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Aleutian Mink Disease Parvovirus Infection of Mink Macrophages and Human Macrophage Cell Line U937: Demonstration of Antibody-Dependent Enhancement of Infection

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Aleutian mink disease parvovirus (ADV) infects macrophages in adult mink. The virulent ADV-Utah I strain, but not the cell culture-adapted ADV-G strain, infects mink peritoneal macrophage cultures and the human macrophage cell line U937 in vitro. However, preincubation of ADV-G with ADV-infected mink serum enhanced its infectivity for U937 cells. The enhancing activity was present in the protein A-binding immunoglobulin G fraction in the serum, but $F(ab')_2$ fragments failed to enhance the infection. On the other hand, the same sera inhibited ADV-G infection of Crandell feline kidney (CRFK) cells. Although U937 cells were not fully permissive for antibody-enhanced ADV-G infection, ADV mRNA expression, genome amplification, and protein expression were identical to those found previously for ADV-Utah I infection of U937 cells. Preincubation of ADV-Utah I with soluble protein A partly inhibited the infection of U937 cells but did not affect infection of CRFK cells. In mink peritoneal macrophages, preincubation with the infected mink serum did not make ADV-G infectious. However, the infectivity for mink macrophages of antibody-free ADV-Utah I prepared from the lungs of infected newborn mink kits was enhanced by ADV-infected mink serum. Moreover, protein A partly blocked ADV-Utah I infection of mink macrophage cultures. These results suggested that ADV-Utah I enters mink macrophages and U937 cells via an Fc receptor-mediated mechanism. This mechanism, antibody-dependent enhancement, may also contribute to ADV infection in vivo. Furthermore, since ADV infection in mink is characterized by overproduction of anti-ADV immunoglobulins, antibody-dependent enhancement may play a critical role in the establishment of persistent infection with ADV in vivo.

Aleutian mink disease parvovirus (ADV) causes a persistent infection associated with a severe disorder of the immune system. The disease in adult mink, known as classical Aleutian disease (AD), is characterized by marked hypergammaglobulinemia, plasmacytosis, arteritis, and fatal immune complex glomerulonephritis (37, 38). Moreover, serum immune complexes that contain ADV from infected adult mink are still infectious (35).

Studies on adult mink infected with ADV indicate that macrophages are target cells for ADV replication but that infection is restricted (25, 32) in that the levels of replicative intermediates are greatly reduced compared with those in permissive infections (4, 10, 14). In order to study the virus-cell interactions in a more controlled system, we recently developed an in vitro system of ADV infection with the human macrophage lineage cell line U937 (26). In these studies, we found that the pathogenic ADV-Utah I strain could infect the U937 cells and that the infection was restricted in a fashion similar to that observed for mink macrophages in vivo (4, 25, 26, 32). However, the nonpathogenic cell culture-adapted ADV-G strain (12) was unable to initiate infection in U937 cells (26). On the other hand, both ADV-G and ADV-Utah I can infect Crandell feline kidney (CRFK) cells (12).

These differences suggest that there may be unique features about the virus-cell interactions. U937 cells are macrophage lineage cells and express Fc receptors (44), and although they are not mink cells, their infection with ADV-Utah I bears similarities to the in vivo situation (4, 25, 26, 32). ADV-Utah I prepared from organ homogenates of infected adult mink (ADV-Utah I/adult) is coated with mink immunoglobulins (Igs) (13, 17), but ADV-G derived from infected CRFK cells is antibody free. These observations suggested that the antibody coating the ADV-Utah I prepared from adult mink might facilitate infection of U937 cells by this virus via binding to Ig receptors on the cell surface.

Antibody-dependent enhancement (ADE) is a phenomenon in which antivirus antibodies enhance entry of virus into cells via Fc- or complement receptor-mediated pathways (39). Several chronic infections of Fc receptor-bearing cells are associated with ADE (39).

In this study, we demonstrated that preincubation of ADV-G with serum containing anti-ADV antibody enhanced its infectivity for U937 cells, whereas the same treatment neutralized ADV-G infectivity for CRFK cells. This enhancing effect of the serum was present in the protein A-binding Ig fractions but not in the $F(ab)_2$ fragments. We also examined a possible role for ADE in ADV infection of mink peritoneal macrophages.

MATERIALS AND METHODS

Cells and viruses. Growth of cell culture-adapted ADV-G in CRFK cells (19) was done as previously described (12). ADV-G was quantified as fluorescence-forming units (FFU) in CRFK cells as previously described (12, 36). Suspensions (10%) of ADV-Utah I/adult (17, 36) were prepared from the spleens of infected adult sapphire mink and contained 10^5 50% adult mink infective doses (ID_{50}s) per ml. ADV-Utah I virions were concentrated by ultracentrifugation in a Beckman SW50.1 rotor at 50,000 rpm for 90 min at 4°C and resuspended

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in 0.1 volume of the supernatant or phosphate-buffered balanced salt solution (PBBS) for serum-free preparation. Suspensions (10%) of ADV-Utah I/kit were prepared from the lungs of infected sapphire mink kits (2).

