VIROLOGY 250, 263–272 (1998)
ARTICLE NO. VY989371

Characterization of Two Avian Reoviruses That Exhibit Strain-Specific Quantitative Differences in Their Syncytium-Inducing and Pathogenic Capabilities

Roy Duncan[1] and Kevin Sullivan

Department of Microbiology and Immunology, Faculty of Medicine, Dalhousie University, Tupper Medical Building, Halifax, Nova Scotia, Canada, B3H 4H7

Received April 10, 1998; returned to author for revision June 11, 1998; accepted August 12, 1998

We previously proposed that the conservation of the nonessential syncytium-inducing phenotype among all reported avian reovirus (ARV) isolates may reflect a mechanism for enhanced virus dissemination *in vivo*, which in turn could contribute to the natural pathogenicity of ARV. Direct testing of this hypothesis has been hampered by the lack of available virus strains with defined differences in their fusion-inducing capability. We now report on the characterization of two ARV strains, ARV-176 and ARV-138, that exhibited strain-specific differences in their fusogenic properties, which correlated with their pathogenic potential in embryonated eggs. Moreover, both virus strains possessed similar replicative abilities in cell culture, suggesting that the weakly fusogenic ARV-138 virus is specifically inhibited in its syncytium-inducing ability. To test the use of these viruses for reassortant studies aimed at assessing the role of cell fusion in viral pathogenesis, a preliminary genetic analysis was undertaken using a monoreassortant that contained nine genome segments from the parental ARV-138 virus and the S1 genome segment from the highly fusogenic and pathogenic ARV-176 parental virus. The monoreassortant possessed the full fusogenic potential of the ARV-176 parental virus and displayed enhanced embryo pathogenicity, providing the first genetic evidence implicating the ARV S1 genome segment in both syncytium formation and viral pathogenesis.

INTRODUCTION

The avian reoviruses (ARVs) represent a distinct species in the genus *Orthoreovirus,* family Reoviridae (Nibert *et al.,* 1996). ARVs are distinguished from the prototype mammalian reoviruses on the basis of their natural pathogenicity in their animal host and by their unusual propensity for inducing cell–cell fusion from within, resulting in extensive syncytium formation in infected cells (Kawamura *et al.,* 1965; Ni and Ramig, 1993; Robertson and Wilcox, 1986; Van der Heide, 1977). Syncytium formation has also been detected in the tissues of infected animals (Kibenge *et al.,* 1985), suggesting that cell fusion could be a contributing factor to viral pathogenesis. Although all characterized ARV isolates induce syncytium formation, the role of syncytium formation in the ARV replication cycle and in viral pathogenesis has not been defined.

Abrogation of ARV-induced cell fusion by the vesicle transport inhibitor brefeldin A demonstrated that the mechanism responsible for syncytium formation is nonessential for virus replication in cell culture (Duncan *et al.,* 1996). Apparently, the ARV fusogenic phenotype does not represent the sequelae of an essential step in the ARV replication cycle. This is contrary to syncytium formation induced by enveloped viruses, which target their fusion proteins to the cell membrane as an essential step in the assembly and release of infectious virions (Petterson, 1991; Stephens and Compans, 1988). Although ARV-induced cell fusion was not required for virus release or virus-induced cell killing, it did significantly increase the rate of both of these processes. These observations lead us to hypothesize that the conservation of the syncytium-inducing capability of ARVs may reflect a competitive advantage conferred on the virus by a mechanism that facilitates rapid dissemination of the infection within an infected animal (Duncan *et al.,* 1996). Such a process for enhanced virus spread could conceivably contribute to the natural pathogenic potential of this group of nonenveloped viruses. A similar correlation between cell fusion induced by some enveloped viruses and viral pathogenesis has also been proposed (Goodman and Engel, 1991; Koot *et al.,* 1993; Park *et al.,* 1994), although the involvement of syncytium formation in pathogenicity remains uncertain (Cloyd and Moore, 1990; Groenink *et al.,* 1993; Yu *et al.,* 1994)

An improved understanding of the relationship among ARV-induced cell fusion, virus replication, and viral pathogenesis would be facilitated by the analysis of different virus strains that exhibit distinct syncytium-inducing and pathogenic properties. Such virus strains can be used to generate genetic reassortants, a procedure extensively applied by the late Bernard Fields and coworkers to identify mammalian reovirus genome segments involved in viral pathogenesis and in numerous

[1] To whom reprint requests should be addressed. Fax: (902) 494-5125. Email: duncanr@tupdean1med.dal.ca.

