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Monoclonal Antibodies against Aleutian Disease Virus Distinguish Virus Strains and Differentiate Sites of Virus Replication from Sites of Viral Antigen Sequestration

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Monoclonal antibodies (mAbs) were used to study antigenic differences among strains of Aleutian disease virus (ADV) and to characterize viral proteins in vitro and in vivo. A number of ADV field strains could be discriminated, and highly virulent Utah I ADV was clearly delineated from the tissue culture-adapted avirulent ADV-G strain. This specificity could be demonstrated by indirect immunofluorescence against infected cultures of Crandell feline kidney cells or against tissues of Utah I ADV-infected mink. Viral antigens were demonstrated in both the nuclei and the cytoplasm of infected tissue culture cells. However, in mink mesenteric lymph node, spleen, and liver, viral antigen was observed only in the cytoplasm. Absence of nuclear fluorescence suggested that the detected antigen represented phagocytized viral antigens rather than replicating virus. This conclusion was supported by the finding that mAbs reactive only against low-molecular-weight polypeptides derived from intact viral proteins gave the same pattern of in vivo fluorescence as mAbs with broad reactivity for large or small (or both) viral polypeptides. The distribution of infected cells was the same as that described for macrophages in these tissues and suggested that cells of the reticuloendothelial system had sequestered viral antigens.

Aleutian disease (AD) is a parvovirus-induced disease of mink characterized by viral persistence, hypergammaglobulinemia, and immune complex-mediated renal and arterial lesions. Affected mink usually die of terminal glomerulonephritis after a mostly subclinical course lasting weeks, months, or years (6, 12, 18, 22, 29, 31).

Immune complexes play a prominent role in the pathogenesis of the renal disease, and although some of the immune complexes contain infectious virus, it is clear that many complexes are too small to contain intact virus (4, 23, 30). Consequently, it is uncertain what viral components actually participate in the generation of immune complexes. Sera from infected animals contain antibody to both structural and nonstructural viral proteins (7). Furthermore, the proteins of AD virus (ADV) seem to be degraded during in vivo infection (3). Thus, a variety of viral proteins could be involved in immune complex development. Furthermore, antibodies to DNA are present in the sera of infected mink, and DNA–anti-DNA complexes may also have a role in the pathogenesis (16, 17). In addition, it is not clear whether virus detected earlier (30) was within cells replicating virus or rather identified cells that had phagocytized viral antigen, and since the reagent used previously to detect virus is polyclonal, it is not clear that only viral components were recognized. The ability to distinguish viral polypeptides immunologically would make it possible to define which viral products were present, both in soluble immune complexes and complexes deposited in tissues.

Another major problem in the study of AD has been the difficulty in differentiating virus strains. Although the relative pathogenicity of various ADV isolates suggests that there are significant differences among strains (14), it is currently not possible to distinguish these strains serologically with certainty (1). Consequently, antigenic differences that might correlate with virulence have not been defined. In the current studies, we developed and characterized monoclonal antibodies (mAbs) that clearly distinguished virulent Utah I ADV from avirulent ADV-G. Furthermore, by using a combination of mAbs, it was also possible to differentiate among certain field strains of ADV. Other mAbs identified epitopes present on proteolytic breakdown products of viral structural proteins that were not recognized on the intact molecules. Indirect immunofluorescence analysis of infected mink tissues with this group of mAbs indicated that low-molecular-weight derivatives of ADV structural proteins were the major viral antigens recognized in mink tissues.

MATERIALS AND METHODS

Virus strains. The strains of ADV-G, Utah I ADV, Pullman ADV, Ontario ADV, and Danish ADV used have been described previously (1, 5, 15, 22, 30).

CIEP. Direct counterimmunoelectrophoresis (CIEP) was performed as described previously, except that 0.7% agarose was used (5).

Preparation of viral antigens for CIEP assay. The Utah I, Danish, and Ontario ADV test antigens were prepared from the spleens and livers of sapphire mink killed 9 to 12 days after intraperitoneal (i.p.) inoculation of 10^6 50% infective doses (ID_{50}s) of the respective virus. The Pullman ADV test antigen was prepared from similar tissues taken from sapphire mink killed 30 days after i.p. inoculation of 10^6 ID_{50}s of Pullman ADV. A control antigen was prepared from the combined spleens and livers of normal sapphire mink. These antigens were purified as described previously (10, 34). A 1-ml portion of concentrated antigen corresponded to 150 g of starting tissue. Antigen from the cell culture-adapted

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ADV-G strain was prepared by infecting 150-cm^2 Corning tissue culture flasks of Crandell feline kidney (CRFK) cells with an ADV-G multiplicity of infection of 1.0 to 10.0 at 31.8°C for 5 to 7 days as described previously (6, 7). Cell pellets suspended in 0.5 ml of phosphate buffered saline per flask were freeze-thawed four times, sonicated, and clarified by centrifugation, and the supernatant was stored at −20°C until used. A simiar antigen was prepared from uninfected CRFK cells. All test antigens were stored at −20°C.

Hybridoma production. For mouse inoculations, 500 μl of ADV (75 g equivalents of combined spleen and liver from mink infected with the appropriate ADV strain) was purified by CsCl gradient centrifugation (8) or by passage through a Sepharose 4B column (2). Gradient fractions positive in CIEP against a standard anti-ADV serum were stored at −70°C until used. Hybridomas were made by inoculating each C57BL/10 mouse recipient with CsCl gradient- or Sepharose 4B-purified virus simultaneously by three routes of inoculation including intravenous, i.p., and subcutaneous. Primary inocula were 100 to 200 μl of purified virus emulsified in complete Freund adjuvant for i.p. and subcutaneous inoculations, and 50 to 100 μl diluted in 500 μl of physiologic buffered balanced salt solution for intravenous inoculation. After 4 to 6 weeks the mice were boosted i.p. and subcutaneously with similar amounts of virus emulsified in incomplete Freund adjuvant. Intravenous inoculations were given as above. At 4 to 7 days after the last inoculation, individual spleens were recovered, and fusion reactions were performed as described previously (9, 11, 19). Supernatants from all wells in which cell colonies grew were tested (usually 10 to 20 days postfusion) by CIEP against the immunizing and control antigens as well as by indirect immunofluorescence against ADV-G-infected CRFK cells. Cells from positive wells were cloned by limiting dilution.

