture, histocultures were fixed in parafomaldehyde and paraffin embedded. Sections (4 μm) were prepared and stained with haematoxylin and eosin (H&E) by standard histological procedures.

To determine viability, histocultures were analysed by confocal microscopy basically as described (Margolis et al., 1995b). Histoculture blocks were harvested on successive days and incubated for 20 min at room temperature in PBS containing 4 μM ethidium homodimer-1 (which stains dead cells red) and 2 μM calcein-AM (which stains live cells green) (Molecular Probes), rinsed in PBS and analysed within 15 min. Confocal microscopy was performed on a Zeiss Axiosvert 135 microscope equipped with a Bio-Rad MRC 1024 scanning system, supplied with a 15 mW krypton/argon laser. This laser produces bands at 488, 568 and 647 nm. First filter block was a triple dichroic filter; second filter block was a 560 nm longpass dichroic filter. Emission filters used were 522DF32 for calcein-AM and 605DF32 for ethidium homodimer-1. The images were acquired and analysed with Lasersharp software (Bio-Rad). The figure was prepared using Corel Draw 8 software.

For a nonviable control at each time-point, a histoculture was incubated in 70% ethanol at room temperature for 30 min, and stained as above. These samples were used to adjust photomultiplier tube gain parameters.

**Immunofluorescence.** The presence of ADV-specific antigens in lymph node histocultures was evaluated by indirect immunofluorescence (IFA). Infected and uninfected histoculture blocks were harvested daily, embedded in O. C. T. compound in a 95% ethanol–dry ice slurry and frozen at −70°C. Cryostat sections (6 μm) were fixed in ice-cold acetone for 10 min. The sections were then blocked for 20 min at room temperature with PBS containing 15% normal swine serum and incubated for 30 min at room temperature with specific antisera diluted in PBS containing 15% normal swine serum. The sections were then washed 2 × 5 min in PBS and incubated for 30 min at room temperature with a 1:15 dilution of FITC-conjugated swine anti-rabbit F(ab)₂ fragments (DAKO) absorbed with mink sera prior to use. The sections were then washed 2 × 5 min in PBS, rinsed with 70% ethanol, air-dried and mounted with anti-fade mounting medium (Vector).

Sera used were rabbit anti-VP2 capsid protein (diluted 1:200) (Clemens et al., 1992; Christensen et al., 1993; Oleksiewicz et al., 1996), and rabbit anti-NS1 (diluted 1:100) and rabbit anti-NS2 (diluted 1:200) (Christensen et al., 1995; Oleksiewicz et al., 1996; Bloom et al., 1982; Porter et al., 1990). Fluorescence microscopy was performed on a...
Nikon Micro-Photo EPI-fl3 microscope with a PI fluorescence attachment equipped with a shortpass FITC-TRITC Exciter and Emission Filter (Chroma). Pictures were taken with a Nikon FX-35DX camera on Kodak 400 ASA Elite Chrome film. The figures were prepared using Corel Draw 8 software.

Negatives controls included uninfected histocultures stained for viral antigens and infected histocultures stained with irrelevant antibodies (rabbit antiserum against mink enteritis virus (diluted 1:300), kindly provided by Åse Utenthal, Copenhagen Fur Center, Denmark).

For a comparison of the histoculture infection with the in vivo infection, the MLN was taken from an adult sapphire mink infected 10 days prior with 1 × 10^7 ID_{so} ADV-Utah. Cryostat sections (6 μm) were fixed and stained.

### Southern blot hybridization.
Whole cell DNA was prepared from frozen histocultures with slight modifications from previous studies (Kanno et al., 1993). Briefly, after analysis of individual histocultures by IFA, the subjacent tissue fragment was excised from the O.C.T. blocks and proteinase K digested at 50 °C for 4–6 h. After phenol–chloroform extraction and ethanol precipitation, the samples were redissolved in 30 μl 10 mM Tris–1 mM EDTA and quantified by agarose gel electrophoresis. Samples of whole cell DNA (1 μg corresponding to approximately 3 × 10^6 diploid cell equivalents) were electrophoresed on a 0.7% agarose gel and Southern blotted onto nylon membrane (Hybond-N, Amersham). Standards corresponding to 100, 10 and 1 ADV genomes in 1 μg whole cell DNA were included as controls. The standard was a restriction fragment purified from a full-length molecular clone representing 93 map units of the ADV genome (Bloom et al., 1990).