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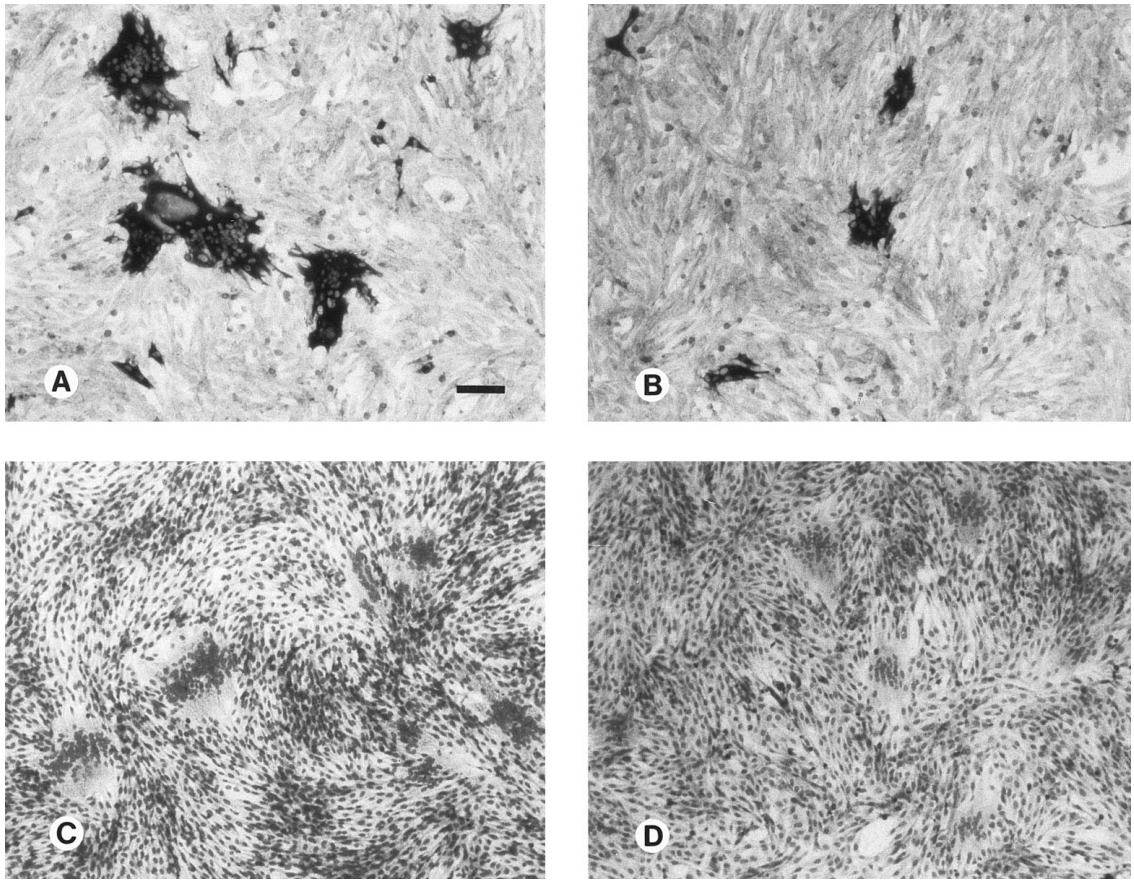

FIG. 1. Isolation of an ARV strain with reduced fusogenic potential. Monolayers of quail cell fibroblasts (QM5 cells) were infected at an m.o.i. of 0.03 with ARV-176 (A and C) or ARV-138 (B and D). Infected monolayers were incubated for 16 h before staining with a reovirus-specific rabbit polyclonal antiserum and goat anti-rabbit IgG conjugated with alkaline phosphatase to detect viral antigens (A and B) or with a water-soluble Wright–Giemsa stain (Diff-Quik) to reveal cell nuclei (C and D).

aspects of virus replication (e.g., Hazelton and Coombs, 1995; Hooper and Fields, 1996; Yin *et al.,* 1996). Although several ARV strains with distinct pathogenic properties have been described (Clark *et al.,* 1990; Hieronymous *et al.,* 1983; Tang *et al.,* 1987), there are no reports describing strain-specific differences in the fusion-inducing potential of different ARV isolates. The absence of a characterized ARV strain with a definable defect in its syncytium-inducing capability has prevented the use of reassortment analysis to define the relationship between virus-induced cell fusion and pathogenesis.

We now report on the characterization of two ARV strains with distinct fusion-inducing and pathogenic properties. Most notably, the fusogenic and pathogenic potential of the viruses was unrelated to their replicative abilities in cell culture, suggesting that the reduced fusogenic ability of the ARV-138 strain is not the consequence of ineffective virus infection or replication. A preliminary genetic reassortment analysis using these two virus strains implicated the S1 genome segment in both virus-induced cell fusion and viral pathogenesis.

RESULTS

ARV strains 176 and 138 exhibit distinct syncytium-inducing properties

Although no strain of ARV defective for syncytium formation has been described, we observed that a particular strain of ARV under investigation in our laboratory, ARV-138, was inefficient at inducing cell fusion. Infection of a quail cell line, QM5, with ARV-176 or ARV-138 at a low multiplicity of infection (m.o.i.) resulted in foci of infection detectable by immunostaining using ARV-specific antiserum (Figs. 1A and 1B). Although both strains induced the formation of multinucleated syncytia, ARV-138 induced syncytial foci that were considerably smaller than those associated with ARV-176 infection under identical experimental conditions. Infected cell monolayers stained with a water-soluble Wright–Giemsa stain clearly demonstrated the polykaryon nature of these infected-cell foci and the reduced fusogenic potential of ARV-138 (Figs. 1C and 1D).

The kinetics of syncytium formation induced by these two ARV strains was quantified by determining the aver-

age number of nuclei per syncytium at various times postinfection (p.i.) (Fig. 2). ARV-176 induced small syncytial foci of infection within 12 h after infection of QM5 cells at a low m.o.i. These syncytial foci rapidly increased in size to an average of 25–30 nuclei per syncytium by 16 h p.i. and to >50 nuclei per syncytium by 22 h p.i. (Fig. 2). Conversely, ARV-138 syncytia were difficult to detect at 12 h p.i. and did not become apparent until 14–16 h p.i.. By 20–22 h p.i., syncytia induced by ARV-138 had increased in size to an average of only ~20 nuclei per syncytium, 2.5- to 3-fold smaller than those induced by ARV-176. In repeated experiments, the difference between the ARV-138 and ARV-176 syncytial indices ranged from 2.3- to 3.2-fold. This represents the first identification of an ARV with a quantifiable difference in its fusion-inducing capability.