Immunoglobulin isotype. The immunoglobulin classes of antibodies produced by hybridoma cultures were determined by gel diffusion in 1% agarose. A 10-fold-concentrated culture supernatant fluid was reacted against rabbit antisera specific for mouse immunoglobulin classes (Litton Bionetics, Kensington, Md.). In none of the subsequent assays could reaction patterns be attributed solely to antibody isotype.

Immunoprecipitation. The techniques for metabolic labeling of ADV-G with [^{35}S]methionine and preparation of [^{35}S]methionine-labeled cell lysates for immunoprecipitation have been described previously (6, 7). For immunoprecipitation, precleared samples corresponding to 5×10^5 CRFK cell equivalents were incubated for 3 h at 4°C with 0.5-ml samples of individual undiluted hybridoma supernatants or 5 to 10 μl of sera. [^{35}S]methionine labeling of Utah I ADV proteins was performed in a manner identical to that used for ADV-G. Utah I ADV or Pullman ADV infection of CRFK cells was accomplished by adding 5 ml of mesenteric lymph node (MLN) supernatant to a subconfluent culture of CRFK cells in a 150-cm^2 Corning tissue culture flask. After 2 to 3 h, the infecting material was replaced with 50 ml of medium, and incubation was continued at 31.8°C for 72 h. Viral proteins were then labeled as described for ADV-G (7). Utah I ADV or Pullman ADV supernatant for infection of cell cultures was prepared by making a 10% homogenate (wt/vol) in physiologic buffered balanced salt solution of individual or pooled MLNs from sapphire mink infected 10 days earlier with 10^6 ID_{50}s of Utah I ADV or 30 days earlier with 10^6 ID_{50}s of Pullman ADV. Homogenates were freeze-thawed four times, sonicated, and clarified by centrifugation. The resulting supernatants were kept at −70°C until needed.

Immunofluorescence assay. Indirect immunofluorescence was done on monolayers of CRFK cells as follows. Microscope slides (25 by 75 mm) were placed in Leighton tubes (Bellco Glass, Inc., Vineland, N.J.) and seeded with 5.0×10^5 CRFK cells in 5 ml of complete medium. The next day the medium was removed, and the subconfluent cultures were infected for 2 h with a dilution of ADV-G that induced virus-specific fluorescence in about 10% of the cells after incubation at 31.8°C for 3 days or with 1 ml of two-fold-diluted Utah I ADV lymph node supernatant prepared as described above. After 3 days, the slides were rinsed in physiologic buffered balanced salt solution, fixed in acetone at −20°C for 60 min, air dried, and stored at −20°C until used. The cell layers were then divided into replicate compartments with Pliobond (Goodyear Tire and Rubber Co., Akron, Ohio). Undiluted hybridoma supernatant (10 μl) was applied to each compartment of the cell sheet, and the slide was incubated at 37°C for 30 min. Slides were washed for 10 min in physiologic buffered balanced salt solution and overlaid with 10 to 20 μl of a 1:300 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin serum (Sigma Chemical Co., St. Louis, Mo.). After 30 min of incubation at 37°C, slides were washed as before, dried, and examined in a Leitz Orthoplan microscope. The reaction of FITC-conjugated anti-ADV mink serum (FITC-MAD) was used as a positive control.

Tissue blocks (3 to 5 mm) of spleen, liver, and MLN were obtained from normal sapphire mink or mink infected 10 days earlier with 10^6 ID_{50}s of Utah I ADV and were snap frozen in liquid N_2. Frozen sections 6 μm thick were cut on an American Optical Histostat microtome. These sections were serially stained with mAb and FITC-goat anti-mouse immunoglobulin G or with FITC-MAD as described above. Snap-frozen blocks could be stored at −70°C in 1-dram (3.7-ml) vials for at least 6 months without loss of reactivity.

Radioimmunoassay. Radioimmunoassay was performed with selected mAbs and various virus preparations. The basic technique has been described previously (2) and was modified to use $F(ab')_2$ fragments from ADV-infected mink sera instead of whole sera as the antibody (28).

RESULTS

Immunofluorescence analysis of ADV-G- and Utah I ADV-infected CRFK cells. A panel of mAbs was reacted against ADV-G-, Utah I ADV-, or Pullman ADV-infected CRFK cells to determine whether any of the mAbs was specific for any of these strains. Most of the mAbs gave bright nuclear and cytoplasmic staining of CRFK cells infected with either virulent Utah I ADV or avirulent ADV-G and were unable to distinguish the two virus strains (mAb 30 in Fig. 1). FITC-MAD gave identical nuclear and cytoplasmic staining of cultures infected with either virus and was also unable to discriminate strains. Some mAbs, however, clearly had preferential reactivity for either Utah I ADV or ADV-G. For example, mAb P11 stained Utah I ADV-infected CRFK cells but failed to react against any ADV-G-infected cells. Both nuclear and cytoplasmic staining was observed, but the predominant pattern was cytoplasmic as shown (Fig. 1). In contrast, mAbs such as 68 gave bright nuclear and cytoplasmic staining of CRFK cells infected with ADV-G but only rarely stained cells in Utah I ADV-infected cultures (Fig. 1). In those instances when mAb 68 stained cells in Utah I ADV-infected cultures, the number of positive cells was <1% the number positive when Utah I ADV-reactive mAbs such as P11 were used. These results indicated that appropriate mAbs distinguished between Utah I ADV and ADV-G

