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Aleutian Mink Disease Parvovirus Infection of Mink Peritoneal Macrophages and Human Macrophage Cell Lines

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Aleutian mink disease parvovirus (ADV) mRNAs are found in macrophages in lymph nodes and peritoneal exudate cells from ADV-infected mink. Therefore, we developed an in vitro infection system for ADV by using primary cultures of mink macrophages or macrophage cell lines. In peritoneal macrophage cultures from adult mink, virulent ADV-Utah I strain showed nuclear expression of viral antigens with fluorescein isothiocyanate-labeled ADV-infected mink serum, but delineation of specific viral proteins could not be confirmed by immunoblot analysis. Amplification of ADV DNA and production of replicative-form DNA were observed in mink macrophages by Southern blot analysis; however, virus could not be serially propagated. The human macrophage cell line U937 exhibited clear nuclear expression of viral antigens after infection with ADV-Utah I but not with tissue culture-adapted ADV-G. In U937 cells, ADV-Utah I produced mRNA, replicative-form DNA, virion DNA, and structural and nonstructural proteins; however, virus could not be serially passaged nor could [^{3}H]thymidine-labeled virions be observed by density gradient analysis. These findings indicated that ADV-Utah I infection in U937 cells was not fully permissive and that there is another restricted step between gene amplification and/or viral protein expression and production of infectious virions. Treatment with the macrophage activator phorbol 12-myristate 13-acetate after adsorption of virus reduced the frequency of ADV-positive U937 cells but clearly increased that of human macrophage line THP-1 cells. These results suggested that ADV replication may depend on conditions influenced by the differentiation state of macrophages. U937 cells may be useful as an in vitro model system for the analysis of the immune disorder caused by ADV infection of macrophages.

Aleutian mink disease parvovirus (ADV) causes a chronic infection associated with a severe disorder of the immune system. The disease in adult mink, known as classical Aleutian disease, is characterized by marked hypergammaglobulinemia, plasmacytosis, arteritis, and fatal immune complex glomerulonephritis (37).

ADV was identified as the etiological agent of Aleutian disease more than 10 years ago (11, 15), and the replication of the nonpathogenic ADV-G strain of virus in Crandell feline kidney (CRFK) cells has been extensively characterized in vitro (6–8, 9–11, 32). Unfortunately, pathogenic strains of ADV, such as ADV-Utah I, cannot be reliably propagated in cell culture (11), and therefore in vitro studies of host cell interactions with pathogenic strains have been limited.

In vivo studies of ADV infection in adult mink have revealed that macrophages are target cells for ADV infection (23) and that the peak of ADV replication occurs approximately 10 days after infection with the pathogenic ADV-Utah I strain of virus (3, 10, 12). At later times after infection, macrophages are still reservoirs of ADV (32) and functional defects in the mononuclear phagocytic system can be demonstrated (28). Furthermore, the infection of macrophages does not seem to be fully permissive when compared with in vitro infections or with infection of mink kits (3, 4).

Generally, parvoviruses require rapidly cycling cells for productive and permissive infection (16). Although macrophages are considered to be terminally differentiated, noncycling cells (47), they have been reported to proliferate under certain circumstances (34, 41). In addition, porcine parvovirus replicates in swine macrophages (21). These findings support the likelihood that macrophages are target cells in ADV infection. Unfortunately, there are no mink macrophage cell lines, and studies in primary macrophage cultures are difficult. However, there are a number of human and murine cell lines exhibiting characteristics of macrophage lineages, and intercellular regulatory interactions in these species are well characterized.

Macrophages and similar cells play a pivotal role in regulating the immune response both by antigen presentation and by cytokine production. ADV infection of macrophages might interfere with one or more of these functions, disrupting normal regulatory interactions and thus leading to the abnormalities of the immune system so characteristic of this disease. One way to examine these ideas would be to develop an in vitro system in which the effect of ADV infection on macrophage function could be directly assessed.

In this study we infected primary cultures of mink peritoneal macrophages with ADV-Utah I in vitro and showed that this infection was not fully permissive. Then we screened several human and murine macrophage cell lines for ADV infection. The human macrophage cell line U937 selectively supported infection by the virulent ADV Utah I strain but not by ADV-G. We characterized the ADV infection of U937 cells compared with that of CRFK cells. Although we could show that ADV-Utah I produced mRNA, replicative-form (RF) DNA, virion DNA, and viral proteins, we could not demonstrate the production of infectious virions in ADV-infected U937 cells.

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MATERIALS AND METHODS

Cells and viruses. Cell-culture-adapted ADV-G was grown in CRFK cells as previously described (6, 17). ADV-G was quantified by fluorescence-forming units (FFU) in CRFK cells as previously described (11, 36). We prepared 10% suspensions of ADV-Utah I (15, 36) from the spleens of infected adult sapphire mink; the suspensions contained 10^7 or 10^5 adult mink 50% infective doses (ID_{50}s) per ml.