The weakly fusogenic ARV-138 strain displays limited embryo pathogenicity

We previously hypothesized that the syncytium-inducing property of ARV may contribute to viral pathogenesis (Duncan *et al.,* 1996). The identification of two strains of ARV with distinct fusogenic properties allowed us to test this hypothesis by determining whether there was a correlation between the syncytium-inducing and pathogenic potential of the viruses. ARV-176 has been described as a highly pathogenic isolate that induces rapid morbidity or mortality in the majority of test animals infected with this virus (Hieronymus *et al.,* 1983; Tang *et al.,* 1987). ARV-138 has not been as extensively characterized, but in a separate report, it was shown to induce no mortality in test animals and exhibited limited ability to induce morbidity (Drastini *et al.,* 1994). We undertook a direct comparison of the pathogenic potential of these two virus strains by using embryonated eggs, a more convenient assay than whole animal studies. Both virus strains were used to inoculate 10-day-old chicken embryos in the chorioallantoic cavity, and embryo mortality was monitored over time (Fig. 3). In all experiments, ARV-176 induced 80–100% mortality by 4–5 days p.i. at a time when embryo mortality induced by ARV-138 was <10% (see also Fig. 8). Equivalent numbers of mock-infected embryos showed <10% mortality (data not shown), similar to the situation with embryos infected with ARV-138. These results demonstrated that the diverse pathogenic properties of the two virus strains observed *in vivo* are reproducible *in ovo.* Furthermore, although the data do not establish a causal link between ARV-induced cell fusion and pathogenesis, the results did provide the first evidence of a correlation between the strain-specific fusogenic and pathogenic properties of ARV.

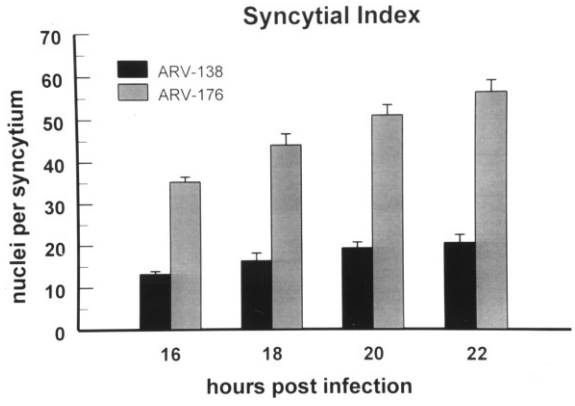

FIG. 2. Quantitative analysis of strain-specific ARV-induced syncytium formation. Duplicate quail cell monolayers were infected with ARV-176 or ARV-138 as described in Fig. 1 and stained with Diff-Quik at various hours p.i. The average number of nuclei contained per syncytium was determined by counting the nuclei per syncytium in five random fields. Results are the mean ± SEM of four separate experiments.

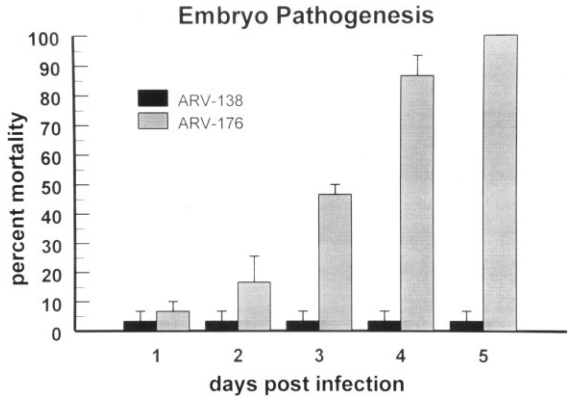

FIG. 3. ARV-176 and ARV-138 exhibit distinct embryo pathogenicities. Ten-day-old chicken embryos were inoculated into the chorioallantoic cavity with 1×10^3 pfu of ARV-176 or ARV-138, with 10 embryos for each strain of virus. Infected embryos were observed daily for embryo mortality as determined by the lack of embryo movement and the appearance of blackened blood vessels indicative of necrosis. Reported results are the mean ± SEM of three separate experiments.

Strain-specific differences in cell fusion and pathogenicity are unrelated to the relative replicative ability of the virus

ARV strains with distinct fusogenic and pathogenic properties may be used for reassortant analysis to evaluate whether any correlation exists between syncytium formation and virus pathogenesis. Such a genetic approach would be facilitated by using a parental virus strain that is specifically altered in its fusion-inducing potential. It was necessary, therefore, to determine whether the decreased fusogenic and pathogenic potential of ARV-138 reflected either an overall decrease in the replication properties of this virus strain or a specific defect in the mechanism responsible for cell fusion. To address this issue, we compared several aspects of the virus replication cycle in cell culture.