To assess the presence of encapsidated virion DNA, we extracted DNase-resistant DNA from infected histocultures, essentially as described (Bloom et al., 1987). The MLN histocultures were cut out of O. C. T. blocks, homogenized in 500 μl 50 mM Tris (pH 8.0), freeze–thawed three times and sonicated. The tissue was digested for 3 h with 40 μg/ml RNase A and 200 μg/ml DNase I, in the presence of 10 mM MgCl₂. The reaction was stopped by adding EDTA to 20 mM and 1 μg of carrier DNA was added. The DNA samples were then extracted and purified as above. One-third of the extracted DNase-resistant DNA from each sample was electrophoresed on 0.7% agarose together with standards and blotted as above.

All blots were then hybridized with 32P-labelled plus-sense single-stranded RNA probes as described (Bloom et al., 1987; Kanno et al., 1993). Plasmids p18-1 (Bloom et al., 1987) and 13W-13 (a plus-sense probe spanning 53–100 map units) were used as templates for the in vitro transcription reaction, utilizing SP6 and T7 polymerase, respectively. These probes together span more than 80% of the viral genome (Bloom et al., 1988, 1990; Alexandersen et al., 1986) and react with both virion DNA as well as duplex replicative forms.

After hybridization and washing, the blots were visualized by autoradiography on Kodak X-OMAT X-ray film. The figures were prepared using Corel Draw 8 software.

### Passage of ADV-Utah in histoculture.
A set of histocultures was infected with 1 × 10^6 ID_{so}/ml ADV-Utah and blocks were harvested at 24, 48, 72, 96 and 120 h, frozen in liquid nitrogen and stored at −70 °C. The frozen histocultures were thawed in 500 μl complete medium in microcentrifuge tubes, homogenized with sterile plastic pestles and freeze–thawed three times. After sonication and centrifugation at 15 000 g for 15 min at room temperature, the lysates were adjusted to 3 ml with complete medium and sterile filtered. The samples were then diluted 1:10 and 1:100 and 1.5 ml of each dilution was used to infect fresh cultures in 24-well plates. After 24 h the histocultures were transferred to freshly hydrated Gelfoam in 5 ml complete medium in six-well plates and incubated for an additional 96 h (120 h total). The fragments were then embedded in O.C.T. compound and stored at −70 °C until sectioning. Cryostat sections (6 μm) were analysed by IFA.

As controls, histocultures were infected with ADV-Utah stock 10-fold serially diluted from 1 × 10^9 ID_{so}/ml to 1 × 10^6 ID_{so}/ml, and harvested at 120 h. Uninfected histocultures were also harvested at 120 h after culturing, prepared and diluted as above.

### Analysis of cells emigrating from the histocultures into the media.
Cells that had emigrated from the histocultures into the media were counted and isolated by centrifugation at 800 g at room temperature. Viability of the emigrated cells was assessed by trypan blue exclusion test. For IFA, emigrated cells were cytocentrifuged at 800 g at room temperature and then fixed in ice-cold acetone for 10 min and stained as detailed above. For Southern blot hybridization, whole cell DNA from emigrated cells was prepared as detailed above.

### Results

#### Characterization of mink MLN histocultures

Fragments of MLN placed on gel matrix supports retained basic cytoarchitecture (Fig. 1a, b) with a clear demarcation between lymph node cortex and medulla. However, over the course of culture, some changes were consistently noted. After 12 h of culture many cells from the medulla and deep cortex in the MLN histocultures had migrated into the medium (data not shown), probably the result of normal lymphocyte trafficking and as a result of compromised structure of the lymph node lobule (Margolis et al., 1995a; Glushakova et al., 1997). The medullary regions of the MLN histocultures were depleted of lymphocytes and there was a loss of clearly defined medullary cords. In the cortical regions, structures compatible with germinal centres as distinguished by morphology became more prominent after 48–72 h of culture, which may have reflected demasking from migration (Glushakova et al., 1997; Grez et al., 1997) or apoptosis of surrounding cells not in direct contact with follicular dendritic cells (Lindhout et al., 1993; Bonnefoy et al., 1993). This morphology was maintained until 120 h of culture without significant changes (Fig. 1b, c). At 120 h only a few cells undergoing necrosis were observed.