Peritoneal cells were collected from adult sapphire mink by peritoneal lavage as reported previously (23). Cells were washed twice and then counted in a hemocytometer after being stained with crystal violet solution. More than 80% of cells had large nuclei and abundant cytoplasm. Peritoneal cells were resuspended in Dulbecco's modified Eagle medium with the indicated concentration of heat-inactivated fetal calf serum (FCS; HyClone Laboratories Inc., Logan, Utah) and seeded in culture vessels coated with 2% gelatin. After a 6-h incubation, nonadherent cells were removed and fresh medium was added. After 3 days of culture at 37°C, more than 95% of cells were nonspecific esterase positive and thus were recognized as macrophages. After various periods, macrophage cultures were infected with ADV and then used for subsequent experiments.

Human macrophage cell lines U937 (44) and THP-1 (46) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS. Murine myelomonocytic cell lines WEHI-3BD$^-$ (31) and P388 (18) were grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS. The cells were collected at 1×10^5 to 3×10^5 cells per ml (greater than 95% viability) and used for ADV infection.

Murine macrophage cell lines J774A (39) and IC-21 (29) were grown in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated FCS. These adherent cells were grown in wells of eight-well chamber slides (Nunc Inc., Naperville, Ill.). The cells were infected with ADV when they reached subconfluency.

Virus infections. Viruses were diluted to the indicated concentration with phosphate-buffered balanced salt solution supplemented with 2% heat-inactivated FCS and adsorbed to cells at the indicated temperature for 3 h. Suspension cultures were washed once and resuspended in growth medium at a density of 10^5 cells per ml. For adherent cell cultures, virus inocula were aspirated after adsorption and fresh growth medium was added.

In some experiments, phorbol 12-myristate 13-acetate (PMA; Sigma Chemical, St. Louis, Mo.) was added to growth medium after adsorption of virus. PMA was dissolved in 95% ethanol at a concentration of 1 mg/ml and stored at −70°C until use.

Immunofluorescence. Cells in suspension cultures were cytocentrifuged onto poly-L-lysine (Sigma)-coated slides. The slides were fixed with acetone at 4°C for 10 min (32) and stained by direct immunofluorescence with fluorescein isothiocyanate-labeled ADV-infected mink serum as previously described (11). Cells showing nuclear staining were counted as positive for ADV infection.

Northern blot analysis. mRNA was extracted from cells by using a Micro Fast Track mRNA preparation kit (Invitrogen, San Diego, Calif.). RNA samples were electrophoresed in 0.7% formaldehyde–agarose gels, subjected to Northern (RNA) blot analysis, and hybridized with ^{32}P-labeled minus-sense ADV RNA probe as previously described (2, 12).

Southern blot analysis. Total cellular DNA was extracted from cells as previously described (43). DNA samples were electrophoresed in 0.7% agarose gels, Southern blotted, and hybridized with ^{32}P-labeled plus-sense ADV RNA probes as previously described (12).

Immunoblot analysis. Cells were directly lysed with Laemmli sample buffer and boiled for 5 min. Lysates were electrophoresed in sodium dodecyl sulfate–7.5% polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were incubated with a 1/100 dilution of ADV-infected adult mink serum and with a 1/4,000 dilution of peroxidase-labeled protein A (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and developed as previously described (8).

Labeling of viral DNA with [^{3}H]thymidine and density gradient centrifugation. [^{3}H]thymidine was added to ADV-G-infected CRFK cells or ADV-Utah I-infected U937 cells at 48 h postinfection to achieve a concentration of 100 μCi/ml. At 20 h later, 3 volumes of fresh medium was added to each culture, and the incubation was continued for a total of 7 days. Cells were collected and resuspended in 1 ml of 0.01 M Tris buffer (pH 7.4) for each culture and lysed by a freeze-thaw cycle. After a 4-h digestion of cellular nucleic acids with 200 μg of bovine pancreas DNase I (Pharmacia, Uppsala, Sweden) per ml and 20 μg of bovine pancreas RNase A (Pharmacia) per ml, virions were purified through a 40% (wt/vol) sucrose cushion by ultracentrifugation in a Beckman SW50.1 rotor at 35,000 rpm for 15 h at 15°C. The pellets were resuspended in 5 ml of 0.05 M Tris–0.005 M EDTA–0.1% Sarkosyl (pH 8.0), and solid CsCl was added to produce a final density of 1.37 g/cm^3. A gradient was formed by ultracentrifugation in a Beckman SW41 rotor at 28,000 rpm for 70 h at 15°C. Fractions (0.4 ml) were collected, and the density of each fraction was estimated with a refractometer as previously described (11). The acid-insoluble radioactivity of each fraction was measured by scintillation counting.