The cell-binding and internalization capabilities of

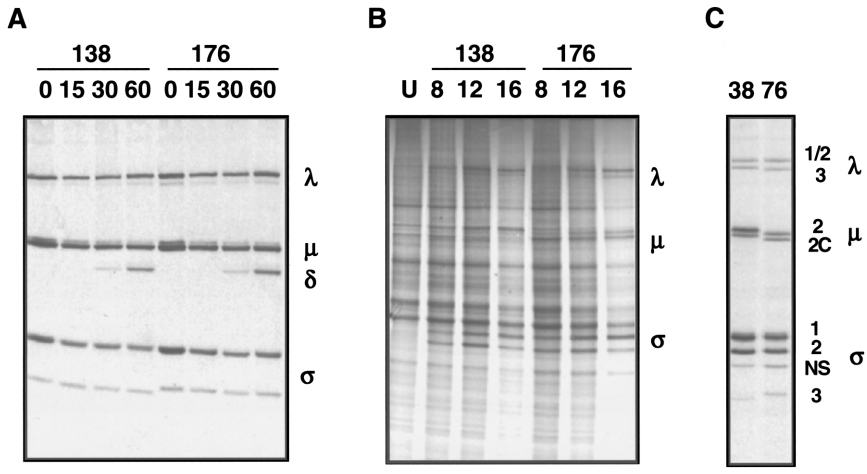

FIG. 4. The extent of syncytium formation and viral pathogenesis is unrelated to virus macromolecular synthesis or cell infection. (A) Radiolabeled, purified virus particles of ARV-176 and ARV-138 were adsorbed to quail cell monolayers at room temperature, followed by extensive washing to remove unadsorbed particles. Cultures were shifted to 37°C to permit virus entry, and at various times after the temperature shift (0, 15, 30, or 60 min), cells were harvested, the lysates were fractionated by SDS–PAGE, and radiolabeled viral proteins were detected by autoradiography. The locations of the major λ, μ, and σ viral structural proteins are indicated along with the location of the entry-specific δ polypeptide, a cleavage product of the μ2C outer capsid protein. (B) Quail cell monolayers were infected with either strain of ARV at an m.o.i. of 10, and at the indicated times (8, 12, or 16 h p.i.), monolayers were pulse-labeled for 30 min with [^{35}S]methionine, harvested in detergent lysis buffer, fractionated by SDS–PAGE, and radiolabeled translation products were detected by autoradiography. The locations of the major λ, μ, and σ virus proteins are indicated. U indicates uninfected cell lysate. (C) Radiolabeled viral proteins present in the 16-h time points from panel B were detected by immunoprecipitation using reovirus-specific antiserum. The immunoprecipitates were analyzed by SDS–PAGE and autoradiography. The locations of the major λ, μ, and σ virus proteins are indicated.

each virus strain were assessed using equivalent numbers of radiolabeled virus particles with comparable specific activities. Bound virus particles were detected by SDS–PAGE and autoradiography (Fig. 4A, 0 time points). Quantitative analysis of the density contained in the major μ and σ capsid proteins detected in scans of the autoradiographs indicated that both viruses possess similar steady-state cell-binding efficiencies under the experimental conditions used in this assay. These results suggested that the receptor-binding function of the σ3 protein (Shapouri *et al.,* 1996) was not impaired in the weakly fusogenic ARV-138 virus. More importantly, the kinetic analysis of virus entry, as determined by the appearance of the δ polypeptide, an entry-specific cleavage product of the major μ2C outer capsid protein (Duncan, 1996; Sturzenbecker *et al.,* 1987), also revealed no obvious difference between the two viruses in the early events after virus–cell interaction (Fig. 4A, 15–60 min time points). The appearance of the ARV-specific δ′ polypeptide, an alternate cleavage product of μ2C, was delayed with both of these virus strains relative to the previously characterized strain S1133 virus (see Duncan, 1996). The entry results suggested that the two viruses were equally capable of infecting cultured cells, which was confirmed using a quantitative focus-forming assay (data not shown), similar to the results presented in Figs. 1A and 1B. The cumulative weight of the attachment and entry results implied that the decreased pathogenic and fusogenic potential of ARV-138 is unlikely to be the result of a diminished capacity to bind or infect cells.

Similarly, an analysis of virus macromolecular synthesis indicated that ARV-138 is not impaired in its ability to direct the synthesis of viral proteins. As shown in Fig. 4B, both ARV strains displayed a similar pattern of viral protein synthesis in infected QM5 monolayers. This was most evident by observing the appearance of the σ-class proteins that were detectable in cells infected with either virus strain by 8 h p.i.. The rate of viral protein synthesis for both viruses increased to a maximum by 12 h p.i. and decreased thereafter, along with host translation levels, due to virus-induced cytopathic effects (Duncan *et al.,* 1996). In addition, immunoprecipitation of infected cell extracts indicated that both strains synthesized a similar complement of viral proteins (Fig. 4C). Therefore, the reduced fusogenic and pathogenic potential of ARV-138 does not appear to be the consequence of a generalized defect in virus macromolecular synthesis.

The reduced pathogenic potential of ARV-138 could not be accounted for by a deficiency in the production of progeny virions. The rate and extent of infectious ARV-138 progeny virus production were not reduced relative to titers obtained after ARV-176 infection (Fig. 5); in actuality, the final yield from cultures infected by ARV-138 was reproducible 3- to 5-fold higher than corresponding cultures infected by ARV-176. The above analyses indicated that the distinct syncytium-inducing properties and pathogenicities of these two ARV strains appear to be unrelated to any of the processes that govern the virus replication cycle at a cellular level and suggested that

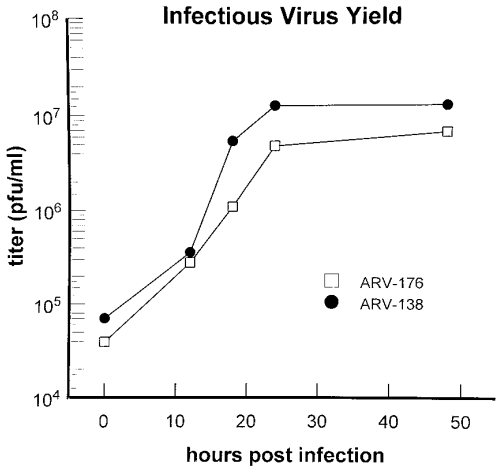

FIG. 5. Strain-specific differences in ARV pathogenicity do not reflect differences in the replicative ability of the virus. Duplicate monolayers of quail cells were infected with ARV-176 or ARV-138 at an m.o.i. of 10, and at various times p.i., the monolayers were harvested, and the yield of infectious progeny virions was determined by plaque assay on QM5 cell monolayers. Results are the average of two separate experiments.

