Ability of Foot-and-Mouth Disease Virus To Form Plaques in Cell Culture Is Associated with Suppression of Alpha/Beta Interferon

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A genetic variant of foot-and-mouth disease virus lacking the leader proteinase coding region (A12-LLV2) is attenuated in both cattle and swine and, in contrast to wild-type virus (A12-IC), does not spread from the initial site of infection after aerosol exposure of bovines. We have identified secondary cells from susceptible animals, i.e., bovine, ovine, and porcine animals, in which infection with A12-LLV2, in contrast to A12-IC infection, does not produce plaques; this result indicates that this virus cannot spread from the site of initial infection to neighboring cells. Nevertheless, A12-LLV2 can infect these cells, but the pathogenic causes and virus loads are significantly reduced compared to those seen with A12-IC infection. Reverse transcription-PCR analysis demonstrates that both A12-LLV2 and A12-IC induce the production of alpha/beta interferon (IFN-α/β) mRNA in host cells. However, only supernatants from A12-LLV2-infected cells have significant antiviral activity. The antiviral activity in supernatants from A12-LLV2-infected embryonic bovine kidney cells is IFN-α/β specific, as assayed with mouse embryonic fibroblast cells with or without IFN-α/β receptors. The results obtained with cell cultures demonstrate that the ability of A12-IC to form plaques is associated with the suppression of IFN-α/β expression and suggest a role for this host factor in the inability of A12-LLV2 to spread and cause disease in susceptible animals.

Foot-and-mouth disease (FMD) is a highly contagious disease of cloven-hoofed animals that is spread by aerosol. It is the most economically important disease of livestock worldwide, and FMD-free countries enforce trade embargos of meat and meat products on countries in which the disease is enzootic (1, 18). FMD is caused by a member of the Aphthovirus genus of the family Picornaviridae. To control and eradicate the disease, a chemically inactivated FMD vaccine is used along with slaughter of infected and exposed animals and strict quarantine measures (5). Although killed vaccines are effective, there are recurrent problems and concerns, including escape of live virus from manufacturing plants, improper virus inactivation, relatively short-lived immunity, and development of a carrier state in some vaccinated animals following contact with FMD virus (FMDV) (2, 13, 27). To overcome some of these difficulties, we have initiated a program to develop live attenuated viruses based on our knowledge of the virus and its mode of replication at the molecular level.

The FMDV genome is a positive-sense single-stranded RNA molecule of about 8,300 nucleotides and codes for four primary translation products, leader (L), P1, P2, and P3, which are processed by the virus-encoded proteinases, L, 2A, and 3C, into the mature structural and nonstructural proteins (26). Translation initiates internally, at the L coding region, in a cap-independent manner directed by the internal ribosome entry site. The first translation product, L, is a papain-like proteinase that cleaves itself from the growing polypeptide chain (9, 10, 15, 23, 25, 31). Like the 2A proteinase of other picornaviruses, the L proteinase is essential for the rapid replication of FMDV because it also cleaves the host initiation factor eIF-4G; this factor is required for the translation of host mRNAs, most of which initiate translation by a cap-dependent mechanism (9, 14, 19, 26). Since the translation of FMDV mRNA is cap independent, the cleavage of eIF-4G results in the shutoff of most host cell protein synthesis and the rapid synthesis of viral proteins. In addition, as a result of the shutoff of host cell protein synthesis, the ability of the host to mount an antiviral response may be compromised.

We have constructed a genetic variant of FMDV lacking a complete L proteinase coding region (22). The leaderless virus (A12-LLV2) replicates slightly more slowly than the wild-type virus (A12-IC) in baby hamster kidney cells (BHK-21 cells) and is slightly attenuated in suckling mice (22). In contrast to A12-IC, A12-LLV2 is nonpathogenic in bovine and porcine animals but replicates and induces a neutralizing antibody response as well as partial protection upon virulent virus challenge (6, 17). After aerosol exposure of cattle to A12-LLV2, only a small number of isolated cells in the respiratory bronchioles were found to be infected, and the virus did not spread systemically to epithelial sites in the oral and pedal regions (4). In contrast, A12-IC caused extensive local infection and rapidly spread to secondary sites (4). Based on these data, we hypothesized that L proteinase is a virulence factor, the presence of which results in uncontested growth of virus because of the inhibition of a host antiviral response and rapid viral protein synthesis.