RESULTS

Characterization of ADV infection of resident peritoneal macrophages of adult mink. We collected peritoneal macrophages from sapphire mink and cultured them on gelatin-coated eight-well chamber slides at 37°C for 3 days in Dulbecco's modified Eagle medium supplemented with 30% FCS. Generally, the yield (0.5×10^7 to 1.5×10^7 macrophage-like cells per mink) was low. We infected the macrophage cultures with ADV-Utah I or ADV-G at 32 or 37°C and examined them by direct immunofluorescence 4 days after infection. Nuclear fluorescence was detected in a small percentage of the mink macrophages infected with ADV-Utah I (Fig. 1), but we could not detect any nuclear fluorescence in macrophages infected with ADV-G (results not shown). Thus, pathogenic ADV-Utah I infected macrophages but ADV-G did not.

We also examined mink alveolar macrophages. However, the number of alveolar macrophages susceptible to ADV-Utah I was smaller than that of peritoneal macrophages (results not shown) and was not as reproducible as that of peritoneal macrophages. Therefore, we focused on mink peritoneal macrophage cultures for the subsequent analysis of ADV-Utah I infection.

To optimize conditions for infection, we varied the temperature of the medium (32 or 37°C) and the FCS concentration in the medium (15, 25, 35 or 45%). Medium supplemented with 25% FCS at 32°C after absorption of virus was optimal for ADV-Utah I infection of mink macrophages (results not shown). We also determined that a 2-day interval between the seeding of macrophages and the challenge with ADV gave the largest number of cells with nuclear fluores-

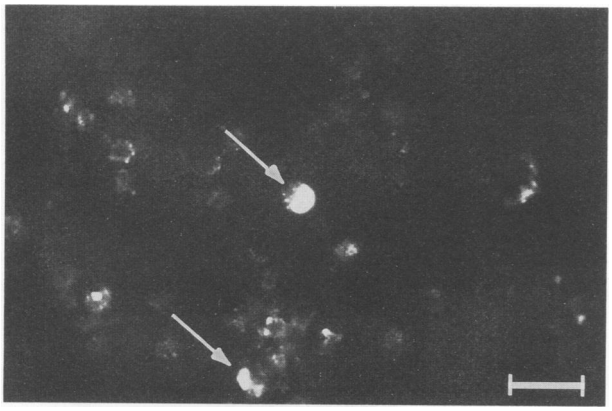

FIG. 1. Expression of viral antigens in mink peritoneal macrophages infected with ADV-Utah I. We seeded 5×10^5 peritoneal resident cells from male sapphire mink into each well of gelatin-coated eight-well chamber slides. At 3 days later the cells were infected with 2×10^5 ID_{50}s of ADV-Utah I and maintained at 32°C for 4 days. Adherent cells were stained with fluorescein isothiocyanate-labeled anti-ADV mink serum. Cells positive for nuclear expression of ADV antigens are indicated (white arrows). Bar, 40 μm.

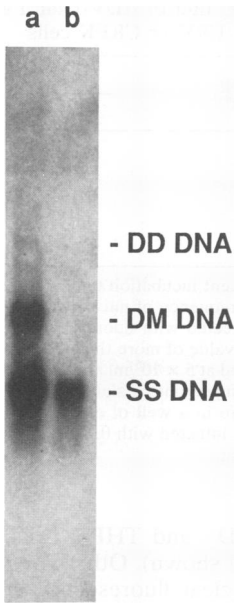

FIG. 2. Southern blot analysis of total cellular DNAs extracted from ADV-infected sapphire mink peritoneal macrophages. We cultured 10^7 peritoneal resident cells at 37°C for 2 days and infected them with 2×10^5 ID_{50}s of ADV-Utah I at 32°C. The cells were cultured at 32°C and collected at the indicated times after infection. DNA was extracted, and the entire samples were applied to wells in 0.7% agarose gel. After the electrophoresis, DNAs were Southern blotted and hybridized with ADV probe as described in Materials and Methods. Lanes: a, mink peritoneal macrophages at 72 h postinfection; b, mink peritoneal macrophages at 12 h postinfection.

cence (results not shown). Under these conditions, about 2% of mink macrophages could support ADV antigen expression.

Next, we performed Southern and Western blot analysis on samples prepared from ADV-Utah I-infected macrophage cultures at various times after infection.

In Southern blot analysis, we clearly detected viral DNA amplification. At 12 h after infection, we detected a signal corresponding to single-stranded virion DNA (SS DNA), probably corresponding to input virus. At 72 h after infection, RF DNAs (4.8-kb duplex monomer RF DNA [DM DNA] and 9.6-kb duplex dimer RF DNA [DD DNA]) were clearly detected in the DNA sample, and amplification of total ADV genomes between 12 and 72 h after infection was obvious (Fig. 2). Moreover, at 72 h after infection, the signal corresponding to virion DNAs was a little stronger than that corresponding to RF DNAs. Thus, amplification and replication of ADV DNA clearly occurred in mink macrophage culture. Although some macrophages showed specific nuclear staining by immunofluorescence, we could not elucidate the specific viral proteins by Western blot analysis (results not shown). These results showed that genomic amplification and protein synthesis, albeit at a low level, were occurring in mink macrophage cultures.