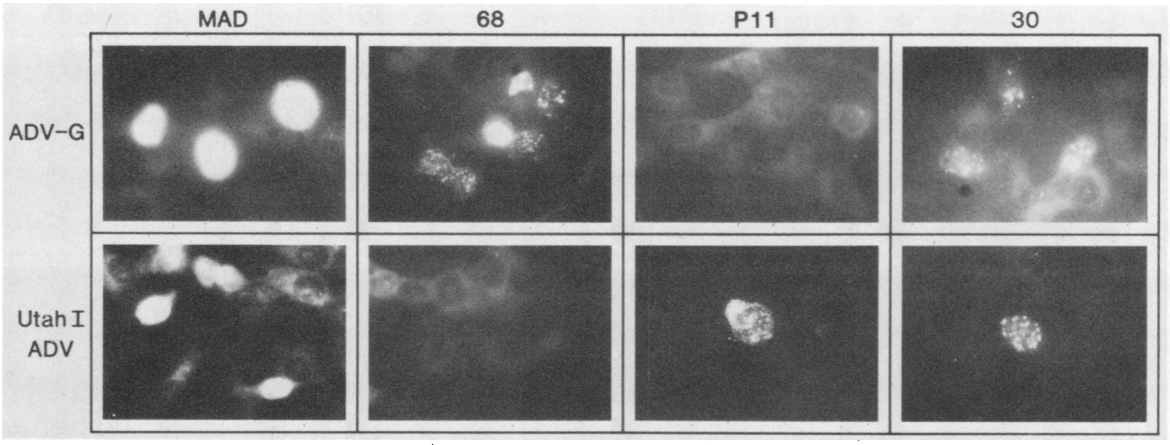

FIG. 1. CRFK cells infected with either ADV-G or Utah I ADV were reacted against FITC-MAD or selected mAbs (68, P11, 30) as detailed in Materials and Methods. MAD is serum from a pool of sera from terminal AD-affected mink. Nuclear fluorescence is depicted in the MAD-positive cells shown. Other cells, however, gave cytoplasmic fluorescence as well (not shown). 68 is an mAb which reacts preferentially against ADV-G. Two cells with nuclear fluorescence and four with cytoplasmic fluorescence are shown. P11 is an mAb which reacts against Utah I ADV. The positive cell shown reflects cytoplasmic fluorescence. A few other cells gave nuclear fluorescence as well (not shown). 30 is an mAb which reacts against either ADV-G or Utah I ADV. Cytoplasmic fluorescence is shown. A few other cells gave nuclear fluorescence as well. All magnifications are ×400.

in infected CRFK cells and identified viral antigens in both the nucleus and cytoplasm. None of the mAbs stained any cells in Pullman ADV-infected cultures, suggesting that detectable virus gene expression did not occur in CRFK cells infected with this virus.

Comparison of kinetics of infection of CRFK cells by ADV-G and Utah I ADV. To confirm that particular immunofluorescence patterns were due to mAb specificity differences and were not a reflection of the kinetics of viral replication in CRFK cells, we examined ADV-G- and Utah I ADV-infected cells at various times after infection. Differences were found between ADV-G and Utah I ADV regarding the appearance of positive cells, but the viral specificity of the various mAbs was apparent anytime positive cells were observed. Cells displaying ADV-G antigens were first apparent at 30 h after infection. During the next 24 h, the number of positive cells increased (maximum of three to eight positive cells per high-power field [×400]), and nuclear fluorescence accumulated in most infected cells. No further change in the number of positive cells or fluorescent pattern occurred during the remainder of a 120-h study interval. In contrast, when CRFK cells were infected with Utah I ADV, positive cells were first observed 72 h postinfection, and only a few positive cells were noted (one positive cell per five high-power fields). The number of positive cells could not be increased even by using very concentrated Utah I ADV virus preparations. Furthermore, nuclear fluorescence was observed in only 5 to 10% of the positive cells. These results showed that Utah I ADV infection of CRFK cells proceeded more slowly than ADV-G infection, involved fewer cells, and only occasionally gave nuclear fluorescence. Since nuclear fluorescence is thought to reflect sites of parvovirus replication and assembly (32, 33), this result also suggested that only a few cells in the Utah I ADV-infected CRFK cells were actually replicating Utah I ADV.

Immunofluorescence analysis of Utah I ADV-infected tissues. Although ADV antigen has been demonstrated in vivo (30), sites of ADV replication have not been convincingly determined. Furthermore, the serologic reagent previously used to demonstrate ADV antigen in tissues was a polyclonal pool of sera derived from infected animals (16, 22, 29) and could have had other than antiviral reactivities. Therefore, selected anti-ADV mAbs were reacted against frozen sections of MLN, spleen, and liver of mink infected 10 days earlier with Utah I ADV. The preferential viral strain reactivity of the mAbs as recognized in ADV-G- or Utah I ADV-infected CRFK cells was extended to the in vivo situation. That is, mAbs such as 68 that recognized ADV-G gave no virus-specific fluorescence of spleen or liver and stained only a few cells in MLNs. However, mAbs with Utah I ADV specificity (such as P11) or broad specificity (such as 30) gave fluorescence of many cells in MLN, spleen (Fig. 2A), and liver (not shown). FITC-MAD gave the same pattern of fluorescence as the Utah I ADV-reactive mAbs and stained similar numbers of cells (Fig. 2A). However, the sections, especially liver, were often more difficult to evaluate due to high background fluorescence with this reagent. The polyvalent nature of MAD may account for some of the high background. Nevertheless, the similar patterns of fluorescence produced by the mAbs and FITC-MAD suggested that most of the FITC-MAD reactivity was indeed directed against viral antigens and that earlier descriptions of viral antigen distribution are valid (30). Since ADV-G is avirulent in mink and does not result in detectable in vivo virus replication upon primary passage in mink (6), converse studies using ADV-G as the infecting virus were not done.