In this study, we have examined the host cell response to FMDV infection in cell cultures by using bovine, ovine, and porcine cells which are susceptible to A12-IC and A12-LLV2 infection but selectively support the spread of only A12-IC. Virus replication was examined with secondary embryonic bovine kidney (EBK), lamb kidney (LK), and porcine kidney (PK) cells, and the induction of a host cell antiviral response in
A12-LLV2-infected cells was demonstrated. The molecular mechanisms responsible for this phenomenon and their implications in disease pathogenesis are discussed.

MATERIALS AND METHODS

Cells and viruses. BHK-21 (clone 13) cells were used to propagate virus stocks and to measure virus titers in plaque assays. Secondary EBK, LK, and PK cells were provided by Carol House (12). Immortalized mouse embryonic fibroblast (129) cells from wild-type mice (+/+) and from mice lacking a subunit of the alpha/beta interferon (IFN-α/β) receptor (−/−) were provided by David E. Levy (16, 20). FMDV A12-IC was derived from the full-length infectious clone pRMCC2 (24), and A12-LLV2 was derived from the infectious clone lacking the Lb coding region, pRM-LLV2 (22). Vesicular stomatitis virus serotype New Jersey (VSV-NJ) and bovine enterovirus serotype 1 (BEV-1) were provided by Carol House and Jim House, respectively. In all assays, the multiplicity of infection (MOI) used was based on titration in BHK-21 cells.

Single-step growth assay. BHK-21, EBK, LK, and PK cells were infected with A12-IC or A12-LLV2 at an MOI of 10 at 37°C. After 1 h of adsorption, cells were rinsed with 150 mM NaCl-20 mM morpholinoethanesulfonic acid (MES) (pH 6) to inactivate unadsorbed input virus and incubated in minimal essential medium (MEM) at 37°C. Supernatants were collected from the infected cell cultures at 1, 2, 4, 6, 7, and 24 h postinfection (hpi) and titrated on BHK-21 cells as described previously (15).

PAGE. EBK, LK, and PK cells were infected with A12-IC or A12-LLV2 for 1 h and radiolabeled with [35S]methionine at various times postseduction as described previously (22). Cells were lysed, and cytoplasmic extracts were analyzed by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis (PAGE) as described previously (22).

Infection inhibition assay. EBK, LK, PK, and BHK-21 cells were infected with A12-LLV2 at MOIs of 0.01, 0.1, 1, and 10 for 1 h. Cells were rinsed as described above to inactivate unadsorbed virus and rinsed with MEM to restore physiological pH. Infection was continued for 4, 7, 24, and 48 h. Supernatants were obtained, centrifuged to remove cellular debris, brought to pH 2 with concentrated HCl, incubated for approximately 24 h at 4°C, and restored to pH 7 with concentrated NaOH (28). Treated supernatants were examined for the presence of FMDV by a plaque assay on BHK-21 cells. The supernatants were serially diluted in MEM and incubated for 24 h with homologous cells. All dilutions were assayed in duplicate. The supernatants were removed, cells were washed with MEM and infected with 50 to 100 PFU of A12-IC, VSV-NJ, or BEV-1, and a plaque assay was performed.

To examine if the ability of FMDV to spread and form plaques was suppressed by antiviral molecules in the supernatants, PK cells were infected with approximately 100 PFU of A12-IC; at 1, 2, 3, and 4 hpi, the supernatants were replaced either with a 1:10 dilution of treated supernatants from A12-LLV2- or mock-infected PK cells or medium with homologous cells. All dilutions were assayed in duplicate. The supernatants were removed, cells were washed with MEM and infected with 50 to 100 PFU of A12-IC, VSV-NJ, or BEV-1, and a plaque assay was performed.

Infective-center assay. EBK, LK, and PK cells were infected with A12-LLV2 or A12-IC at an MOI of 10. Cells were stained at 48 h postinoculation.