Finally, we tried to determine whether ADV-Utah I could be propagated in mink peritoneal macrophages. Cultures were infected with ADV-Utah I and harvested after a 4-day incubation at 32°C. Cultures were lysed by freeze and thaw, concentrated by ultracentrifugation (300,000 × *g* for 1 h), and used to reinfect fresh mink macrophages or CRFK cells. However, we were unable to detect ADV antigen and concluded that ADV was not being propagated in mink macrophages in vitro.

These results suggested that ADV-Utah I infection of mink macrophage culture was not fully permissive.

Screening of macrophage cell lines for ADV infection. Although we could demonstrate ADV replication in mink primary macrophage cultures, the limited cell numbers available make this an inconvenient system in which to analyze the details of ADV infection of macrophages. Thus, for further analysis, we screened several macrophage cell lines as a potentially more useful in vitro model of ADV infection. We infected human (U937 and THP-1) and murine (WEHI-3BD$^-$, P388, J774A, and IC-21) cell lines with ADV-G or ADV-Utah I at 32 or 37°C and assayed them for viral gene expression by direct immunofluorescence for ADV antigens 3 or 4 days after infection. We observed strong nuclear fluorescence in U937 cells (Fig. 3) and weak nuclear fluores-

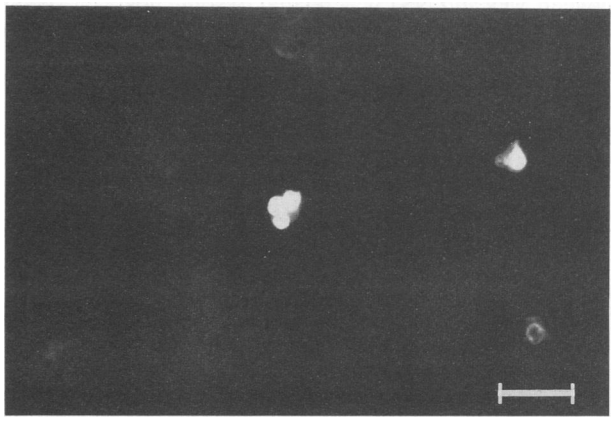

FIG. 3. Expression of ADV proteins in nuclei of ADV-Utah I-infected U937 cells. We infected 5×10^5 U937 cells with 5×10^4 ID_{50}s of ADV-Utah I and cultured them at 37°C for 4 days. Then 300 μl of U937 cell culture was cytocentrifuged on a slide and stained with fluorescein isothiocyanate-labeled anti-ADV mink serum. Bar, 40 μm.

TABLE 1. FFU titer of ADV-Utah I and ADV-G in U937 or CRFK cells

ADV strain	Temp (°C)[a]	FFU/ml[b]	
		U937[c]	CRFK[d]
Utah I[e]	32	1.4×10^6	4.2×10^5
	37	1.7×10^6	1.5×10^5
G	32	$<5 \times 10^2$	2.0×10^7
	37	$<5 \times 10^2$	4.7×10^6

[a] Infection and subsequent incubation temperature.
[b] Calculated from the frequency of nuclear expression of viral antigens on 3 days after infection detected with fluorescein isothiocyanate-labeled anti-ADV mink serum. Mean value of more than three independent experiments.
[c] Cells were resuspended at 5×10^5/ml and infected with ADV. Then 300 μl of culture was cytocentrifuged onto a slide for staining.
[d] A subconfluent culture in a well of eight-well chamber slides (approximately 2×10^5 cells) was infected with 0.1 ml of ADV solution.
[e] Present at 10^7 ID_{50}s/ml.

cence in WEHI-3BD$^-$ and THP-1 cells after ADV-Utah I infection (results not shown). Other lines were negative. We could not detect nuclear fluorescence in any of these cells infected with ADV-G. Therefore, because U937 is a permanent monocytic cell line (44), we selected U937 as a model cell line to study ADV-Utah I infection of macrophages.

Characterization of ADV infection of U937 cells. We compared ADV-G and ADV-Utah I infection in U937 and CRFK cells. ADV-G induced viral protein expression in CRFK cells but not in U937 cells. On the other hand, ADV-Utah I induced viral protein expression in both CRFK and U937 cells; however, the number of ADV-Utah I-positive cells was almost 10-fold larger for U937 cells than for CRFK cells (Table 1).

By Western blot analysis, we identified VP1, VP2, and NS1 proteins in ADV-Utah I-infected U937 cells (Fig. 4, lane d). However, a difference in the relative ratio among species was noted when compared with CRFK cells permissively infected with ADV-G (11). In ADV-G-infected CRFK cells, the VP bands were stronger than the NS1 band (lane b), whereas in ADV-Utah I-infected U937 cells, the VP bands were weaker than the NS1 band (lane d). Since the VP bands were stronger than the NS1 band in ADV-Utah I-infected CRFK cells (lane e), the difference in the relative ratio between VPs and NS1 was related to some host cell-specific differences and not to a difference between the two strains of virus.