Positive fluorescence in Utah I ADV-infected organs as detected with mAbs was of a powdery to granular appearance and was limited to the cytoplasm. In MLNs, positive cells were in clusters located in cortical areas of the nodes (Fig. 2B). In some instances, germinal centers were intensely stained, especially in peripheral regions. In the spleen, positive cells were in clusters uniformly distributed throughout the section, and in the liver positive cells were localized to sinusoids. Only cytoplasmic fluorescence was observed in any of the positive cells, suggesting that the positive cells were not sites of viral replication but instead represented sites of viral antigen sequestration.

Immunoprecipitation of viral proteins by mAbs. Earlier work shows that ADV-G and Utah I ADV induce synthesis of two related major virion polypeptides having molecular weights of approximately 85,000 and 75,000 (p85 and p75)

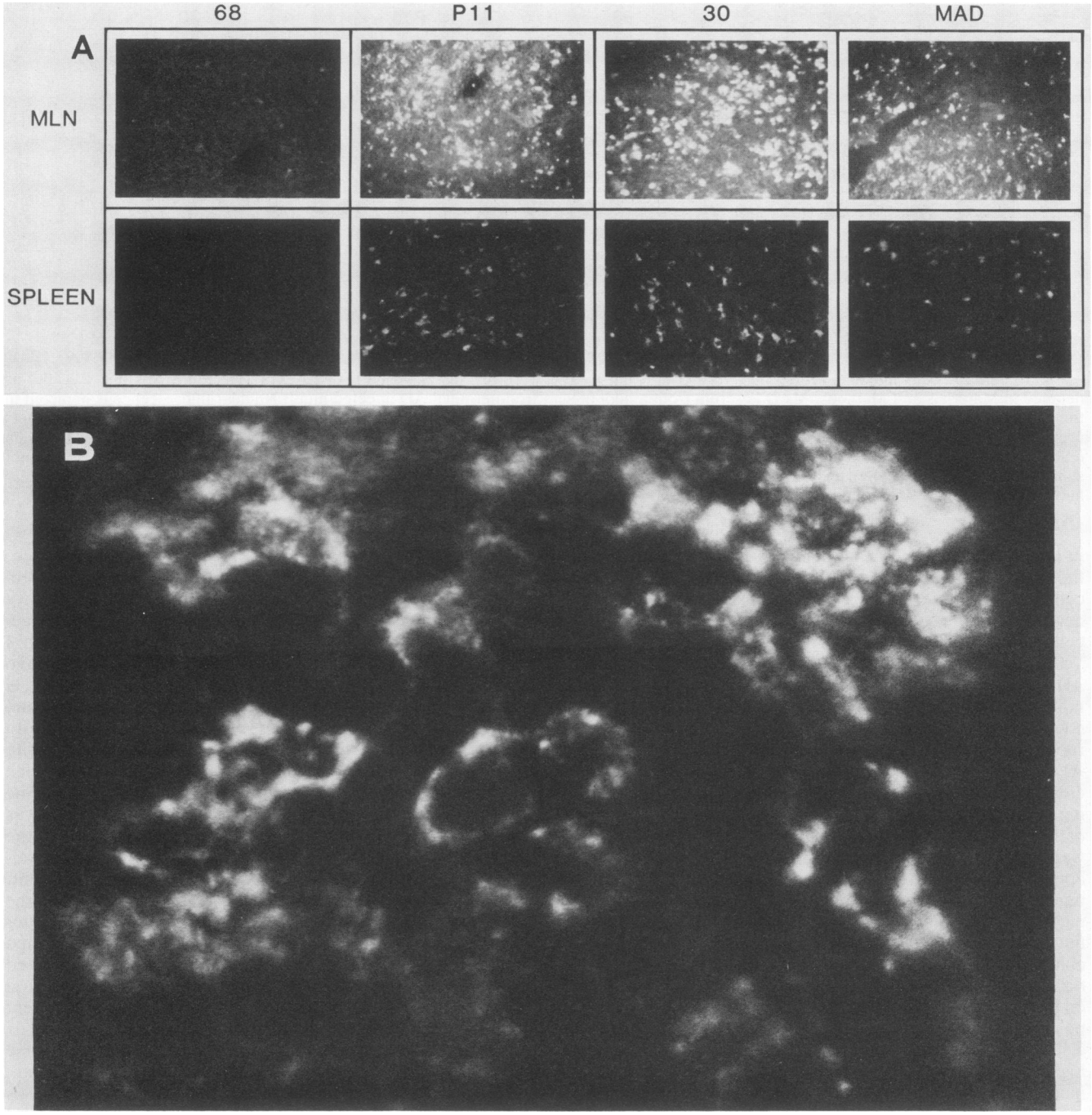

FIG. 2. (A) Cryostat sections of MLN and spleen from a sapphire mink killed 10 days after infection with Utah I ADV were reacted against the indicated mAbs (68, P11, 30) or against FITC-MAD. No virus-specific fluorescence was observed when the mAbs or MAD were tested against similar tissues from normal mink. Furthermore, mAbs with specificity for Friend leukemia viral antigens (9) were negative against tissues from ADV-positive or -negative mink. Mab specificities are described in the legend to Fig. 1. All magnifications are ×83. (B) Cryostat section of MLN from the same sapphire mink used in panel A reacted against mAb 30. At this magnification (×400), cellular detail and cytoplasmic fluorescence can be appreciated.

and an unrelated nonvirion protein having a molecular weight of 71,000 (p71) (6, 7). To determine which viral proteins were recognized by the mAbs, we tested them against lysates of [^{35}S]methionine-labeled CRFK cells infected with ADV-G or Utah I ADV. Most mAbs precipitated both p85 and p75 from both ADV-G and Utah I ADV lysates, thus confirming the close relationship between the two structural proteins suggested by peptide mapping studies (7). In addition, the strain specificity of some of the mAbs was extended to the level of the viral structural proteins. Thus, mAbs 68 and 73 precipitated only the structural proteins of ADV-G (Fig. 3A), whereas mAbs P2, P11, and O33 precipitated only the structural proteins of Utah I ADV (Fig. 3B).