Resident peritoneal cells were collected from adult sapphire mink by peritoneal lavage as reported before (25). Cells were washed twice and then counted in a hemacytometer after staining with crystal violet solution. More than 80% of the cells had large nuclei and abundant cytoplasm. Peritoneal cells were resuspended in Dulbecco's modified Eagle's medium (DME) with 25% heat-inactivated fetal calf serum (FCS; HyClone Laboratories Inc., Logan, Utah) and seeded in eight-well chamber slides coated with 2% gelatin. After 6 h of incubation at 37°C, nonadherent cells were removed and fresh medium was added. After 2 days of culture at 37°C, more than 95% of the cells were nonspecific esterase positive and thus were recognized as macrophages. These macrophage cultures were used for ADV infection.

Human macrophage lineage cell line U937 (44) was grown in RPMI 1640 medium supplemented with 10% heat-inactivated FCS. Cells were collected at 1×10^5 to 3×10^5 cells per ml (>95% viability) and used for ADV infection.

Mink sera and Ig fractions. Serum samples from ADV-Utah I-infected mink (60 days postinoculation) and uninfected mink were obtained from adult sapphire mink, heat inactivated at 56°C for 30 min, and ultracentrifuged in a Beckman SW50.1 rotor at 50,000 rpm for 90 min at 4°C. The supernatants were saved and used as mink sera in the following experiments. These sera do not induce ADV antigen expression in CRFK or U937 cells. The anti-ADV titer of the infected mink sera, as assayed by counterimmunoelectrophoresis, was 1:1,024, while that of the normal sera was <1:4 (13).

Protein A-binding IgG fractions were purified from mink sera by ammonium sulfate precipitation followed by affinity column chromatography with protein A-agarose (Bio-Rad Laboratories, Richmond, Calif.) as described previously (24). The IgG was analyzed by nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and greater than 95% was found in a single band (results not shown) and adjusted to a concentration of 0.3 mg/ml in Hanks' balanced salt solution (HBSS). Only three classes of Igs (IgG, IgM and IgA) have been identified in mink (18), and the only protein A-binding Ig is IgG (17a).

$F(ab')_2$ fragments were prepared by dialyzing the IgG into 0.05 M NaCl–0.07 M acetate buffer (pH 4.0) and digesting with pepsin (Worthington Biochemical Corp., Freehold, N.J.) at a ratio of 150 μg of pepsin per 15 mg of IgG. After a 15-h incubation at 37°C, the reaction mixture was dialyzed against 0.14 M phosphate buffer (pH 8.0) and applied to a protein A column. The passthrough fraction containing $F(ab')_2$ was collected and adjusted to a concentration of 0.2 mg/ml in HBSS. The pepsin digestion was monitored by nonreducing SDS-PAGE and produced single uniform Fc and $F(ab')_2$ species. The $F(ab')_2$ preparation was adjusted to the same molarity as the IgG fraction.

Assay for the effects of sera and Ig fractions on ADV infection. Serial fourfold dilutions of serum, IgG, or $F(ab')_2$ were mixed with an equal volume of ADV diluted in PBBS supplemented with 2% heat-inactivated FCS. After 1 h of incubation at 37°C, 0.1 ml of the mixture was applied to a subconfluent culture of CRFK cells eight-well chamber slides (approximately 2×10^6 cells per well) or mixed with 10 μl of U937 cell suspensions (5×10^4 cells per well) in eight-well chamber slides. After 3 h of incubation, 4 volumes of fresh medium were added, and the cells were cultured for 4 days. After the medium was aspirated, U937 cells were cytocentrifuged onto slides. The slides were fixed with acetone at 4°C for 10 min and stained by direct immunofluorescence with fluorescein isothiocyanate (FITC)-labeled ADV-infected mink serum as described previously (12). Cells showing nuclear fluorescence were considered positive for ADV infection.

Nucleic acid analysis. mRNA was extracted from cells with the Fast Track mRNA preparation kit (Invitrogen, San Diego, Calif.). RNA samples were electrophoresed in 1% formaldehyde–agarose gels, Northern blotted (RNA blotted), and hybridized with the ^{32}P-labeled minus-sense ADV RNA probe as described previously (3, 14).

Total cellular DNA was extracted from cells as described previously (41). DNA samples were electrophoresed in 0.7% agarose gels, Southern blotted, and hybridized with ^{32}P-labeled plus-sense ADV RNA probes as described previously (3, 14).

Immunoblot analysis. Cell pellets were directly lysed with Laemmli sample buffer and boiled for 5 min. Lysates were electrophoresed in SDS–7.5% polyacrylamide gels and blotted onto nitrocellulose membranes. Membranes were incubated with a 1:100 dilution of ADV-infected mink serum and with a 1:4,000 dilution of peroxidase-labeled protein A (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.) and developed as described previously (11).

Lipofection of ADV-G in U937 cells. ADV-G solution (57 μl, 10^6 FFU) or PBBS was mixed with 10 μl of Lipofectin (GIBCO BRL Life Technologies Inc., Gaithersburg, Md.) and incubated at room temperature for 15 min. Then, 33 μl of each mixture was added to 1 ml of a U937 suspension in DME (5×10^6 cells), and the cells were incubated at 37°C in six-well tissue culture plates. After 15 h, 4 ml of fresh medium was added to each well, and incubation was continued at 32 or 37°C for 4 days. A 300-μl amount of culture was cytocentrifuged onto poly-L-lysine-coated slides, and the cells were examined for ADV antigen expression by immunofluorescence as described above. Cells were collected from the remaining 4 ml of culture and used for total DNA extraction.