By Southern blot analysis, we could detect RF DNAs (DM DNA and DD DNA) in ADV-Utah I-infected U937 cells (Fig. 5, lane d). However, a difference in the relative ratio was also noted. Although the signal from SS virion DNA was stronger than that from RF DNAs in ADV-G- or ADV-Utah I-infected CRFK cells, the RF DNA signal was much stronger than the SS virion DNA signal in ADV-Utah I-infected U937 cells (Fig. 5); i.e., there was a relative depression in the amount of SS DNA.

By Northern blot analysis of mRNA from ADV-Utah I-infected U937 cells, we could detect three major bands which were also observed in mRNA from ADV-G-infected CRFK cells (Fig. 6, lanes a and b). However, in U937 cells, the ADV mRNA which corresponded to the 2.8-kb band in CRFK cells was split. The smaller species was estimated to be about 0.3 kb smaller than the larger (lane c). Interestingly, in ADV-Utah I-infected CRFK cells, the 2.8-kb band seemed to be single (lane d). Thus, this smaller transcript was probably related to some host cell-specific differences in RNA processing and not to a difference between the two strains of ADV.

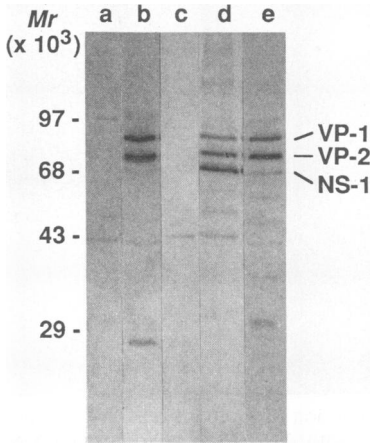

FIG. 4. Western blot analysis of proteins extracted from ADV-infected cell lines. We infected 5×10^5 U937 cells with 5×10^5 ID_{50}s of ADV-Utah I, and we infected subconfluent cultures of CRFK cells in a six-well tissue culture plate (approximately 2×10^6 cells) with 5×10^5 ID_{50}s of ADV-Utah I or 4.2×10^4 FFU of ADV-G per ml. Cells were cultured at 37 or 32°C, collected at the indicated times, and lysed with 150 μl of Laemmli sample buffer. A 50-μl portion of each sample (2.8×10^4 infected-cell equivalents in U937 cells and 7×10^3 ADV-Utah I-infected-cell equivalents or 1.4×10^4 ADV-G-infected-cell equivalents in CRFK cells) was applied to each well in gels. After the electrophoresis, proteins were transferred onto nitrocellulose membrane, incubated with ADV-infected mink serum, and visualized as described in Materials and Methods. Molecular weight markers are indicated (*Mr*). Lanes: a, uninfected CRFK cells; b, CRFK cells infected with ADV-G at 32°C, 96 h postinfection; c, uninfected U937 cells; d, U937 cells infected with ADV-Utah I at 37°C, 96 h postinfection; e, CRFK cells infected with ADV-Utah I at 32°C, 96 h postinfection.

Nonpermissivity of ADV-Utah I infection of U937 cells. The above studies showed that viral mRNA, RF DNA, and viral proteins were induced in ADV-Utah I-infected U937 cells. Therefore, we next tried to determine whether ADV could be propagated in U937 cells.

U937 cell cultures were infected with ADV-Utah I (multiplicity of infection, 0.3 to 1.5). After a 4-day incubation at 32 or 37°C, cells were harvested by centrifugation and lysed by a freeze-thaw cycle. The lysate was concentrated by ultracentrifugation (300,000 × *g* for 1 h) and used to reinfect U937 cells. We passaged ADV-Utah I in this way twice in U937 cells at 32 or 37°C and checked the FFU titer in both CRFK and U937 cells after each passage. However, we were unable to detect ADV gene expression in either cell line (results not shown) and concluded that ADV was not being serially propagated in U937 cells.

We also tried to demonstrate incorporation of [^{3}H]thymidine into virions by density gradient centrifugation. However, although we could easily detect a radioactive peak in mature virions (1.43 g/cm^3) in the samples from ADV-G-infected CRFK cells, we could not detect any radioactive peak in fractions from ADV-Utah I-infected U937 cells (Fig. 7).

These results indicated that although ADV-Utah I gene expression occurred in U937 cells, the infectious process was not fully permissive and that DNA-containing virus particles were not being produced at a detectable level.