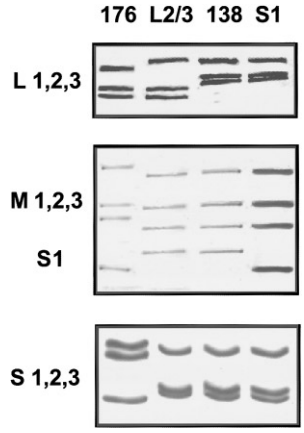

FIG. 6. Isolation of an S1 genome segment monoreassortant. Reassortants were isolated among the progeny obtained after coinfection of quail cells with the parental ARV-176 and ARV-138 viruses. Genomic dsRNA was isolated from a plaque-purified S1 genome segment monoreassortant (S1) and from a reassortant containing the strain 176-L2 and -L3 genome segments (L2/3). The genome segments of the parental viruses and the reassortants were resolved by SDS–PAGE and detected by silver staining. A 10% polyacrylamide gel was used to resolve the L, M, and S1 genome segments (top two panels), whereas the remaining S genome segments were resolved on a 7% polyacrylamide gel (bottom panel). The identities of the various genome segments are indicated on the left.

ARV-138 is specifically impaired in its syncytium-inducing ability.

The ARV-176 S1 genome segment is implicated in efficient cell fusion and enhanced embryo pathogenicity

The apparently specific nature of the fusogenic defect in ARV-138 suggested that this virus strain may represent a useful parental virus for the genetic investigation of the relationship between cell fusion and pathogenesis and to identify viral gene products that influence these properties. To test the use of these two viruses in reassortant studies, we isolated a small panel of reassortants among the progeny generated after coinfection of quail cells with ARV-176 and ARV-138. In a first screen of the progeny produced from such a coinfection, we identified six reassortants, including a monoreassortant containing nine genome segments from ARV-138 and the S1 genome segment from ARV-176 (Fig. 6). Because the σ3 protein of ARV is a product of the S1 genome segment (Schnitzer, 1985; Varella and Benavente, 1994) and has been implicated in syncytium formation (Theophilos *et al.,* 1995), we tested this monoreassortant for its syncytium-inducing and pathogenic capability.

The syncytium-inducing ability of the S1 monoreassortant was compared with that of the two parental viruses and an L2/3 reassortant, one of only two reassortants isolated that did not contain the ARV-176 genome segment, using the quantitative syncytium-forming assay (Fig. 7). The S1 monoreassortant possessed the complete fusogenic ability of the ARV-176 parental virus-generating syncytial indices that ranged from 2.1- to 3.2-fold greater than those induced by ARV-138 or the L2/3 reassortant. Moreover, both reassortants exhibited replicative abilities similar to that of the ARV-138 parental virus (data not shown) indicating that syncytium formation was not influenced by altered virus replication. The clear segregation of the ARV-176 syncytial phenotype with the S1 genome segment suggested a direct involvement of S1-encoded gene product or products in ARV-induced cell fusion and supported the proposal that ARV-138 is specifically impaired in its fusion-inducing ability.

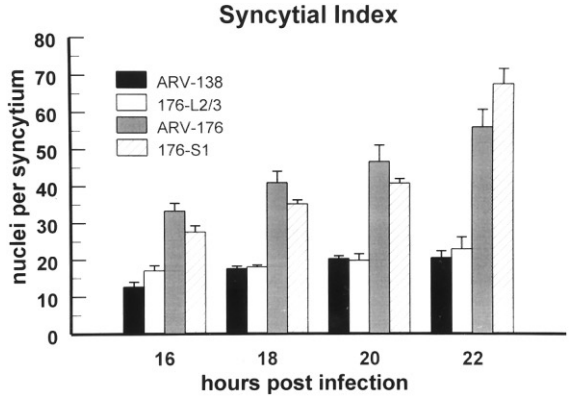

FIG. 7. The ARV-176 S1 genome segment is responsible for extensive syncytium formation. The parental viruses (ARV-176 and ARV-138) and the two reassortants (176-S1 and 176-L2/3) were used to infect quail cell monolayers at an m.o.i. of 0.03, and the syncytial index for each virus was determined as outlined in Fig. 2. Results are the mean ± SEM from three separate experiments.

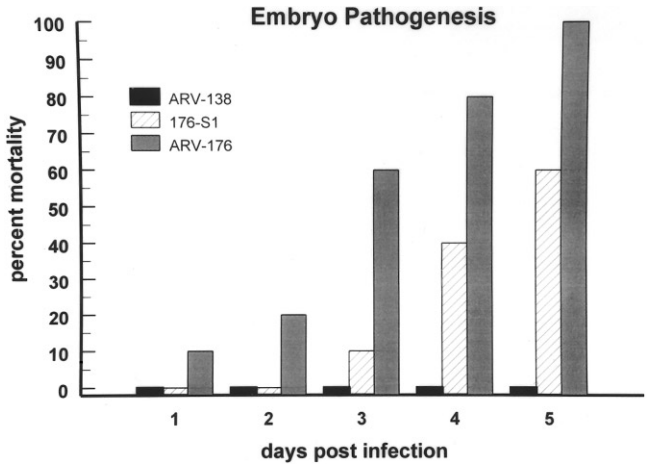

FIG. 8. The S1 genome segment significantly influences ARV pathogenesis. The parental viruses (ARV-176 and ARV-138) and the S1 monoreassortant (176-S1) were used to infect embyonated eggs as outlined in Fig. 3 (10 embryos per treatment). Infected embryos was observed daily, and the percent mortality was determined. Results are the average of two separate experiments.