Assay for effects of protein A on ADV-Utah I infection. The serum-free ADV-Utah I/adult strain was incubated at 37°C for 1 h with an equal volume of various concentrations of protein A (Pharmacia, Uppsala, Sweden) in HBSS. Cells were infected with this mixture and examined for ADV antigen expression by immunofluorescence as described above.

RESULTS

Effects of infected and uninfected mink sera on ADV infection of U937 and CRFK cells. Our previous studies showed that ADV-Utah I/adult infects both CRFK and U937 cells, whereas ADV-G infects only CRFK cells (26). Because U937 is a macrophage lineage cell line and expresses Fc receptors, one possible explanation for these phenomena was that mink antibodies complexed to the virus made ADV-Utah I/adult infectious for U937 cells. Therefore, we looked to see whether infected mink sera made ADV-G infectious for U937 cells.

Preincubation of ADV-G with serum from ADV-infected mink (AD serum) clearly enhanced the number of U937 cells infected (Fig. 1 and 2a). Furthermore, dilution of AD sera had a zonal effect, i.e., 256- to 4,096-fold dilutions of sera induced optimal enhancement (Fig. 2a), although some enhancemcnt was noted even at greater serum dilutions. This result resembled an equivalence phenomenon characteristic of antigen-antibody reactions. Moreover, preincubation with normal mink serum did not enhance the infection (Fig. 2a). These experiments were repeated several times. Although the maximum level of enhancement varied among the experiments, the effect of AD sera was consistent. These results suggested that AD

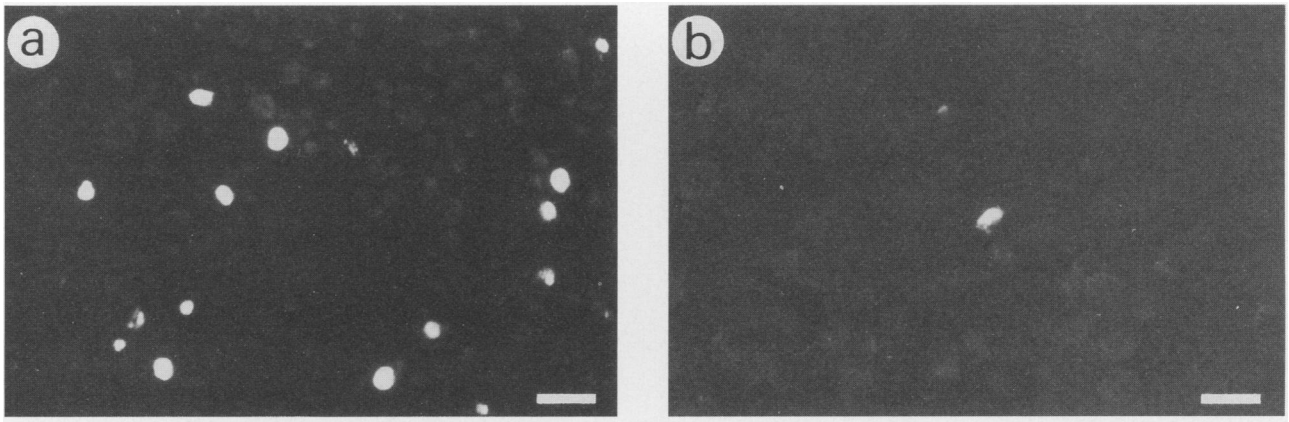

FIG. 1. Expression of ADV proteins in ADV-G-infected U937 cells. ADV-G (10^6 FFU) was mixed with an equal volume of (a) 250-fold-diluted AD serum in PBBS or (b) PBBS alone. After 1 h of incubation at 37°C, the mixtures were mixed with an equal volume of U937 cell suspensions (5×10^5 cells) in PBBS with 2% FCS. After 3 h of incubation at 32°C, the U937 cells were washed once, suspended in growth medium at a density of 10^5 cells per ml, and cultured for 4 days. Then, 400 μl of U937 cell culture was cytocentrifuged and stained with FITC-labeled anti-ADV mink serum. (a) In the presence of AD serum, up to 8% of the cells were positive for ADV protein expression (b) In the absence of AD serum, less than 0.1% of the cells were positive. Bars, 50 μm.

serum enhanced the infection of U937 cells by ADV-G, as is found in ADE models.

In contrast, the same AD sera had an opposite effect on ADV-G infection of CRFK cells. In this case, as reported before (42), the infectivity of ADV-G was specifically inhibited by up to 90% with 4- to 16-fold dilutions of AD sera. Thus, ADV-infected mink sera had an obvious neutralizing effect on ADV-G infection of CRFK cells, although the neutralizing effect was diluted out more quickly than the enhancing effect. Preincubation of ADV-Utah I/adult with these sera did not significantly alter the titer of ADV for U937 or CRFK cells.

The effects of mink sera on ADV infection of U937 and CRFK cells are summarized in Table 1.

Effects of IgG and $F(ab')_2$ fractions on ADV infection of U937 and CRFK cells. In order to show that the enhancing activity in AD sera was mediated by Igs, we repeated the experiments with IgG fractions.