Effect of differentiation state on ADV-Utah I infection of

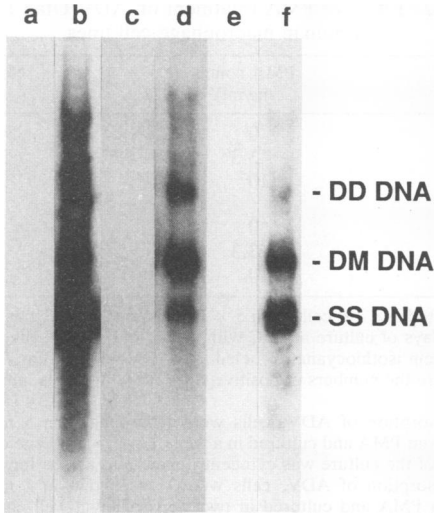

FIG. 5. Southern blot analysis of total cellular DNAs extracted from ADV-infected cell lines. Cells were cultured and infected with ADV as described in the legend to Fig. 4. DNA was extracted, and one-sixth of the extracted DNA samples (1.4×10^4 infected-cell-equivalents in U937 cells and 3.5×10^3 ADV-Utah I-infected-cell equivalents or 7×10^3 ADV-G-infected-cell equivalents in CRFK cells) was applied to each well in 0.7% agarose gels and analyzed as described in the legend to Fig. 2. Lanes: a, CRFK cells infected with ADV-G at 32°C, 6 h postinfection; b, CRFK cells infected with ADV-G at 32°C, 96 h postinfection; c, U937 cells infected with ADV-Utah I at 37°C, 6 h postinfection; d, U937 cells infected with ADV-Utah I at 37°C, 96 h postinfection; e, CRFK cells infected with ADV-Utah I at 32°C, 6 h postinfection; f, CRFK cells infected with ADV-Utah I at 32°C, 96 h postinfection.

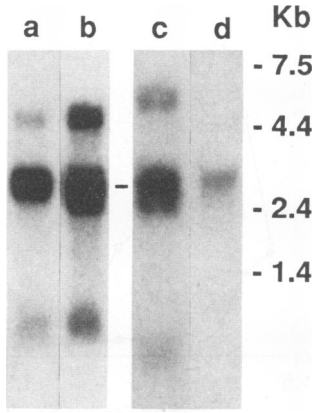

FIG. 6. Northern blot analysis of mRNAs extracted from ADV-infected cell lines. We infected 5×10^6 U937 cells with 5×10^6 ID_{50}s of ADV-Utah I, and we infected subconfluent cultures of CRFK cells in 60-mm tissue culture dishes (approximately 5×10^6 cells) with 1.4×10^6 ID_{50}s of ADV-Utah I or 1.2×10^5 FFU of ADV-G. Cells were cultured at 37 or 32°C and collected at 48 h postinfection. Half the amount of extracted mRNAs from ADV-Utah I-infected CRFK cells (3×10^4 infected-cell equivalents) and one-fourth of the other preparations (2.1×10^5 infected-cell equivalents in U937 cells infected with ADV-Utah I and 3×10^4 infected-cell equivalents in CRFK cells infected with ADV-G) were applied to each well, Northern blotted, and hybridized with ADV probe as described in Materials and Methods. RNA size markers are indicated on the right. Lanes: a, CRFK cells infected with ADV-G at 32°C, 22-h exposure at −70°C; b, U937 cells infected with ADV-Utah I at 37°C, 22-h exposure at −70°C; c, cells from lane b, 7-h exposure at −70°C, d; CRFK cells infected with ADV-Utah I at 32°C, 22-h exposure at −70°C.

macrophage cell lines. As stated above, macrophage cell lines from the same species varied in their permissivity for ADV infection. Furthermore, in adult mink, only a small portion of macrophages are permissive for ADV gene expression in vitro and in vivo (23, 32). These findings suggested that the state of macrophage differentiation might influence permissivity for ADV.

To examine this possibility, we infected the macrophage cell lines U937 and THP-1 with ADV-Utah I and then incubated them with various concentrations of PMA. These concentrations of PMA are sufficient to induce macrophage differentiation in both cell lines (22, 45), and in fact, both cell lines became adherent after PMA treatment. When we looked at immunofluorescence following this treatment, it was apparent that PMA had different effects on the two cell lines. PMA treatment of U937 cells caused a decrease in the number of cells positive for ADV antigen expression, whereas PMA treatment of THP-1 cells caused an apparent increase in the number of positive cells (Table 2).

DISCUSSION

Macrophages are targets for ADV replication in adult mink (23), and our previous studies indicate that at the cellular level this infection is not fully permissive (3). It is, however, unclear how infection of macrophages might contribute to the constellation of immunological aberrations that characterize infections with pathogenic strains of ADV. Investigation of the pathogenesis of ADV infection in macrophages has been hampered by the lack of a suitable in vitro model. In this paper, we describe studies of ADV infection of primary mink macrophages and several macrophage cell lines.