Vital staining was employed to confirm that the histocultures retained viability (Fig. 1d). At 120 h the cultures stained green by calcein-AM, with only few dead cells staining red by ethidium homodimer-1. A control culture, killed with ethanol, showed a uniform red appearance (Fig. 1e) thus confirming the assay.

In summary, at 120 h the general picture of the MLN histoculture revealed a clear delineation of cortex, with structures morphologically compatible with lymph follicles and germinal centres, and medulla, partially emptied of cells (Fig. 1b, c). By 172 h of incubation, the histocultures generally lost distinct anatomic features (data not shown), and as a result we terminated our experiments at 120 h.

Taken together, these preliminary studies indicated that gel-supported histocultures derived from mink MLN remained viable and exhibited similar characteristics to those described for other species (Margolis et al., 1995a; Glushakova et al., 1997; Nilsson, 1971).
Identification of ADV antigens in infected histocultures

In order to see if ADV would replicate in this system, we incubated histoculture blocks with ADV-Utah and examined expression of viral antigens. ADV antigens were first noted after 72 h of incubation. Cells staining positive for capsid protein VP2 were scattered throughout the periphery of the cortical region of the culture fragments (Fig. 2a). The number of capsid-protein-positive cells was 10–50 per section at 72 h increasing to more than 100 per section at 120 h post-infection. Most of the cells positive for capsid protein showed cytoplasmic staining but nuclear staining was readily observed in 5–10% of the positive cells.

Because the presence of nonstructural proteins is indicative of active virus replication, we stained sections with antibodies specific for NS1 and NS2. NS1- and NS2-positive cells were
readily identified at 72 h in the same areas as the capsid-protein-positive cells. However, the NS-positive cells were fewer in number and were limited to one or two discrete areas within the section, in a location suggestive of germinal centres. In each section 5–15 NS1- and NS2-positive cells were observed. There was no obvious increase in number of NS1- and NS2-positive cells at 120 h of infection (Fig. 2b, c). Both cytoplasmic and nuclear staining were observed for non-structural proteins.

The cells staining positive for ADV proteins resembled both macrophages and lymphocytes. No staining was observed in uninfected cultures or in infected cultures stained with a rabbit serum directed against mink enteritis virus (data not shown).

For comparison with in vivo infection, a mink was infected with ADV-Utah, killed at day 10 post-infection, and cryostat sections of the MLN were stained as above. As previously reported, cells positive for capsid proteins were present and widespread in distribution over the cortical and subcortical areas (Fig. 2e). The staining was exclusively cytoplasmic. Cells positive for the nonstructural proteins were not observed (Fig. 2f, g).

Identification of replicative forms of ADV DNA in infected histocultures

During ADV replication the single-stranded DNA genome (SS-DNA) is converted to monomeric and dimeric double-stranded DNA replicative forms (DM-DNA and DD-DNA, respectively) (Cotmore & Tattersall, 1987; Bloom et al., 1987, 1994). Thus, the presence of these forms would be a strong indication that replication is occurring. In order to determine if ADV replicative forms could be detected in the infected histocultures, we performed Southern blots on whole cell DNA extracted from infected histocultures (Fig. 3a). SS-DNA, DM-DNA and DD-DNA were first noted at 72 h and were increased at 120 h (Fig. 3a). No bands were observed at 6, 12 and 24 h, although a faint band of SS-DNA could be seen at 48 h. Uninfected control histocultures were negative for ADV DNA. These findings clearly indicated that ADV DNA replication occurred as early as 48 h and continued to increase until the end of the experiment at 120 h.

The ratio between SS-DNA and DM-DNA resembled that seen in in vivo infection (Bloom et al., 1985, 1987), with SS-DNA about 10 times more abundant than DM-DNA. Based on comparison with the standards, at 120 h the amount of SS-DNA was estimated to be more than 100 copies/total cell and the amount of DM-DNA and DD-DNA to be about 10 copies/total cell and 2–3 copies/total cell respectively.