The IgG fraction from infected mink sera (AD IgG) enhanced ADV-G infection of U937 cells (Fig. 2b) to at least a concentration of 0.3 μg/ml, a value that corresponds approximately to a 16,384-fold serum dilution. IgG prepared from uninfected mink sera did not enhance infection (Fig. 2b). On the other hand, AD IgG had an opposite effect on ADV-G infection of CRFK cells, because preincubation inhibited the infection of CRFK cells by ADV-G up to 70% at a concentration of 75 to 19 μg of IgG per ml. The IgG fraction from uninfected mink sera did not affect infectivity (results not shown). These results suggested that the IgG fraction from infected mink sera enhanced ADV-G infection of U937 cells but neutralized ADV-G infection of CRFK cells.

Next, we wished to determine whether the enhancing effect of AD IgG was a result of Fc receptor-mediated entry. Thus, we prepared $F(ab')_2$ fragments from the IgG fractions and examined their effects in similar experiments.

Incubation of ADV-G with $F(ab')_2$ fragments from AD serum did not enhance ADV-G infection of U937 cells (results not shown), suggesting that an intact Fc portion was essential. $F(ab')_2$ fragments of IgG from uninfected mink serum also did not change ADV-G infectivity for U937 cells. Since U937 cells are macrophage lineage cells and express surface Fc receptors (9, 44), these results suggested that ADV–anti-ADV immune complexes infect U937 cells via an Fc receptor-mediated entry mechanism, i.e., ADE might play a role in ADV-G infection of U937 cells. When the same $F(ab')_2$ preparation was tested on CRFK cells, it still inhibited ADV-G infection up to 70% at a concentration of 50 to 13 μg/ml. We could not demonstrate specific binding of mink Igs on CRFK cells by indirect immunofluorescence with rabbit anti-mink Igs and subsequent FITC-labeled goat anti-rabbit Igs (results not shown). This is not surprising, because CRFK cells are a renal epithelial cell line and would not be expected to show Fc receptor expression. This set of results implied that the Fc portion was not essential for the neutralizing activity of AD IgG on CRFK cells.

Characterization of ADV-G infection of U937 cells mediated with AD sera. The data presented above showed that preincubation of ADV-G with AD sera enhanced ADV antigen expression in U937 cells. However, we wanted to define the ADE-mediated infectious cycle of ADV in U937 cells and compare it with ADV-G infection in CRFK cells.

Neither ADV-G nor ADV-Utah I/adult could be serially passaged in U937 cells in either the presence or absence of anti-ADV antibodies (results not shown), indicating that ADE-mediated ADV-G infection of U937 cells as well as ADV-Utah I infection (26) was not fully permissive.

Northern blot analysis of mRNA from ADV-G-infected U937 cells mediated by ADE revealed the three major bands detected in ADV-G-infected CRFK cells (Fig. 3A). However, in U937 cells, the band which corresponds to a 2.8-kb mRNA in CRFK cells was broadened and split (Fig. 3A, lane c). A similar finding was made previously for ADV-Utah I/adult-infected U937 cells (26), indicating that the difference in transcript pattern between permissive and restricted infections is clearly dependent on some host cell-specific conditions and not due to some features peculiar to the virus.

By Southern blot analysis, we could detect replicative form (RF) DNAs (4.8-kb duplex monomer RF DNA and 9.6-kb duplex dimer RF DNA) in ADV-G-infected U937 cells mediated by ADE (Fig. 3B). However, differences in the relative ratio between species of DNA and in the level of amplification of DNA were noted. Although the signal from single-stranded (SS) virion DNA was similar to that from RF DNAs in ADV-G-infected CRFK cells (Fig. 3B, lane f), the RF DNA signal was much stronger than the SS DNA signal in ADV-G-infected U937 cells (Fig. 3B, lane b). The same result was