The pathogenic ADV-Utah I strain infected mink peritoneal macrophages as defined by the induction of viral proteins by immunofluorescence. By this criterion, up to 2% of the cells were infected. In addition, although amplification of ADV DNA and the presence of RF DNA were demonstrated, detectable virus could not be serially propagated in macrophage cultures. Thus, the in vitro infection of mink macrophages resembled the restricted infection observed in vivo (3), in which levels of obligate replicative intermediates are severely reduced. These results suggested that in vitro infection of mink macrophages might be a suitable model; however, intensive analysis of this system was not practical because of low cell recovery from animals and the limited number of animals available for study. Consequently, several macrophage cell lines were screened.

The human macrophage cell line U937 was found to be susceptible to infection by ADV-Utah I. When conditions were optimized, approximately 4% of the U937 cells scored positive for ADV antigens after ADV-Utah I infection. Furthermore, at a given multiplicity of infection the number of U937 cells positive for infection was greater than that of CRFK cells (Table 1). Under similar conditions, no evidence of ADV-G replication or gene expression could be demonstrated in U937 cells.

As was the case for primary mink macrophage cultures, the infection of U937 cells by ADV-Utah I was not fully permissive. Although we could identify ADV mRNA, genome amplification, and ADV proteins, we could not serially propagate virus or demonstrate incorporation of nucleic acid

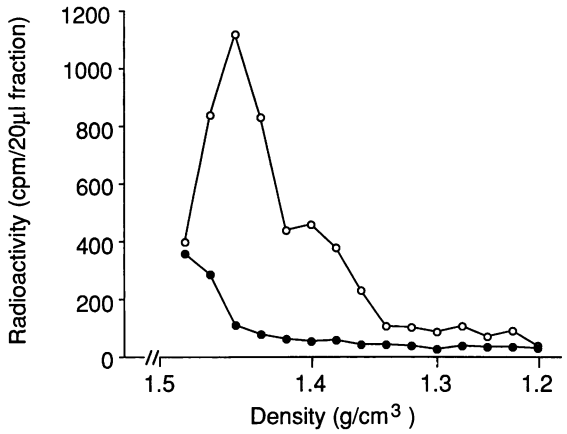

FIG. 7. CsCl density gradient fractionation of lysates of ADV infected cells. We infected 2×10^6 U937 cells with 2×10^6 ID_{50}s of ADV-Utah I and maintained them at 37°C; we also infected a 150-cm^2 subconfluent culture of CRFK cells (approximately 10^7 cells) with 1.3×10^6 FFU of ADV-G and maintained them at 32°C. Cultures were labeled with 100 μCi of [^{3}H]thymidine per ml at 48 h postinfection, and after 4 days of culture, virions were purified and analyzed by equilibrium density gradient centrifugation as detailed in Materials and Methods. Incorporation of [^{3}H]thymidine into ADV virions was assayed by determining the acid-insoluble radioactivity in 20-μl samples of each fraction. Symbols: ●, lysate from U937 cells infected with ADV-Utah I, ○, lysate from CRFK cells infected with ADV-G.

TABLE 2. Effect of PMA treatment on ADV-Utah I infection of human macrophage cell lines

Cell line[a]	PMA concn (ng/ml)	No. of positive cells[b]
U937	0[c]	4.2×10^4
	3.3[d]	3.8×10^3
	10[d]	2.9×10^3
THP-1	0	$<1 \times 10^2$
	3.3	2.6×10^3
	10	2.7×10^3

[a] We infected 5×10^5 cells with 5×10^5 ID_{50}s of ADV-Utah I.

[b] After 3 days of culture at 37°C with or without PMA, cells were stained with fluorescein isothiocyanate-labeled anti-ADV mink serum. The numbers in the table are the numbers of positive cells in 5×10^5 cells initially infected with ADV.

[c] After adsorption of ADV, cells were resuspended in 5 ml of growth medium without PMA and cultured in a well of a six-well tissue culture plate. Then 300 μl of the culture was cytocentrifuged onto a slide for staining.

[d] After adsorption of ADV, cells were resuspended in 1 ml of growth medium with PMA and cultured in two wells of eight-well chamber slides coated with 2% gelatin.

precursors into virions. Therefore, we suggest that the cross-species use of the U937 macrophage cell line represents a relatively faithful model of the ADV infection of mink macrophages.

A number of interesting observations about the ADV-Utah I-infected U937 cells were made, and the results may be relevant to the restricted in vivo infection of macrophages. By Western blot analysis, the relative ratio between the amount of capsid proteins (VP1 and VP2) and NS1 nonstructural protein in ADV-Utah I-infected U937 cells differed from that observed in CRFK cells permissively infected with ADV-G (10, 11). For a given amount of NS1 protein, the level of the capsid proteins was depressed. Not only were the level of capsid proteins reduced, but also the level of SS DNA relative to RF DNA was depressed in comparison with that in ADV-G-infected CRFK cells or ADV-Utah I-infected mink kit lung tissue (1). A marked deficiency in the synthesis of capsid proteins and SS DNA genomes would limit the number of virus particles produced by an infected cell and lead to a low-level restricted infection.