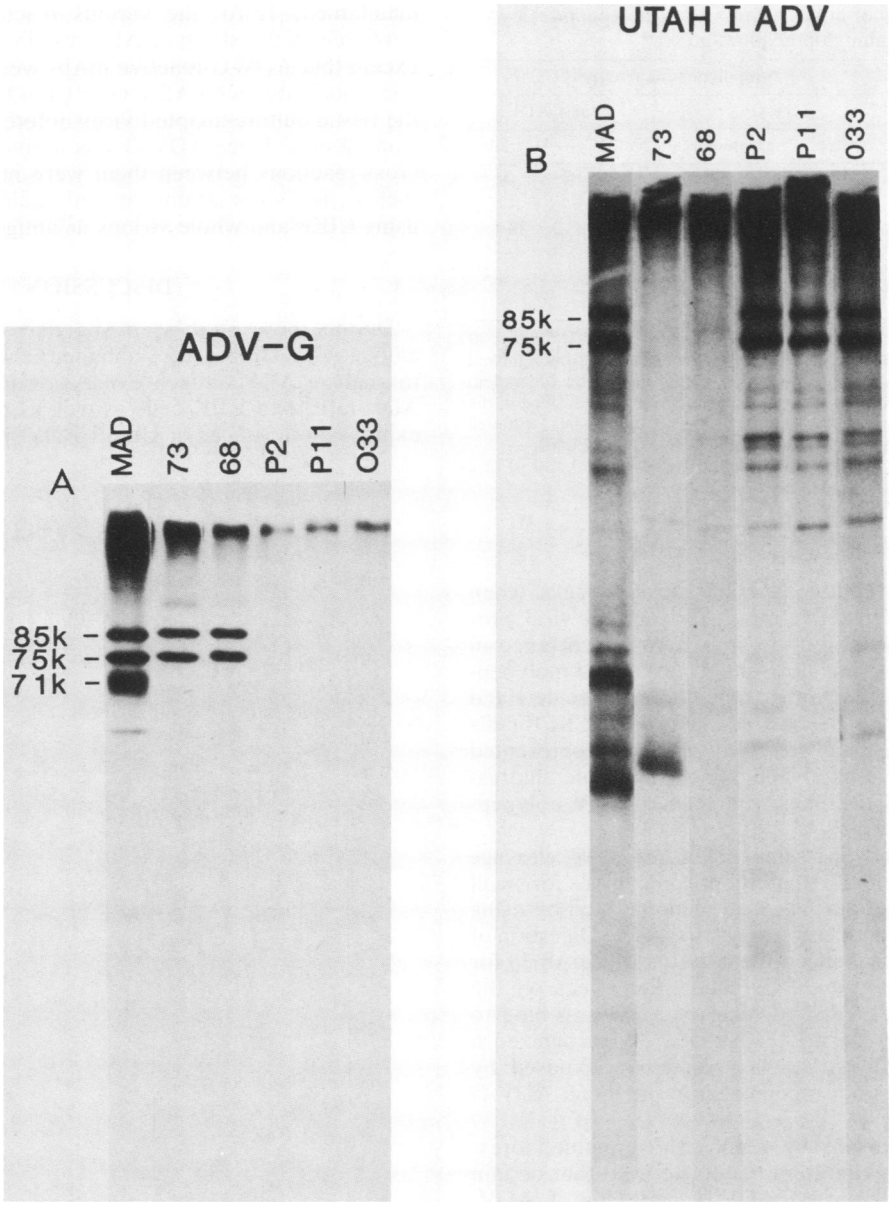

FIG. 3. (A) CRFK cells infected with ADV-G were labeled 72 h later with [^{35}S]methionine. Cell lysates prepared as detailed in Materials and Methods were reacted against a pool of sera from AD terminal mink (MAD) or against selected (73, 68, P2, P11, and O33) mAbs. Kilodalton (k) designations along the vertical axis localize the ADV structural proteins p85 and p75 and the ADV-induced nonstructural protein p71. (B) CRFK cells infected with Utah I ADV were labeled, prepared, and reacted as indicated for panel A.

mAbs specific for viral antigens exposed by trypsin. It has been suggested that low-molecular-weight polypeptides rather than ADV structural proteins predominate in the tissues of mink infected with natural AD strains and may be important in the generation of ADV-associated immune complexes (3). Therefore, we tried to mimic an in vivo situation by testing a panel of mAbs by immunoprecipitation against ADV-G or Utah I ADV exposed to trypsin (Table 1). mAbs that immunoprecipitated ADV-G or Utah I ADV structural proteins (p85 and p75) always reacted with smaller proteins of 60, 50, 27, and 24 kilodaltons produced by trypsin treatment of the lysates (mAb 30 against ADV-G in Fig. 4 and mAbs P2 and O33 against Utah I ADV in Fig. 5). If trypsin-like enzymes degrade ADV in vivo as has been speculated (3), then these results imply that the antigen detected in vivo by immunofluorescence could have been either intact p85 and p75 or low-molecular-weight polypeptides derived from these proteins. Importantly, four mAbs (36, O4, O6, O8) reacted preferentially with low-molecular-weight proteolytic cleavage products but not with intact virion structural polypeptides (mAb 36 against ADV-G in Fig. 4 or mAb O8 against Utah I ADV in Fig. 5). These mAbs stained lymph node and spleen cells with the same pattern as was seen with the mAbs reactive against either intact or cleaved ADV structural proteins. Thus, the antigen detected in infected mink tissues probably represented proteolytic cleavage products of viral structural proteins. When these same mAbs specific for the low-molecular-weight polypeptides were tested against ADV-G- or Utah I ADV-infected CRFK cells, no positive cells were apparent