As a first step toward assessing the influence of the fusion-inducing S1 genome segment on virus pathogenesis, the pathogenic potential of the two parental viruses was compared with that of the S1 monoreassortant in embryonated eggs (Fig. 8). As previously described (Fig. 3), ARV-138 induced no embryo mortality by 5 days p.i. versus the 100% mortality induced by ARV-176. In two separate experiments, the monoreassortant exhibited an intermediate pathogenic potential, inducing mortality in 60% of the embryos by 5 days p.i. (Fig. 8). These results, although preliminary, did provide the first genetic evidence of the involvement of the ARV S1 genome segment in viral pathogenesis. In addition, the data suggested that although syncytium formation may be primarily, if not exclusively, influenced by the S1 genome segment, ARV pathogenesis appears to reflect a multigenic phenomenon.

DISCUSSION

Characterization of an ARV with reduced fusogenic and pathogenic potential

Although distinct pathogenicities have been described for numerous ARV isolates (e.g., Clark *et al.,* 1990; Hieronymous *et al.,* 1983; Tang *et al.,* 1987), this represents the first report of a quantifiable difference in the syncytium-inducing ability of different ARV strains. Although ARV-138 is not a syncytium-negative virus strain, it is clearly impaired in its syncytium-inducing ability in fibroblasts (Figs. 1 and 2). In addition, ARV-138 was significantly reduced in its pathogenic potential in embryonated eggs compared with the highly fusogenic ARV-176 strain (Figs. 3 and 8), pathogenic phenotypes that mimic those induced in infected birds (Clark *et al.,* 1990; Drastini *et al.,* 1994; Hieronymous *et al.,* 1983; Tang *et al.,* 1987).

Similar to these results, Takase et al. (1987) reported a strong correlation in strain-specific differences between ARV pathogenesis in whole animals versus embryonated eggs. Moreover, the degree of virus pathogenesis correlated with the extent of cytopathic effect induced in cell cultures after the first passage of clinical isolates, although no attempt was made to describe the extent of cell fusion (Takase *et al.,* 1987). Our characterization of an ARV with impaired fusogenic and pathogenic potential and the preliminary genetic analysis of this virus represent the first demonstrated correlation between the conserved fusogenic potential of ARVs and their natural pathogenicity. Establishing the significance of this correlation will require a comparative analysis of the rate and extent of virus dissemination and histopathology induced in infected animals by these two parental viruses and a comprehensive group of reassortants.

Equally important was our demonstration that the reduced pathogenic and fusogenic potential of ARV-138 was unrelated to the replicative abilities of the virus in cell culture (Figs. 3–5). The current data suggest that ARV-138 is specifically defective in the mechanisms responsible for cell fusion that are not the result of general deficiencies in other aspects of the virus replication cycle at a cellular level. These results corroborated our previous studies demonstrating that the processes governing cell fusion are unrelated to other aspects of the virus replication cycle (Duncan *et al.,* 1996). Consequently, the genetic identification of genome segments involved in syncytium formation and viral pathogenesis using these two parental viruses is unlikely to be complicated by viral gene products that merely influence virus replication.

Role of the S1 genome segment in syncytium formation

Previous reports have suggested the possible involvement of either the major outer capsid proteins, μ2C and/or σ2, or the cell attachment protein, σ3, in ARV-induced syncytium formation (Ni and Ramig, 1993; Theophilos *et al.,* 1995). Our current quantitative analysis of cell fusion induced by the parental viruses and the two reassortants implicated the S1 genome segment in ARV-induced cell fusion; although based solely on the monoreassortant data, this assignment must still be viewed as tentative. For instance, it has been demonstrated that specific mutations in certain genome segments of mammalian reovirus may be required to generate "acceptance signals" that permit virus particles to package heterologous genome segments (Roner *et al.,* 1995). Consequently, alterations in the coding sequence of other genome segments in the S1 monoreassortant that influence syncytium formation cannot be excluded. This scenario, however, seems unlikely because it requires that a mutation in an ARV-138 genome segment

that is required for "acceptance" of the ARV-176 S1 genome segment happens, coincidentally, to confer the full fusogenic potential of ARV-176 on the monoreassortant. In addition, one of only two reassortants isolated that contained the ARV-138 S1 genome segment behaved identically to the parental ARV-138 virus in fusion assays (Fig. 7), which is in accord with our prediction that the ARV-176 S1 genome segment is required for enhanced syncytium formation. These results suggest that mutations in other genome segments that are required to permit the formation of reassortants are unlikely to contribute to enhanced syncytium formation.

The genetic analysis implicating the ARV S1 genome segment in cell fusion has recently been confirmed using transfection assays. Our analysis indicates that a predicted small open reading frame encoding a 10kDa protein previously detected in the sequence of the ARV-S1133 S1 genome segment (Shapouri *et al.,* 1995) is both necessary and sufficient for virus-induced cell fusion (Shmulevitz and Duncan, unpublished observations). Therefore, the results obtained from both genetic and molecular analyses indicate the direct involvement of the S1 genome segment in virus-induced cell fusion.

Neither of these experimental approaches, however, eliminates a potential role for other genome segments in virus-induced cell fusion. In the case of the genetic analysis, the phenotype of genetic reassortants can be influenced by the nature of the recipient genetic background (Chen *et al.,* 1989), which can complicate assignment of particular phenotypes to specific genome segments. Further characterization of an extended group of reassortants is required to determine the role, if any, of other genome segments in influencing the extent of ARV-induced cell fusion.

Role of the S1 genome segment in viral pathogenesis

Our initial genetic survey provided the first evidence implicating the ARV S1 genome segment in virus pathogenesis. The data also suggested that the S1 genome is not solely responsible for the pathogenicity of ARV-176 (Fig. 8), indicating, not surprisingly, that ARV pathogenesis is influenced by several genome segments. The preliminary genetic analysis demonstrated the use of these two ARV strains in investigating the viral factors influencing virulence and the relationship between ARV-induced cell fusion and pathogenicity. Clearly, a larger panel of reassortants is required to confirm the direct involvement of the S1 genome segment in ARV pathogenesis and to identify additional virulence determinants.