RESULTS

Replication of A12-IC and A12-LLV2 in secondary cells. Secondary EBK, LK, and PK cells as well as BHK-21 cells are highly susceptible to A12-IC (Fig. 1) (12, 22). In contrast, no plaques were observed after infection of EBK, LK, and PK cells with A12-LLV2 (Fig. 1 and 2). However, in BHK-21 cells, A12-LLV2 grew to high titers and formed plaques, although A12-LLV2 infection resulted in slightly lower yields than A12-IC infection (Fig. 1 and 2) (22). To determine if A12-LLV2 could replicate in EBK, LK, and PK cells, single-step growth experiments were performed, and samples were titrated on BHK-21 cells. A12-LLV2 grew much more slowly than A12-IC, and the yield was significantly lower in these cells than after A12-IC infection (Fig. 3).

Cytopathic effects (CPE) were observed in A12-IC-infected EBK cells beginning at 3 hpi, and the cell sheet was completely destroyed by 8 and 24 hpi (Fig. 4). In contrast, CPE were first observed in A12-LLV2-infected EBK cells at 4 hpi, but by...
24 hpi, only approximately 15 to 20% of the cell sheet was destroyed. The remainder of the sheet was intact, although it appeared altered compared to that of mock-infected cells; i.e., the cells had a darker appearance and the monolayer had lost its characteristic swirling pattern (Fig. 4). Similar results were obtained with LK and PK cells. A12-IC infection of BHK-21 cells was similar to that of other cell types, while A12-LLV2 infection resulted in delayed CPE (apparent at 6 hpi). However, in contrast to the results for A12-LLV2-infected secondary cells, by 24 hpi CPE were observed in 90 to 95% of the cell sheet (data not shown).

To demonstrate that the difference in CPE between A12-IC- and A12-LLV2-infected EBK and LK cells was not the result of different binding efficiencies of these viruses, an infective-center assay was performed. EBK and LK cells were infected with A12-IC and A12-LLV2 at an MOI of 10 (based on titration in BHK-21 cells) for 1 h and acid treated to remove unadsorbed virus, 10-fold dilutions of cells were inoculated onto a BHK-21 cell monolayer for 1 h, and a plaque assay was performed to quantitate the number of cells harboring virus. We found that both viruses actually infected only 30% of EBK cells and that A12-IC and A12-LLV2 infected 50 and 70% of LK cells, respectively, demonstrating that these viruses have similar binding efficiencies.

**Protein synthesis in infected cells.** We previously showed that in A12-LLV2-infected BHK-21 cells, host cell protein synthesis shutoff and viral protein synthesis were delayed compared to those in A12-IC-infected cells (22). In A12-IC-infected EBK cells, viral proteins were first observed between 2 and 3 hpi, reached a maximum between 3 and 4 hpi, and declined by 4 to 5 hpi, concomitant with the destruction of the cell sheet (Fig. 5). The shutoff of host cell protein synthesis in A12-IC-infected cells correlated with the increase in viral protein synthesis (Fig. 5, compare lane 1 with lanes 2 through 6). In contrast, only a very low level of viral protein synthesis was observed in A12-LLV2-infected cells, and there was a delay in the shutoff of host cell protein synthesis (Fig. 5, compare lanes 3 and 8) prior to the global inhibition of translation, as we previously showed for BHK-21 cells (22) and as has been documented for other picornavirus-infected cells (3, 8, 21). Identical results were obtained with infected LK and PK cells (data not shown).