TABLE 1. Analysis of mAbs against ADV polypeptides by immunoprecipitation[a]

mAbs represented	Antipolypeptide reactivity			
	ADV-G		Utah I ADV	
	Native	Degraded	Native	Degraded
68, 73	+	+	−	−
P2, P11, O33	−	−	+	+
36, O4, O6, O8	−	+	−	+
10, 30, seven others	+	+	+	+
13 mAbs	−	−	−	−
MAD[b]	+	+	+	+

[a] Each of 31 mAbs was tested by immunoprecipitation against a lysate of ADV-G- or Utah I ADV-infected CRFK cells as indicated in Materials and Methods. Each lysate was tested both before (native) and after (degraded) digestion for 60 min at 37°C with 10 μg of trypsin per ml as described in the text. Native proteins had molecular weights of 85,000 and 75,000. Degraded proteins had molecular weights of 60,000, 50,000, 27,000 and 24,000.

[b] Represents a pool of sera derived from ADV-infected mink. 10 μl of a 1:100 dilution of MAD was used per reaction.

in preparations that contained many positive cells when tested against mAbs reactive against structural viral proteins. Thus, proteolysis was not a common occurrence in infected CRFK cells, and low-molecular-weight polypeptides probably contributed little of the fluorescence detected in vitro. Therefore, fluorescence seen in infected CRFK cells when appropriate mAbs were used probably represented intact structural proteins, while the cytoplasmic fluorescence observed in tissues likely represented ADV polypeptides degraded in vivo.

Preferential reactivity of some mAbs for ADV cleavage products. The preferential reactivity of some mAbs for small ADV-derived polypeptides was also demonstrated by using a radioimmunoassay and a representative mAb. Digestion of ADV-G with trypsin enhanced the reactivity of mAb 36 for this virus by a factor of 20 but had little effect on the reactivity of mAbs 10 and 24 which are known to bind to intact viral proteins (Fig. 6). Thus, mAb 36 recognized a determinant on ADV-G that was created or exposed by proteolysis which was not recognized on the intact ADV-G molecule.

Analysis of ADV strains by CIEP. The immunofluorescence and immunoprecipitation results showed that certain mAbs distinguished between ADV-G and Utah I ADV. However, other strains of ADV exist which have been less well characterized and not adapted to cell culture growth. Therefore, we sought to differentiate among some of these strains by using CIEP. A set of viral antigens was prepared from tissues of mink infected with Utah I ADV, Pullman ADV, Danish ADV, and Ontario ADV. In addition, a cell culture ADV-G antigen was made. mAbs were developed from mice by using each of the above strains individually as antigen, and the entire panel of mAbs thus obtained was reacted against each antigen. Several distinct reaction patterns were observed (Table 2), and a number of observations were made. First, all five strains could be distinguished from one another by using appropriate combinations of mAbs and viral antigens. For example, ADV-G could be identified with a group VI reagent; Pullman ADV could be recognized by using a group III mAb with a group IV mAb; Utah I ADV was recognized by using a group III mAb with a group VI mAb; and so forth. Second, although considerable cross-reactivity existed among the mAbs, the virus strain preferences of certain mAbs as demonstrated in other assays were maintained. Third, the various reaction patterns seemed unrelated to the strain of ADV used to immunize the mice, except that ADV-G-reactive mAbs were obtained only from mice inoculated with ADV-G or Utah I ADV, indicating that the tissue culture-adapted virus differed from those derived from tissues. Since ADV-G was derived from Utah I ADV, cross-reactions between them were not surprising. Nevertheless, the virus strains studied could be distinguished by using CIEP and whole virions as antigen.

DISCUSSION

During these studies, mAbs that clearly distinguished among ADV strains were obtained, characterized, and used to analyze ADV antigen expression in ADV-G- or Utah I ADV-infected CRFK cells as well as to study viral antigen expression in tissues of Utah I ADV-infected mink.

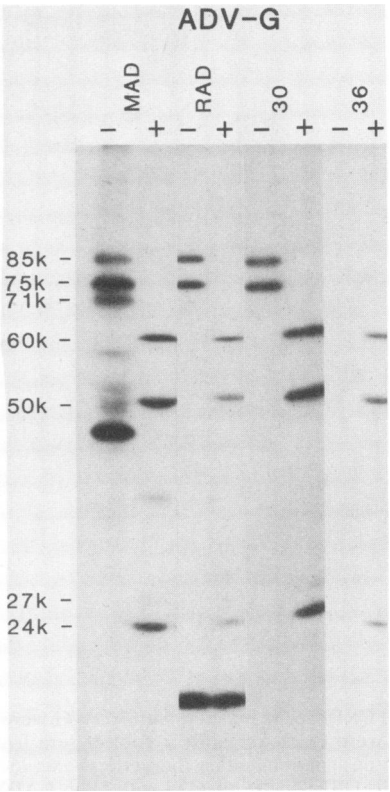

FIG. 4. ADV-G-infected CRFK cells were labeled with [^{35}S]methionine 72 h after infection as detailed in Materials and Methods. Cell lysates were divided into two equal aliquots and handled identically except that one aliquot was incubated with 10 μg of trypsin per ml for 30 min at 37°C. Trypsin was inactivated with 20 μg of soybean trypsin inhibitor per ml, and an equal volume of twice-concentrated lysing buffer (7) was added. Samples (0.5 ml) of the trypsin-treated and untreated lysates were individually reacted in immunoprecipitation with sera from infected mink (MAD), sera from AD-inoculated rabbits (RAD), or the indicated mAbs (30 and 36). ADV-specific proteins were not seen if lysates were reacted against sera from normal mink, normal rabbits, or mAbs made against non-ADV viruses. + indicates that the lysate was exposed to trypsin. − indicates that the lysate was not exposed to trypsin. Proteins specific for ADV are indicated by molecular weight designations along the vertical axis (k, × 10^3). Bands not labeled along the vertical axis were present in uninfected but otherwise similarly treated controls and are not ADV specific.