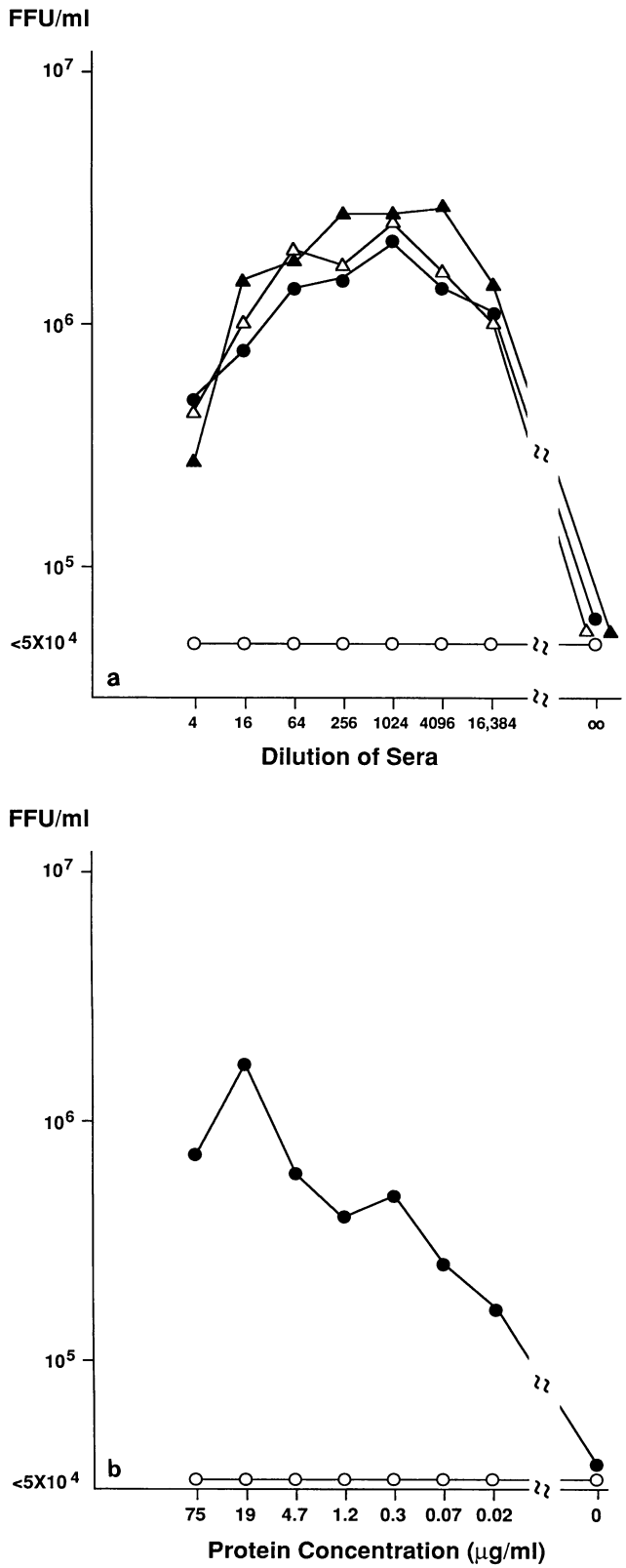

FIG. 2. Effects of sera and IgG on ADV-G infection of U937 cells. ADV-G (1.5×10^5 FFU) was preincubated (a) with three AD sera (●, ▲, △) and one normal serum (○) or (b) with IgG from AD serum (●) or normal serum (○) and used to infect 5×10^4 U937 cells at 32°C. Cells were cultured at 32°C for 4 days. Titers were calculated from the frequency of nuclear expression of ADV antigens detected with FITC-labeled anti-ADV mink serum. Mean values for duplicate wells are shown.

TABLE 1. Titer of ADV-G and ADV-Utah I/adult in U937 and CRFK cells in the absence and presence of ADV-infected and uninfected mink sera

ADV strain	Addition	Titer (FFU/ml)	
		U937[a]	CRFK[b]
ADV-G[c]	None	$<5 \times 10^4$	3×10^7
	AD serum	3×10^6	3×10^6
	Uninfected serum	$<5 \times 10^4$	3×10^7
ADV-Utah I/adult[d]	None	4×10^5	2×10^5
	AD serum	4×10^5	2×10^5
	Uninfected serum	4×10^5	2×10^5

[a] Cells were cultured for 4 days at 32°C after adsorption for ADV-G infection or at 37°C for ADV-Utah I/adult infection.
[b] Cells were cultured for 4 days at 32°C after adsorption.
[c] Prepared from a lysate of infected CRFK cells.
[d] Prepared from organ homogenates of infected adult mink; 10-fold concentrate of the standard preparation of 10^5 ID_{50}s/ml.

observed in ADV-Utah I/adult infection of U937 cells, as reported previously (26). Furthermore, the total amplification of whole ADV DNA between 6 and 96 h postinfection in ADV-G-infected U937 cells (Fig. 3B, lanes a and d) was less than that in ADV-G-infected CRFK cells (Fig. 3B, lanes e and h).

By Western blot (immunoblot) analysis, we identified VP1, VP2, and NS1 proteins in ADV-G-infected U937 cells mediated by ADE (Fig. 3C). However, a difference in the relative ratio was also noted. In ADV-G-infected CRFK cells (Fig. 3C, lane d), the VP bands were stronger than that of NS1, whereas in ADV-G-infected U937 cells (Fig. 3C, lane b), the VP bands were weaker than that of NS1, as observed previously for ADV-Utah I/adult infection (26). A 28-kDa band that represents a minor degradation product of the capsid proteins (1) was noted in the ADV-G-infected CRFK cells but not in infected U937 cells, probably because the overall level of capsid protein in the U937 cells was too low for this species to be apparent.

These results clearly indicated that although de novo ADV mRNA expression, ADV genome amplification, and ADV protein synthesis were occurring in U937 cells, infectious virions were not generated at detectable levels.

Liposome transduction of ADV-G into U937 cells. The results presented above showed that anti-ADV antibody made ADV-G infectious for U937 cells via Fc receptor-mediated entry. We wondered whether the infection was exclusively dependent upon antibody or whether antibody simply transported virus inside the cell. To elucidate this point, we made liposomes containing ADV-G and incubated them with U937 cells.

After 4 days of incubation, we detected clear nuclear expression of ADV antigens in these cells by immunofluorescence, up to 0.5% of total cells. We also detected ADV genome amplification at between 0 and 96 h of incubation by Southern blot analysis (results not shown). These results clearly indicate that virus entry via a non-Fc receptor-mediated pathway could induce ADV infection. Thus, although antibody definitely promoted virus uptake into U937 cells, antibody enhancement was not absolutely required for subsequent viral gene expression.