Viral genomes encapsidated within virions are resistant to DNase digestion (Bloom et al., 1987). In order to define if the de novo produced SS-DNA was encapsidated in virions, we performed Southern blot on DNase-resistant DNA isolated from infected histocultures. Virion DNA was not demonstrable at 24 and 48 h of culturing but was observed at 120 h (Fig. 3c). Thus, it was likely that SS-DNA contained within capsids was being produced.
Table 1. Serial passage of ADV-Utah in mink mesenteric lymph node histocultures

<table>
<thead>
<tr>
<th>Dilution</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
<th>120 h</th>
<th>ADV-Utah*</th>
<th>Mock</th>
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<tr>
<td>1:10</td>
<td>fi</td>
<td>fi</td>
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<td>1:100</td>
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* Histocultures were infected as first passage cultures as described above. Cryostat sections were stained for capsid protein and positive cells were scored.

Serial passage of ADV-Utah in histoculture

Finally, we attempted to see if the virus produced in histocultures was infectious for new histocultures. First passage cell lysates were prepared from histocultures infected with ADV-Utah for 48, 72, 96 and 120 h. Fresh cultures were incubated with a 1:10 and a 1:100 dilution of the first passage lysates. The second round cultures were harvested after 120 h and examined for evidence of ADV infection by IFA.

The histocultures infected with the 48 h lysates were negative for ADV antigens, whereas histocultures infected with the 72, 96 and 120 h preparations were positive for both cytoplasmic and nuclear staining (Table 1). Histocultures infected with the 72 h preparation were positive only with the 1:10 dilution whereas the histocultures infected with the 96 and 120 h preparations also were positive at the 1:100 dilution. The histocultures that were positive for capsid protein were also positive for nonstructural proteins with both cytoplasmic and nuclear stain (data not shown). The parallel histocultures infected directly with the ADV-Utah stock were positive at both concentrations whereas uninfected histocultures were negative.

The results of this experiment strongly suggested that ADV-Utah gives rise to a productive infection in the histocultures and that the virus can be serially passaged in fresh histocultures.

Analysis of cells emigrating from the histocultures to the media

As noted, cells emigrated from the lymph node fragments over the culture period. The emigrating cells were viable as
assessed by trypan blue exclusion test. The cells had a morphology resembling both lymphocytes and macrophages. The number of emigrating cells was between 25,000 and 50,000 cells/24 h with a sudden increase at 120 h where \( \approx 100,000 \) cells emigrated from the histocultures to the media.

In order to determine if cells emigrating from infected histocultures were positive for viral antigen, we collected the cells daily and stained them for ADV proteins. No ADV-positive cells were noted until 120 h, when fewer than 0.01% of cells showed cytoplasmic capsid protein staining. These few positive cells had a morphology compatible with macrophages (data not shown). The finding probably reflected phagocytosis and sequestration of virus or viral proteins by macrophages emigrating from the lymph node histoculture. No cells stained positive for nonstructural proteins.

To further determine if the emigrating cells contained viral DNA, we performed Southern blot on whole cell DNA. SS-DNA was observed at 6, 12 and 24 h but not at later timepoints. Furthermore, no replicative forms were observed. Emigrating cells from uninfected histocultures were also negative for ADV DNA. The experiment suggested that cells emigrating during the inoculation period take up virus from the inoculum but do not support replication. Once the inoculum was removed, virus DNA was detectable only within the histoculture fragment, suggesting that the emigrant cell population does not support ADV replication to detectable levels.

**Discussion**

A continuing problem in the study of ADV pathogenesis in the adult mink is the elucidation of target cells for viral infection. Previous studies have identified cells in lymphoid tissues that contain ADV DNA, mRNA and capsid proteins (Alexandersen et al., 1988a; Mori et al., 1991a, b). However, the infection is restricted and the characteristics of these infected cells differ from those supporting permissive virus replication. Furthermore, the identification of primary target cells is confounded by the sequestration of virus-containing immune complexes and by the infection of phagocytic cells via an antibody-mediated mechanism (Mori et al., 1991a; Kanno et al., 1992, 1993; Dworak et al., 1997). In the present study we have circumvented these problems by infecting lymph node sections in gel-supported histocultures.