**Host cell antiviral response.** To investigate the factors involved in the inability of A12-LLV2 to spread in secondary cells, we examined supernatants from infected EBK, LK, PK, and BHK-21 cells for antiviral activity. Cells were infected with A12-LLV2 at various MOIs and for various lengths of time. Supernatants were obtained, treated at pH 2 for 24 h, and neutralized. No virus was detected in treated supernatants by a plaque assay on BHK-21 cells. Fresh cells were incubated for 24 h with treated supernatants from homologous cells and infected with a known amount of A12-IC, and a plaque assay was performed. The supernatants harvested from A12-LLV2-infected EBK, LK, and PK cells inhibited A12-IC plaque formation, while the supernatants harvested from A12-LLV2-infected BHK-21 cells and all mock-infected cells had no antiviral activity (Table 1). The ability of A12-LLV2 to spread from the initial site of infection to form plaques in BHK-21 cells correlates with the absence of a host cell antiviral response in these cells, as measured in our assay. Supernatants from A12-IC-infected cells contained either no antiviral activity (PK cells) or considerably less antiviral activity than those.
from A12-LLV2-infected cells (EBK and LK cells) (Table 1). The maximum inhibitory effect was obtained with supernatants from cells infected with A12-LLV2 at an MOI of 1 or 10 after 24 h of infection. Supernatants from cells infected at a lower MOI, i.e., 0.01 or 0.1, or from cells infected for shorter or longer periods of time, i.e., 4, 7, or 48 h, yielded reduced or no antiviral activity. Supernatants from A12-LLV2-infected EBK cells also inhibited plaque formation of VSV-NJ and BEV-1, and supernatants from A12-LLV2-infected PK cells inhibited plaque formation of VSV-NJ (Table 1).

The ability of A12-IC to spread and form plaques in PK cells was clearly suppressed by supernatants from A12-LLV2-infected PK cells. A12-IC formed small or poorly defined plaques when incubated with these supernatants, and the suppression was apparent even after the A12-IC infection had progressed for 4 h prior to treatment. Supernatants from mock-infected PK cells or medium alone did not have any effect. The suppression of A12-IC infection in the presence of A12-LLV2-infected PK cell supernatants also resulted in a significant reduction (approximately 600- to 2,000-fold) in virus yield (Fig. 6).

**The antiviral response is IFN-α/β specific.** The production of IFN-α/β is one of the initial host responses to viral infections (32). To determine if IFN-α/β mRNA was induced in infected EBK cells, RT-PCR was performed. As shown in Fig. 7, IFN-α and IFN-β mRNAs were induced in both A12-IC- and A12-LLV2-infected EBK cells but not in mock-infected cells. Similar results were obtained with infected LK, PK, and BHK-21 cells (data not shown).

In contrast to A12-IC infection, we have demonstrated that the continuation of host cell protein synthesis in A12-LLV2-infected EBK, LK, and PK cells allows the host to express an antiviral response that could be involved in the inhibition of the spread of virus to neighboring uninfected cells. The stability of antiviral activity present in supernatants after pH 2 treatment and its ability to inhibit the spread of other viruses, including VSV-NJ and bovine enterovirus, are characteristic of IFN-α/β. In addition, we found that antibodies against porcine or human IFN-α partially inhibited the antiviral activity present in supernatants of A12-LLV2-infected PK or EBK cells, respectively (data not shown). To further demonstrate that this antiviral activity is IFN-α/β specific, we used fibroblast cell lines derived from wild-type mice (+/+ 129) or IFN-α/β receptor-negative mice (−/− 129) (16, 20). In this system, +/+ 129 but not −/− 129 cells respond via a signal transduction pathway to IFN-α/β and become resistant to viral infection.
However, antiviral activity that is not IFN-α/β specific affects both cell lines equally.

In preliminary experiments, we found that 129 cells were refractory to FMDV type A12. To overcome this problem, we assayed treated supernatants from infected EBK cells on 129 cells, since IFN-α/β activity has been demonstrated in heterologous systems (11). We selected VSV-NJ as the test virus for antiviral activity, since we found that VSV-NJ can replicate in EBK cells, since IFN-β/2 cells can induce the production of IFN (30). It was suggested that the lack of virulence in cattle was correlated with increased IFN production (28). However, no studies of these viruses at the genome level have been performed to identify the mutation(s) responsible for attenuation and increased IFN induction.

To understand the molecular basis of the events involved in the inhibition of the spread of A12-LLV2, we screened in vitro cell culture systems for the inability of A12-LLV2 to form plaques on cells which are highly susceptible to A12-IC and PK. Although IFN-α/β mRNA in EBK, LK, PK, and BHK-21 cells upon infection with either virus in a qualitative RT-PCR assay demonstrates that these viruses are IFN inducers.