Immunofluorescence analysis of CRFK cells infected with ADV-G or Utah I ADV revealed that most of the mAbs had reactivity for either virus. However, some mAbs had exclusive and others preferential reactivity for only one strain, a distinction not possible with previously available reagents. Cytoplasmic localization of virus antigen was common in CRFK cells infected with either ADV-G or Utah I ADV, but the presence of nuclear antigen was consistent in most positive cells only in ADV-G infections. Apparently only a small number of cells was productively infected with Utah I ADV compared with the number infected with ADV-G. Furthermore, the time course of viral antigen expression was slower in CRFK cells infected with Utah I ADV than in those infected with ADV-G, suggesting that Utah I ADV replication was suppressed at some level since parvovirus replication and assembly occurs in the nucleus (33). It is not clear whether failure to infect a large number of cells and to cause nuclear antigen formation is due to the virus or the recipient cell. Utah I ADV may be a mixture of viruses only a small percentage of which may be capable of growth in CRFK cells. Host-range mutants of other parvoviruses are a frequent finding (27, 32, 35), and in fact ADV-G may be a host-range mutant of Utah I ADV that developed during cell culture (4a). The few positive cells seen in CRFK cells infected with Utah I ADV may have represented the generation of such mutants in single cells. Virus-strain-specific mAbs should provide us with better reagents to study how different viruses replicate in these cells and a way to identify mutants as they arise.

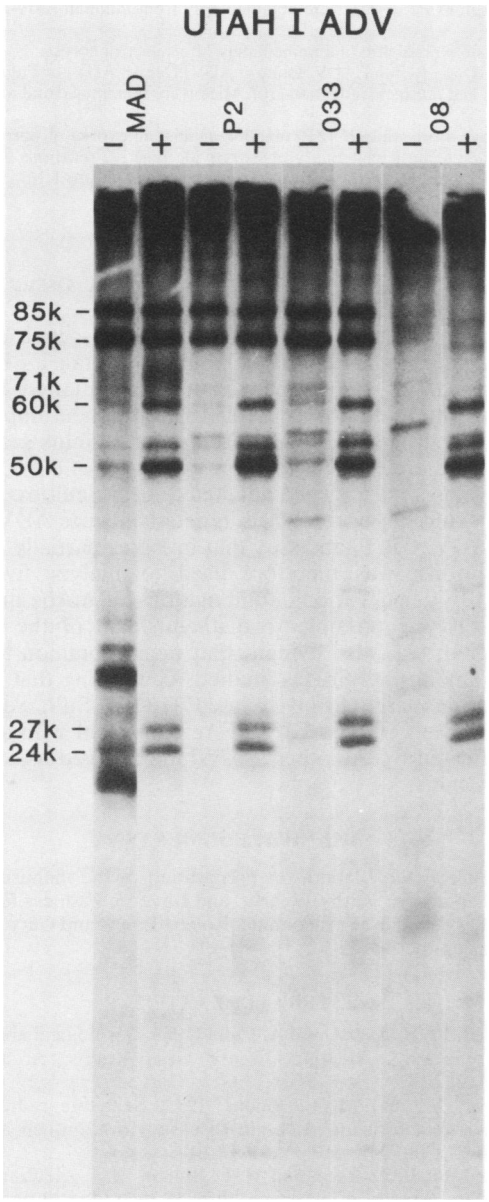

FIG. 5. Utah I ADV-infected CRFK cells were processed as indicated for ADV-G in the legend to Fig. 4. Trypsin-treated and untreated lysates were reacted against sera from AD-positive mink (MAD) and the Utah I ADV-reactive mAbs P2, O33, and O8. + indicates that the lysate was exposed to trypsin. – indicates that the lysate was not exposed to trypsin. Proteins specific for ADV are indicated by molecular weight designations (k, × 10^3) along the vertical axis.

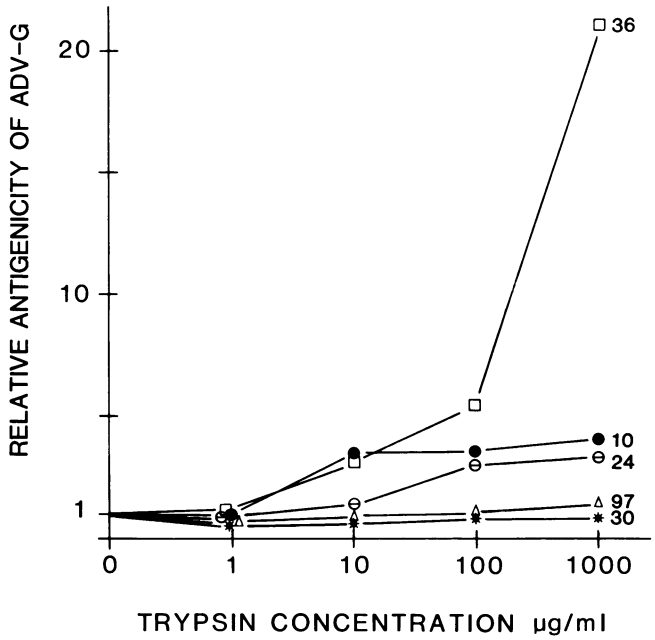

FIG. 6. Radioimmunoassay reactivity of several mAbs was analyzed against trypsinized ADV-G samples. The F(ab')$_2$ fragments from sera derived from ADV-G-infected mink were absorbed onto plastic pearls as described previously (2). This solid-phase antibody matrix was then reacted with ADV-G previously exposed to various concentrations of trypsin as indicated (Difco Laboratories, Detroit, Mich.). Reaction time was 3 h at 37°C followed by two washes. Pearls were then reacted against 1:10 dilutions of the indicated hybridoma culture supernatants for 3 h, washed, and then reacted against 50,000 cpm of ^{125}I-protein A, washed, and counted. mAb 36, which does not react against intact ADV-G, did react against trypsinized ADV-G as shown. Reactivity was increased 21-fold by trypsinization, indicating that mAb 36 reacts with an epitope exposed or created by proteolysis.