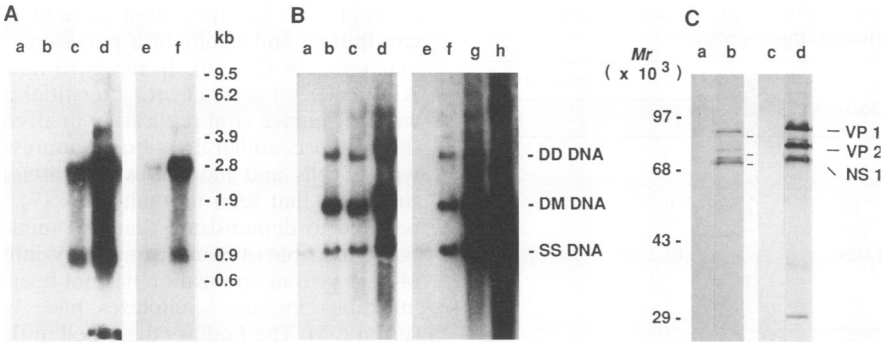

FIG. 3. Characterization of ADV-G infection of U937 cells mediated with AD sera. (A) Northern blot analysis. U937 cells (2×10^7) were infected with 4×10^7 FFU of ADV-G in either the presence or absence of AD serum as described in the text. Subconfluent cultures of CRFK cells in three 150-cm^2 tissue flasks (approximately 10^8 cells) were infected with 10^7 FFU of ADV-G at 32°C. Cells were cultured at 32°C and collected at 66 h postinfection. mRNAs at 5 μg (lanes a, b, and c) and 1 μg (lane d) were applied to each well, Northern blotted, and hybridized with the ADV probe as described in Materials and Methods. RNA size markers are indicated on the right. Lanes: a, uninfected U937 cells; b, U937 cells infected with ADV-G without AD serum; c, U937 cells infected with ADV-G with AD serum; d, CRFK cells infected with ADV-G. Lanes: a through d, 43 h of exposure at −70°C; e, 6-h exposure of lane c sample at −70°C; f, 6-h exposure of lane d sample at −70°C. (B) Southern blot analysis. U937 cells (10^6) were infected with 2×10^6 FFU of ADV-G preincubated with AD serum. Approximately 2×10^6 CRFK cells a six-well tissue culture plate were infected with 2×10^5 FFU of ADV-G at 32°C, and aliquots were collected at the indicated times. DNA was extracted, and 1/6 of the DNA samples from U937 cells and 1/12 of the DNA samples from CRFK cells were electrophoresed in 0.7% agarose gels, Southern blotted, and hybridized with an ADV probe. Lanes: a through d, U937 cells infected with ADV-G in the presence of AD serum for 6 (a), 24 (b), 48 (c), and 96 (d) h; e through h, CRFK cells infected with ADV-G for 6 (e), 24 (f), 48 (g), and 96 (h) h. DM, duplex monomer RF DNA; DD, duplex dimer RF DNA. (C) Western blot analysis. Cells were cultured and infected with ADV-G as described for panel B. Cells were lysed with 150 μl of Laemmli sample buffer, and a 50-μl portion of each sample was applied to each well. After electrophoresis, proteins were transferred to a nitrocellulose membrane, incubated with ADV-infected mink serum, and visualized as described in Materials and Methods. The positions of molecular size markers are indicated (in kilodaltons). Lanes: a, uninfected U937 cells; b, U937 cells infected with ADV-G in the presence of AD sera, 96 h postinfection; c, uninfected CRFK cells; d, CRFK cells infected with ADV-G, 96 h postinfection.

Blocking of ADV-Utah I infection of U937 cells by preincubation with protein A. The ADV-Utah I/adult strain is coated with mink antibodies (13, 17). Consequently, in vitro incubation of ADV-Utah I/adult with AD serum might not further alter the infectivity.

One way to circumvent this problem would be to strip the complexed antibodies from ADV-Utah I virions. Therefore, we treated ADV-Utah I/adult at low pH, a maneuver that does not lower in vivo infectivity (17). Although this procedure abolished the infectivity of ADV-Utah I/adult for U937 cells, it also abolished the infectivity for CRFK cells (results not shown). As a result, it was impossible to evaluate the contribution of ADE to ADV-Utah I/adult infection of U937 cells by this strategy.

As an alternative, we incubated ADV-Utah I/adult with soluble protein A before infection. Since protein A binds to the Fc portion of IgG (22, 31, 40), protein A might bind the Fc portion of the antibodies coating ADV-Utah I and compete with the Fc receptors on U937 cells. In fact, preincubation of ADV-Utah I/adult with protein A reduced the infectivity of ADV-Utah I for U937 cells by up to 80% (Fig. 4). However, even at higher concentrations of protein A, we could not completely abolish the infectivity of ADV-Utah I/adult for U937 cells.

On the other hand, preincubation of ADV-Utah I/adult with protein A did not change its infectivity for CRFK cells (results not shown).