Although IFN-α/β mRNA was induced in infected BHK-21 cells, the absence of antiviral activity in the supernatants, as we (Table 1) and others (30) have reported, suggests a defect in some aspect of the IFN signal transduction pathway and/or IFN-regulated cellular proteins in these cells. We have shown that A12-LLV2 can replicate to high titers and form plaques in BHK-21 cells. The high yield and the ability of A12-LLV2 to spread from its initial site of infection to form plaques in not spread systemically to highly susceptible sites in the oral and pedal regions (4). Since efficacy studies of A12-LLV2 in cattle and swine demonstrated only low neutralizing antibody titers by 3 days postinoculation (unpublished observations), the inability of this virus to spread after aerosol exposure must be the result of mechanisms other than the presence of antibody. Other workers have shown that FMDV strains modified by passage in alternate hosts or repeated passage in cell cultures are avirulent in cattle and, in contrast to animal-virulent virus, can induce the production of IFN (30). It was suggested that the lack of virulence in cattle was correlated with increased IFN production (28). However, no studies of these viruses at the genome level have been performed to identify the mutation(s) responsible for attenuation and increased IFN induction.

Table 1. Antiviral activity

<table>
<thead>
<tr>
<th>Cell supernatant source</th>
<th>Inducing virus</th>
<th>MOI</th>
<th>Antiviral units</th>
</tr>
</thead>
<tbody>
<tr>
<td>EBK</td>
<td>None (mock infection)</td>
<td>1 2</td>
<td>&lt;2*</td>
</tr>
<tr>
<td>A12-LLV2</td>
<td>10</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>A12-IC</td>
<td>10</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>A12-LLV2</td>
<td>10</td>
<td>&gt;64</td>
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</tr>
<tr>
<td>A12-IC</td>
<td>10</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>LK</td>
<td>None (mock infection)</td>
<td>1 2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>A12-LLV2</td>
<td>10</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>A12-IC</td>
<td>10</td>
<td>4</td>
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<tr>
<td>A12-LLV2</td>
<td>10</td>
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<td></td>
</tr>
<tr>
<td>A12-IC</td>
<td>10</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>PK</td>
<td>None (mock infection)</td>
<td>1 2</td>
<td>&lt;2</td>
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<tr>
<td>A12-LLV2</td>
<td>10</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>A12-IC</td>
<td>10</td>
<td>&lt;2</td>
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<td>A12-LLV2</td>
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<td>32</td>
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</tr>
<tr>
<td>A12-IC</td>
<td>10</td>
<td>&lt;2</td>
<td></td>
</tr>
<tr>
<td>BHK</td>
<td>None (mock infection)</td>
<td>1 2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>A12-LLV2</td>
<td>10</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>A12-IC</td>
<td>10</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>A12-LLV2</td>
<td>10</td>
<td>&lt;2</td>
<td></td>
</tr>
<tr>
<td>A12-IC</td>
<td>10</td>
<td>—</td>
<td></td>
</tr>
</tbody>
</table>

a Highest dilution of supernatant needed to reduce the number of A12-IC plaques by 50%, unless otherwise indicated. ND, not done; —, cells died within 6 to 8 h, and supernatants were not harvested.

b This value also applies to VSV-NJ and BEV-1 plaques.

c The values for VSV-NJ and BEV-1 plaques were 128 and 8, respectively.

d This value also applies to VSV-NJ plaques.

e The value for VSV-NJ plaques was 8.
BHK-21 cells correlate well with the absence of antiviral activity in these cells.

We demonstrated an antiviral response in secondary EBK, LK, and PK cells upon A12-LLV2 infection; this response was significantly greater than that induced by A12-IC, reflecting the rapid inhibition of host cell protein synthesis in A12-IC-infected cells. No antiviral activity was found in A12-IC-infected PK cell supernatants; however, when exposed to antiviral molecules present in A12-LLV2-infected PK cell supernatants, the A12-IC phenotype could be altered toward that of A12-LLV2, i.e., reduced spread and yield. The antiviral activity present in the supernatants of A12-LLV2-infected EBK cells is IFN-α/β.