The implication of the ARV S1 genome segment in viral pathogenesis is similar to the situation with mammalian reovirus. The role of the mammalian reovirus S1 genome appears to reflect primarily the involvement of the encoded σ1 protein in receptor attachment (Kauffman *et al.,* 1983; Lee *et al.,* 1981; Tyler *et al.,* 1986; Weiner *et al.,* 1977, 1978; Wilson *et al.,* 1994). Even though the ARV S1 genome segment encodes the viral cell attachment protein σ3 (Shapouri *et al.,* 1996), the ARV-138 σ3 protein does not appear to adversely influence steady-state receptor interactions or, more importantly, the efficiency of virus infection of cultured cells (Fig. 4A). This observation suggests that receptor interactions alone may not be responsible for the enhanced pathogenic potential contributed by the ARV-176 S1 genome segment. Confirmation of this conjecture requires more extensive receptor-binding studies performed on primary cell cultures obtained from any tissues that display strain-specific alterations in virus infection or replication after infection in whole animals.

The segregation of both enhanced fusogenic and pathogenic properties with the S1 genome segment, in a replication-independent manner, offers an alternative hypothesis to explain the role of the S1 genome segment in ARV pathogenesis. The impaired fusogenic ability of ARV-138 in cell culture suggests that a similar situation occurring *in vivo* could reduce the rate of virus dissemination and the extent of tissue destruction within an infected animal contributing to the reduced pathogenic potential of this virus strain. Several additional lines of evidence support this hypothesis. First, the observation that ARV-induced cell fusion is not restricted to cells in culture but also occurs in the tissues of infected animals (Kibenge *et al.,* 1985) suggests that cell fusion may affect virus–host interactions *in situ,* although the extent of cell fusion *in vivo* has never been rigorously examined. Second, although syncytium formation is not essential for virus release or virus-induced cytopathology, it does enhance the rate of both of these processes by accelerating a lytic-type infection that contributes to the progression of the infection in cell culture (Duncan *et al.,* 1996). Third, we previously demonstrated that the mechanisms responsible for syncytium formation are distinct from those involved in virus replication in cell culture (Duncan *et al.,* 1996), consistent with our present results indicating the more robust replicative ability of the weakly fusogenic ARV-138 virus (Fig. 4). Therefore, syncytium formation, or the lack thereof, may influence virus dissemination independent of virus replication at a cellular level.

A similar correlation between syncytium formation and viral pathogenesis has been observed with several enveloped viruses. For example, a herpes simplex virus type 1 variant capable of extensive syncytium formation exhibits significantly increased pathogenic potential *in vivo* (Goodman and Engel, 1991). The syncytial and pathogenic phenotypes were mapped to a single region near the carboxyl terminus of glycoprotein B, and most importantly, this alteration did not affect viral replication, suggesting a correlation between syncytium formation and pathogenesis (Engel *et al.,* 1993). Similarly, the appearance of syncytium-inducing strains of HIV-1 corre-

lates with an unfavorable prognosis (Bozzette *et al.,* 1993; Groenink *et al.,* 1993; Koot *et al.,* 1993).

The current analysis of ARV has provided the first genetic evidence implicating the S1 genome segment in both syncytium formation and pathogenesis. A more detailed kinetic analysis of ARV pathogenesis *in vivo* using the two ARV strains characterized in this report and including an expanded group of reassortants should contribute to an improved understanding of the relationship between the unusual syncytium-inducing ability of this group of nonenveloped viruses and their pathogenic potential.

MATERIALS AND METHODS

Virus strains and cells

ARV-176 has been previously described (Duncan *et al.,* 1996). Strain SK138a (ARV-138) was originally isolated in chicken embryos inoculated with the extract from the hock joint of an infected chicken in New Brunswick (Canada). We obtained ARV-138 from Frederick Kibenge (Atlantic Veterinary College, University of Prince Edward Island) after six low-multiplicity passes in Vero cells (Drastini *et al.,* 1992). Both strains were plaque purified and amplified to pass four using an m.o.i. of 0.01 in a continuous quail cell line, QM5, a clonal derivative of QT6 cells that exhibits a very low spontaneous fusion index (Antin and Ordahl, 1991). QM5 cells were grown in Medium 199 supplemented with 10% tryptose phosphate broth, 10% fetal bovine serum, and penicillin–streptomycin (50 U/ml and 50 μg/ml, respectively).

Staining cell monolayers and determination of a syncytial index

To quantify the relative extent of virus-induced syncytium formation, a syncytial index was calculated as previously described (Duncan *et al.,* 1996). Monolayers of QM5 cells were infected with the various virus isolates at an m.o.i. of 0.03 (to generate foci of infection) and incubated for varying lengths of time. At the indicated times p.i., medium was removed from duplicate wells, and the cells were washed once with PBS, fixed with methanol and stained using a water-soluble Wright–Giemsa stain (Diff-Quik) according to the manufacturer's protocol (VWR Scientific). The average number of nuclei per syncytium was determined by counting five random fields from duplicate samples at 100$\times$ magnification using bright-field microscopy. The number of nuclei contained in a minimum of 30 syncytia were counted to determine the average number of nuclei per syncytium.

A similar procedure was used in a focus-forming assay to detect viral foci of infection, except that after fixation, monolayers were stained using polyclonal rabbit antiserum raised against virus structural proteins as previously described (Duncan *et al.,* 1996). Foci were detected using a secondary goat anti-rabbit IgG conjugated with alkaline phosphatase (Life Technologies) according to standard protocols (Harlow and Lane, 1988).