Viral antigens were also demonstrated in tissues of Utah I ADV-infected mink. The distribution of viral antigen was the same as that previously detected in immunofluorescence studies with FITC-conjugated sera from ADV-infected mink (30) and indicated that the earlier reagent, although polyclonal (16, 22, 29), nevertheless accurately detected viral components.

The preferential viral strain reactivities of various mAbs

TABLE 2. Analysis of anti-ADV mAbs by CIEP[a]

Group designation	mAb prototype(s)	Immunogen(s)[b]	Reactivity with the following antigen[c]:				
			Pullman[d]	Danish (DK)[d]	Ontario[d]	Utah I[d]	ADV-G[e]
I	10, 30	Utah I, ADV-G	+	+	+	+	+
II	36	ADV-G, Pullman, Danish, Ontario	+	+	+	+	−
III	P2, P11	Pullman	+	−	−	+	−
IV	24	Utah I	−	−	−	+	+
V	O8, O33	DK, Ontario	−	+	+	+	−
VI	68, 73	ADV-G, Utah I	−	−	−	−	+

[a] This table is representative of a much larger panel of mAbs. All reactivities, however, fell within the six groups represented. Only mAbs mentioned in the text are shown in the mAb prototype column.

[b] This column lists the viral strain antigens inoculated into mice which gave rise to mAbs with the indicated reaction pattern. For example, mAbs such as 10 or 30 were obtained when mice were immunized with either Utah I ADV or ADV-G. mAbs such as P2 or P11 were obtained only from Pullman ADV-inoculated mice, etc.

[c] + indicates a visible precipitin line immediately after electrophoresis; − indicates no visible precipitin line immediately after electrophoresis.

[d] Antigens were extracted from the combined spleens and livers of mink infected with 10^6 ID_{50}s of Utah I ADV, Ontario ADV, Danish ADV, or Pullman ADV and purified by Freon (E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) extractions and ultracentrifugation (10). Mice were immunized and mAbs were obtained as detailed in Materials and Methods.

[e] Antigen was prepared from ADV-G-infected CRFK cells. No hybridoma supernatant gave a precipitin (CIEP) reaction against an extract of normal CRFK cells or against spleen-liver extract from normal mink, nor did hybridoma supernatants made against leukemia viruses provide positive reactions against the antigens indicated. CIEP was performed by reacting hybridoma supernatants against twofold dilutions of the antigens indicated (undiluted to 1:16).

for ADV-infected CRFK cells were valid in vivo as well as in vitro. Thus, mAbs that reacted against Utah I ADV-infected CRFK cells also reacted against infected cells in tissues of Utah I ADV-infected mink, while mAbs with reactivity for ADV-G gave either no fluorescent staining or stained only a rare tissue cell. Again, the few positive cells may reflect an ADV-G-like population within the Utah I ADV stock or, alternatively, an epitope common to both viruses that was expressed quantitatively differently in the two viruses. Perhaps antigenic variants arise during ADV infection as well, and this could be one mechanism by which the virus escapes containment by the immune response. Such variants develop during a number of other persistent virus infections and are thought to play a prominent role in pathogenesis (13, 20, 21, 24, 25, 32, 35). Anti-ADV mAbs make it possible to search for such variants in infected tissues.

The pattern of tissue fluorescence suggested that cells of the lymphoid and reticuloendothelial system were involved. In fact, the cytoplasmic localization of viral antigen and the distribution of antigen-positive cells correlated well with an earlier study describing antigen capture by macrophages in lymphoid tissues (26). It seems likely, therefore, that the viral antigen detected in vivo represented ADV polypeptides, possibly complexed, that were phagocytized and sequestered by cells of the reticuloendothelial system. It is also likely that the detected antigen represented low-molecular-weight polypeptides since mAbs with specificity for proteolytic products of viral structural proteins were strongly positive against these tissues. Since we have no mAb specific for only intact p85 and p75, we could not formally exclude serologically the presence of some intact ADV structural proteins in these cells. However, no nuclear fluorescence was observed in infected tissues, which suggested that the viral antigen detected was not in cells actually replicating ADV. This observation is consistent with a simultaneous study (4a) that showed that ADV replicative-form DNA is a minor component of viral DNA in spleen, liver, or MLN. In marked contrast, nuclear fluorescence was detected in both ADV-G- and Utah I ADV-infected CRFK cells by mAbs reactive against intact structural proteins, whereas mAbs reactive only against breakdown polypeptides did not react against infected CRFK cells. These results suggested further that proteolytic degradation of virus is not a significant occurrence in the tissue culture situation but rather an in vivo occurrence (3).

In immunoprecipitation and immunofluorescence analyses mAbs clearly distinguished ADV-G from Utah I ADV, but we also wished to extend strain differentiation to field isolates. Differentiation of strains has practical importance since some strains are highly virulent for all mink genotypes while others are virulent for only certain ones (14). Most of these strains have not been adapted to tissue culture growth, so they could not be analyzed in detail as were ADV-G and Utah I ADV. CIEP, an assay that involves partially purified intact virions, was therefore used to analyze five virus strains. By using various combinations of mAbs and viral antigens it was possible to distinguish all of the strains. However, it was also evident that many common antigens existed among the viruses studied, confirming that various ADV strains are closely related (1, 4). Still, reactivity patterns observed with CIEP were consistent with specificities indicated by the other assays and allowed us to differentiate strains.

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