FIG. 6. Reduction of virus yield in the presence of antiviral activity. PK cells were infected with approximately 100 PFU of A12-IC; at 1, 2, 3, and 4 hpi, the supernatants were replaced with a 1:10 dilution of treated supernatants from A12-LLV2-infected PK cells (■), mock-infected PK cells (□), or medium alone (○) for a total of 48 h. The growth of A12-IC was determined in a subsequent plaque titration assay on BHK-21 cells.

FIG. 7. RT-PCR for IFN-α and IFN-β mRNAs. EBK cells were infected with A12-IC or A12-LLV2 or mock infected for 6 h and used in RT-PCR as described in Materials and Methods. Aliquots from RT reactions were used in three separate PCR assays with IFN-α, IFN-β, and β-actin primers. RT-PCR products from EBK cells infected with A12-IC (IC) or A12-LLV2 (LLV) or mock infected (M) in the presence or absence of reverse transcriptase (RT) are shown. IFN-α (A), IFN-β (B), and β-actin (B) RT-PCR products are 379, 452, and 890 bp, respectively. Lanes MW are 1-kb ladder DNA molecular weight markers.
specific, since it is biologically active after pH 2 treatment, inhibits the replication of wild-type FMDV as well as other viruses and, most importantly, is demonstrated only on cells with IFN-α/β receptors, as assayed in the 129 cell system.

Deletion of the coding region for the L proteinase of FMDV has resulted in the inability of A12-LLV2 to spread from the initial site of infection in the secondary cells examined. At least two factors have contributed to this phenomenon, including the slow replication of A12-LLV2 and the induced expression of IFN-α/β in A12-LLV2-infected cells. The inability of this virus to inhibit host cell protein synthesis at early times after infection results in competition between viral and host mRNAs for the protein synthesis machinery and, as a consequence, a slower rate of virus replication compared to that seen with A12-IC infection. However, competition alone is not sufficient to prevent the spread of A12-LLV2 from its initial site of infection to form plaques in a cell line (BHK-21) that lacks a detectable antiviral response. We have also demonstrated that A12-IC can become attenuated in PK cells in the presence of antiviral activity, further supporting the observation that differences in the rate of replication between A12-IC and A12-LLV2 alone cannot account for the differences in their infectivity. Thus, the expression of IFN-α/β, resulting in the development of an antiviral state in neighboring cells, is necessary for a higher degree of attenuation of A12-LLV2. A low level of antiviral activity is also detected in A12-IC-infected EBK and LK cells (Table 1). However, the small quantity of antiviral activity produced in wild-type virus-infected cells in the presence of levels of virus significantly higher than those produced in A12-LLV2-infected cells (Fig. 3) is apparently insufficient to provide resistance to infection.

The rapid onset of FMDV infection is generally attributed to the ability of the virus to shut off host cell cap-dependent protein synthesis, thereby diverting the host cell machinery to virus production. In this study, we have demonstrated that, as a consequence of host cell protein synthesis shutoff, the virus also prevents the host from expressing IFN-α/β. The combination of these two events results in rapid virus growth and spread. Deletion of the L proteinase eliminates the ability of the virus to inhibit the host cell response and consequently attenuates the virus. However, the possibility that host factors besides IFN-α/β may also be involved in virus attenuation cannot be ruled out. Studies with both our in vitro cell culture system and susceptible animals should lead to a better understanding of the host cell response to FMDV infection and may allow the development of improved disease control strategies.

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TABLE 2. Heterologous antiviral activity in the 129 cell system

<table>
<thead>
<tr>
<th>Inducing virus</th>
<th>Units of antiviral activity* on the following 129 cells:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+/+</td>
</tr>
<tr>
<td>None (mock infection)</td>
<td>&lt;2</td>
</tr>
<tr>
<td>A12-LLV2</td>
<td>32</td>
</tr>
<tr>
<td>A12-IC</td>
<td>&lt;2</td>
</tr>
</tbody>
</table>

* The supernatant source was EBK cells.

** Highest dilution of supernatant needed to reduce the number of VSV-NJ plaques by 50%.

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are virulent in mice. J. Virol. 67:5139–5145.