Contribution of ADE to ADV infection of mink peritoneal macrophages. Macrophages in lymph nodes support ADV mRNA expression in ADV-infected mink (25). Moreover, mink peritoneal macrophage cultures can be infected with ADV-Utah I/adult but not with ADV-G and can support ADV DNA amplification (26). Therefore, we examined the role of ADE in ADV infection of mink peritoneal macrophages in vitro.

Infection of mink macrophages with ADV-G could not be demonstrated with AD serum or IgG (results not shown). Therefore, as an alternative, we used ADV-Utah I/kit as the antibody-free ADV preparation (2) instead of ADV-G. Preincubation of ADV-Utah I/kit with these antibody sources

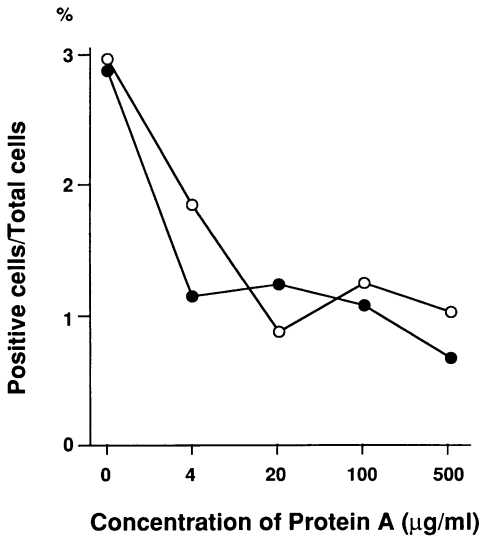

FIG. 4. Effects of protein A on ADV-Utah I infection of U937 cells. 2×10^5 ID_{50}s of Serum protein-free ADV-Utah I (2×10^5 ID_{50}s) was mixed with the indicated concentration of protein A. After 1 h of incubation at 37°C, 5×10^5 U937 cells were infected with the mixture, cultured at 37°C, and stained as described in the legend to Fig. 1. Experiments were repeated two times (experiment 1, ○; experiment 2, ●). Mean values for duplicate slides are shown.

TABLE 2. ADE in ADV-Utah I/kit infection of mink peritoneal macrophages[a]

Addition[b]	Dilution (fold)	No. of infected cells	
		Expt 1	Expt 2
None		0	0
AD sera	64	6	7
	256	7	15
	1,024	10	9
NI sera	64	0	ND[c]
	256	0	ND
	1,024	0	ND
AD IgG	4	7	14
	16	11	36
	64	13	27
NI IgG	4	1	ND
	16	1	ND
	64	1	ND

[a] Resident peritoneal cells (2.5×10^5) were seeded onto eight-well chamber slides coated with 2% gelatin. After 2 days of culture at 37°C, cells were infected with 1.7×10^3 FFU (in CRFK cells, at 32°C) of ADV-Utah I/kit preincubated with Ig samples. After 4 days of culture at 32°C, cells were stained with FITC-labeled anti-ADV serum. Values are means for duplicate wells.

[b] NI, noninfected.

[c] ND, not done.

induced the infection of mink macrophage cultures (Table 2). ADV-Utah I/kit alone was not infectious to mink macrophages, and preincubation with uninfected antibody sources did not induce ADV-Utah I/kit infection (Table 2). Since the effects of anti-ADV antibodies on ADV-Utah I/kit infection of U937 cells were almost the same as those on ADV-G infection of U937 cells (results not shown), ADE might play a role in ADV-Utah I/kit infection of mink macrophages in vitro.

We also examined the effects of protein A on ADV-Utah I/adult infection of mink macrophage cultures. Preincubation of ADV-Utah I/adult with soluble protein A reduced the virus titer in a dose-dependent manner (Table 3).

These results suggest that ADE may play a role in ADV-Utah I infection of mink macrophages in vitro.

DISCUSSION

Antibody is central to the pathogenesis of Aleutian disease. Adult mink infected with ADV develop a very high level of antiviral antibody and die of immune complex-mediated diseases (13, 37, 38). In spite of these high antibody levels, ADV can initiate and maintain a persistent, restricted infection in macrophages (25, 26). In newborn mink, on the other hand, ADV causes a severe acute interstitial pneumonitis associated with permissive viral replication in alveolar type 2 cells (5). In this instance, antiviral antibody suppresses the infection in the type 2 cells and modulates the disease into a chronic one similar to that seen in adult mink (7). Finally, although it is possible to demonstrate that AD mink serum contains antibodies capable of neutralizing ADV infectivity for CRFK cells (42), in vivo neutralization has not been observed, and in fact, infectious immune complexes have been demonstrated in serum (35). The findings described in this article may begin to reconcile some of these apparently paradoxical observations.

TABLE 3. Protein A blocking of ADV-Utah I/adult infection of mink peritoneal macrophages[a]

Protein A (μg/ml)	No. of infected cells	
	Expt 1	Expt 2
500	22	10
100	32	8
20	53	59
4	63	59
0	113	91

[a] Resident peritoneal cells (2.5×10^5) were seeded onto eight-well chamber slides coated with 2% gelatin. After 2 days of culture at 37°C, cells were infected with 4×10^4 ID_{50}s of ADV-Utah I/adult preincubated with the indicated concentrations of protein A. After 4 days of culture at 32°C, cells were stained with FITC-labeled anti-ADV mink serum. Values are means for duplicate wells.