Using histocultures prepared from mink MLN, we demonstrated unequivocal evidence of ADV replication by IFA (Fig. 2a–c). Southern blot (Fig. 3a, c) and serial passage of infectious virus (Table 1). Viral antigen and replicative forms of ADV DNA were easily detected by 72 h, a finding that is in marked contrast to infected lymphoid tissue from infected adult mink. Because we did not study the infected histocultures with in situ hybridization, we cannot yet conclude whether the infection at the cellular level is fully permissive or restricted. It will be interesting to address this issue with in situ hybridization.

The number of cells positive for capsid proteins in the histocultures was much lower than in lymph node sections prepared from ADV-infected mink (Fig. 2e). Because capsid protein staining in vivo largely represents sequestration of virus (Race et al., 1986), this finding suggested that sequestration does not occur in the histoculture to the same extent as in vivo. In addition, NS proteins were readily demonstrable in the histocultures, whereas they have not been clearly observed in vivo (Fig. 2f, g). Failure to detect the NS proteins in sections from infected mink might be due to technical reasons or might reflect a lower level of expression in the restricted in vivo infection.

Immunohistochemical analysis localized positive cells to the peripheral cortical regions (Fig. 2a–e). This location corresponds with findings from the in vivo infection, where infected cells identified by in situ hybridization are located within germinal centres and subcortical sinuses of the lymph node (Mori et al., 1991a; Alexandersen et al., 1988a; Kanno et al., 1992). Thus, the infected cells in the histocultures had a similar distribution to infected cells in vivo.

The cells positive for ADV antigens in the histoculture resembled both macrophages and lymphocytes, an observation consistent with the proposition that the target cells are either B cells or macrophages (Alexandersen et al., 1988a; Mori et al., 1991a, b). The macrophages within the germinal centre, the so-called tingible body macrophages (TBM), are interesting candidates for an ADV target cell. The TBM phagocytize and remove apoptotic lymphocytes (Tabe et al., 1996) and down-regulate the germinal centre reaction in mice (Smith et al., 1998). A disruption of this regulatory function may partially explain several key features of AD. ADV infection of the TBM could impair the down-regulation of the germinal centre reaction and lead to the hypergammaglobulinaemia observed during AD by permitting unrestricted expansion of B cell populations. In addition, the relatively low frequency of TBM (1 TBM to every 350–450 B cells in mice; Smith et al., 1991), might explain the relatively low frequency of cells positive for ADV replicative intermediates in the adult mink infection. The identity of the infected cells in the MLN histocultures is presently under study using recently developed and characterized markers for mink leukocytes (Chen et al., 1997; Brodersen et al., 1998).

The kinetics of ADV replication in the infected histocultures were significantly more rapid than in vivo ADV infections in lymphoid tissues. Both ADV DNA and proteins were observed at 72 h as compared to the in vivo situation where DNA is not detected until 5 days (Alexandersen et al., 1988a) and ADV antigens not until 8–10 days (Porter et al., 1969) post-infection. Reasons for this difference are unclear, but there are several potential explanations. First, virus is directly applied to the histoculture fragments, in contrast to in vivo infection where virus must concentrate in lymphoid tissues before contacting target cells. Alternatively, the ex vivo culture system may somehow enhance either the appearance or the susceptibility.
of target cells to infection. An additional explanation could be that the absence of a systemic immune response in the explant cultures somehow permits more rapid replication (Alexandersen et al., 1988c, 1994).

During the initial phases of culture, lymphocyte emigration into the culture medium was apparent in the histocultures. Studies on emigrated cells revealed several interesting points. First, the emigrant cells were positive only for capsid protein at a low level in the cytoplasm (< 0.01%) and no RF-DNA forms could be detected. In addition, SS-DNA could be detected only from 6 to 24 h, but not after removal of the inoculum (Fig. 3b). The morphology of these few positive cells was compatible with macrophages and no positive cells had a morphology compatible with lymphocytes. This suggests that a proportion of emigrating macrophages take up the inoculum but do not support ADV replication, and that the infected cells remain fixed in the histocultures.

We conclude that the histoculture system has greatly expanded information about ADV infection in lymphoid tissue, and offers an ideal method to identify target cell populations in more precise detail. With this system, it will now be possible to perform kinetic studies on the transcriptional programme of ADV in target tissue. Furthermore, detailed studies of subcellular localization of ADV proteins and DNA during ADV infection can be investigated under in vitro like conditions. The system may also allow propagation of replication-defective molecular clones of ADV that do not grow in cell cultures.

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