Embryo pathogenesis analysis

Fertilized eggs were obtained from a specific pathogen-free flock screened for ARV and maintained at the Agriculture Canada Research Center (Ottawa). The eggs were incubated at 37°C in a moist environment and allowed to develop to 10 days postfertilization. The embryos were checked for viability and stage of development by candling, and the locations of the embryo and air sac were marked. The titrated virus stocks were diluted to 1×10^3 pfu/0.1 ml in tissue culture medium (minus fetal bovine serum) and inoculated into the chorioallantoic cavity using a 23-gauge needle. Control samples were inoculated with 0.1 ml of dilution medium. The puncture holes were sealed with vacuum grease, and the embryonated eggs were returned to the incubator. Eggs were candled daily and scored for embryo viability by a lack of embryo movement and darkened blood vessels indicative of necrosis.

Viral protein synthesis and immunoprecipitation

The kinetics and extent of viral protein synthesis were analyzed in infected QM5 cells (m.o.i. of 10) by [^{35}S]methionine pulse-labeling and SDS–PAGE as previously described (Duncan *et al.,* 1996). Infected and uninfected, radiolabeled cell extracts were immunoprecipitated using a combination of a rabbit antiserum prepared against purified ARV as previously described (Duncan, 1996; Duncan *et al.,* 1995) and a chicken antiserum obtained after virus infection. The chicken antiserum recognizes virus nonstructural proteins in addition to the structural proteins recognized by the rabbit antiserum. Both antisera (1:400 dilution) were incubated with detergent-disrupted cell lysates for 60 min at room temperature, followed by the addition of rabbit anti-chicken Ig (DAKO) and an additional 60-min room temperature incubation. Immune complexes were precipitated using IgGsorb (The Enzyme Center) and released by boiling in SDS protein sample buffer (Laemmli, 1970) before SDS–PAGE.

Radiolabeled virus purification and cell binding assays

[^{35}S]Methionine-labeled virus particles were purified by CsCl gradient centrifugation and used to assess the efficiency of virus attachment to, and entry into, quail cells as previously described (Duncan, 1996). Monolayers of cells in 12-well cluster plates (1×10^6 cells/well) were infected at room temperature using $\sim$100,000 cpm (10^{10} virus particles). Assuming $\sim 10^5$ receptors per cell (Armstrong *et al.,* 1984; Tardieu *et al.,* 1982), the number of virus particles per well was well below the concentration required to saturate the cell receptors. After ab-

sorption and washing, cultures were incubated at 37°C for various times before harvesting. The extent of virus attachment and entry was assessed by SDS–PAGE and autoradiography followed by quantification of the intensity of virus bands on the scanned x-ray film. The specific activity of both virus strains was 80,000–170,000 cpm/10^{10} particles and both viruses displayed similar particle-to-pfu ratios in quail cells as determined by plaque assays.

Virus growth curves

The rate and extent of ARV replication in QM5 cells were determined by infecting cells at an m.o.i. of 10 for 1 h at 37°C. After absorption, the inoculum was removed, and the monolayers were washed three times with warm PBS and overlaid with warm Medium 199 containing 1% fetal bovine serum. At various times p.i., the cells from duplicate wells were scraped into the culture medium, harvested, and disrupted by three freeze–thaw cycles. The total infectious progeny virus titer was determined by plaque assay on QM5 cells as previously described (Duncan *et al.,* 1996).

RNA analysis

Viral dsRNA genome segments were isolated from concentrated virus stocks. The concentrated virus was resuspended in TMN (10 mM Tris, pH 8.0, 10 mM $MgCl_2$, 150 mM NaCl) and treated with 10 μg/ml RNase A and DNase to remove contaminating extra-virion nucleic acid, and the virus particles were disrupted with 1% SDS at 37°C for 30 min. The liberated double-stranded RNA (dsRNA) was isolated by phenol–chloroform extraction and ethanol precipitation and resuspended in TNE (10 mM tris, pH 7.5, 150 mM NaCl, 1 mM EDTA). The resuspended dsRNA was mixed with Laemmli's electrophoresis sample buffer, heated to 65°C for 5 min, and fractionated by SDS–PAGE (Laemmli, 1970) using 0.75-mm-thick, 20-cm-long 7% or 10% acrylamide gels to resolve various size classes of the dsRNA. The gels were fixed and silver stained according to the procedure of Blum et al. (1987).

Isolation of ARV reassortants

Reassortant viruses were isolated from QM5 cells infected simultaneously with ARV-176 (m.o.i. of 5) and ARV-138 (m.o.i. of 1), similar to previous reports (Ni and Kemp, 1990, 1992). The progeny virions obtained from coinfections were isolated by plaque assay, and individual plaques were picked and amplified by a single passage in QM5 cells. The RNA was isolated from pass 1 virus stocks and analyzed by SDS–PAGE. Virus reassortants, containing various genome segments from both parents, were plaque purified a second time and grown to pass 2, and their RNA genome segment profiles were rechecked by SDS–PAGE. One such reassortant, containing nine genome segments from ARV-138 and the S1 genome segment from ARV-176, was used in this study to determine the role of the S1 genome segment in syncytium formation. A second reassortant containing the L2 and L3 genome segments of ARV-176 in an ARV-138 genetic background was used for comparison purposes.

ACKNOWLEDGMENTS

This work was supported by grants from the Medical Research Council of Canada and the Natural Sciences and Engineering Research Council. The authors thank Zhaoxia Chen and Jingyun Shou for technical assistance and Kevin Coombs and Maya Shmulevitz for useful suggestions on the manuscript.

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