In the presence of antibody from infected mink, ADV-G infected the macrophage lineage cell line U937, and this infectivity required a functional Fc portion of the immunoglobulin molecule. ADV-Utah I/adult infected U937 cells, and preincubation with antibody did not enhance its level of infectivity, probably because the virus is already complexed with antibodies (13, 17). This idea received support from the observation that incubation with protein A decreased the infectivity of ADV-Utah I/adult. The relevance of the results with U937 cells was apparent when similar experiments were performed with mink macrophages. The infectivity for mink macrophages of antibody-free ADV-Utah I/kit (2, 6) was clearly dependent upon serum. Interestingly, the nonpathogenic strain ADV-G did not infect mink macrophages regardless of whether this virus was incubated with serum, and this finding may correlate with the severely impaired ability of this virus to replicate in mink in vivo (12). Thus, in macrophages and a macrophage cell line, ADV infectivity was enhanced by antibody, that is, the phenomenon known as ADE was involved in ADV infection.

ADE has been reported for a wide range of virus infections, such as dengue virus (21, 30), lactate dehydrogenase virus (15, 16, 43), feline infectious virus (34), influenza A virus (33, 45), and respiratory syncytial virus (28) infection, but this is the first report of ADE in a parvovirus infection.

In the present study, we showed that ADV-G infection of U937 cells was restricted in the presence of anti-ADV antibodies, although ADV-G can induce fully permissive infection in CRFK cells (12). In addition, ADV-Utah I infection of U937 cells is also restricted (26), although it can initiate fully permissive infections of type 2 alveolar cells in newborn mink (5). Thus, these results suggest that the limited permissivity of U937 cells for ADV does not depend on the strain of virus but on cellular factors that determine the outcome of the infection. The precise nature of the restriction in vivo has yet to be determined. In an infected lymph node or in macrophages prepared from infected mink, a relatively large number of macrophages have sequestered ADV virion SS DNA, but only a small number of them contain ADV mRNA (4, 25, 32), indicating that even in vivo, only a small proportion of cells exposed to ADV actually initiate infection. Furthermore, in vitro infection of mink macrophages does not yield detectable levels of infectious virus (26). Clearly, the restriction to ADV replication in macrophages is complex, but although U937 is a human cell line, studies on infection in these cells may be useful for the analysis of ADV infection. Mapping the precise nature of the restriction in the U937 cells will provide a framework to see whether similar mechanisms are operative in infected mink.

As noted above, a relatively small population of macrophages support ADV mRNA expression in lymph nodes and peritoneal exudate cells in infected mink in vivo (25, 32). This

observation suggests that the differentiation and/or activation state of macrophages may have a relationship to susceptibility for ADV infection. The expression of Fc receptors is one indicator of macrophage differentiation that might relate to ADE. Low levels of Fc receptor expression might limit the number of macrophages susceptible to ADV infection via ADE. In fact, human gamma interferon enhances ADE infection of U937 cells with dengue virus through the increased expression of Fc receptors (27). However, pretreatment with phorbol myristate acetate, which induces increased expression of Fc receptors on U937 cells (23), does not enhance ADV-Utah I/adult infection (26), and only a small fraction of ADV DNA-containing cells support ADV mRNA expression in vivo (25). Therefore, certain steps after the entry of virus that are controlled by macrophage differentiation and/or activation may also regulate the susceptibility for ADV infection.

Antibodies do not neutralize ADV infection in vivo. Although the masking of an antigenic site of ADV with phospholipids has been suggested as an explanation for this observation (42), the mechanism for the absence of neutralizing antibodies for ADV in vivo is still unclear. In this study, we used polyclonal antibodies and demonstrated their enhancing effects on ADV infection of mink macrophages and U937 cells. However, although some antibodies might enhance the entry of virus into cells, others might inhibit subsequent steps in replication, i.e., work as neutralizing antibodies. Consequently, we might just be observing the sum of these opposite effects in our infection system. In order to clarify this point, it would be valuable to determine whether monoclonal anti-ADV antibodies can inhibit ADV infection in macrophage lineage cells even after virus entry. In fact, antibodies to West Nile virus block penetration of virus from endosomes to the cytosol without affecting attachment or endocytic internalization and then cause neutralization (20). The ADE model system with ADV-G and U937 cells presented in this report might also be useful for the analysis of these aspects.

In addition to ADE, virus-antibody complexes can contribute directly to the pathogenesis of viral diseases. For example, ADE in respiratory syncytial virus infection can be demonstrated in U937 cells (8). In this system, the addition of anti-respiratory syncytial virus antibodies stimulates the release from U937 cells of leukotrienes (8), which act as a bronchoactive mediator of inflammation. It is still unclear whether a similar effect of ADV-containing complexes contributes to the pathogenesis of AD. However, since the phagocytic activity of the mononuclear phagocytic system in mink with AD is impaired (29), secondary alterations of macrophage functions might also contribute to the development of AD.

Since ADV infection in vivo is characterized by the stimulation of B-cell maturation and the overproduction of antibodies (38), ADE in ADV infection could induce a self-perpetuating cycle between infection and antibody production. Therefore, ADE in ADV infection might promote the establishment of persistent infection.

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