The relatively low level of capsid proteins might be related to inefficient *trans*-activation of the promoter for capsid protein mRNA (p36) by NS1, or, alternatively, there might be aberrant transcription in the U937 cells. The latter hypothesis is particularly intriguing because the transcription program in U937 cells showed definite differences from that in the CRFK cells. Specifically, the ADV-Utah I-infected U937 cells contained a 2.5-kb transcript, which was not detected in ADV-G or ADV-Utah I-infected CRFK cells or in ADV-Utah I-infected mink kit lung tissue (24). We speculated that this 2.5-kb mRNA may be an aberrant form of one of the two species found in the 2.8-kb band, possibly resulting from alternative splicing. The 2.8-kb mRNA band has two species; R3 mRNA uses the p36 promoter and codes for the two capsid proteins (VP1 and VP2), and R2 mRNA uses the p3 promoter and codes for nonstructural protein (NS2) (2). In fact, NS2 influences the replication of the parvovirus minute virus of mice in a cell-type-specific fashion (13, 33), and a defective NS2 transcript might affect the permissiveness of ADV infection in certain cell types.

On the other hand, we can propose a mechanism by which an altered R3 transcript might lead to low-level capsid synthesis and restricted infection. If the R3 transcript were rendered nonfunctional by improper processing or splicing, synthesis of the capsid proteins VP1 and VP2 would be abrogated, but this would have to be reconciled with the observation that the capsid proteins are observed, albeit at low levels, in infected U937 cells. However, the transcription map of ADV (2) indicates that the 2.8-kb R2 transcript, initiating at p3, contains all the coding information required for the capsid proteins in addition to NS2. If synthesis of VP1 and VP2 initiated on the R2 mRNA inefficiently by a leaky scanning mechanism (25) or by attenuation of the NS2 ATG (5), low levels of VP1 and VP2 could be accounted for in the absence of an intact R3 mRNA. We are currently evaluating mRNA from ADV-infected U937 cells with transcript-specific oligonucleotide hybridization to identify this novel transcript and clarify its structure.

Although ADV-G gave a high FFU titer in CRFK cells, it produced no nuclear fluorescence in U937 cells, whereas ADV-Utah I induced antigen expression in both U937 and CRFK cells. These observations suggest that the mechanism responsible for ADV entry and/or subsequent gene expression may differ between U937 and CRFK cells. The fact that U937 cells are macrophage lineage cells and express Fc receptors has suggested to us that a mechanism of antibody-dependent enhancement of virus infection (38) may play a role in ADV-Utah I infection of U937 cells. Antibody-dependent enhancement is observed in dengue virus (20) and lactate dehydrogenase virus (14) infection and might contribute to the establishment of persistent infections. Moreover, ADV-Utah I was prepared from a spleen homogenate from infected mink and is therefore coated with mink immunoglobulins (35). On the other hand, ADV-G was prepared from CRFK tissue-cultured cell lysates and is therefore antibody free. We are currently examining a role for antibody-dependent enhancement in ADV infection of macro-

phages. In preliminary experiments, we have induced nuclear expression of ADV antigens in U937 cells infected with ADV-G by adding immunoglobulins prepared from ADV-infected mink (24).

The state of maturation and activation is known to influence the susceptibility of macrophages to several viruses (19, 26, 48). Some of our findings suggested that the state of macrophage differentiation or activation might be related to ADV susceptibility. U937 cells supported ADV-Utah I infection much more efficiently than THP-1 cells did, although the two cell lines have nearly identical characteristics such as nonadherence, phagocytosis, Fc receptor expression, and esterase activity (43, 46). Furthermore, PMA treatment is known to increase several parameters indicative of macrophage activation in both cell lines (22, 45). However, with PMA treatment, the two cell lines reacted differently to ADV infection; the susceptibility of U937 cells decreased whereas that of THP-1 clearly increased. Thus, not only was the state of macrophage differentiation related to the level of ADV susceptibility but also subtle factors not reflected in functional activities must be involved.

That FCS concentration and temperature of incubation also affected the level of ADV infection of primary mink macrophage cultures suggested that similar factors relating to macrophage differentiation or metabolic state may exist in vivo. This might explain why although many macrophages contain ADV DNA in vivo, only a small fraction are engaged in ADV gene expression at any one time (23).

U937 cells could not support the production of infectious ADV. However, U937 is a macrophage lineage cell line, and macrophages are target cells in ADV infection in vivo (23). Therefore, since U937 cells clearly supported ADV mRNA expression, ADV genome amplification, and ADV protein expression, this cell line may be useful as a cell model for the analysis of the functional alterations induced in macrophages by ADV infection. Even a restricted infection might be able to induce inappropriate production of cytokines by infected macrophages or to alter macrophage effector functions. Further analysis in this model may contribute to the study of interrelationships between parvovirus infections and subsequent immune disorders, not only in mink but also in humans (27, 40, 42).

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