Antiviral Activities Directed Against Wild-type and Interferon-sensitive Mengovirus

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SUMMARY

Two distinct antiviral activities can be detected in L cells treated with low levels of interferon and infected with a one-step interferon-sensitive mutant of mengovirus (is-1). The first antiviral activity (AVA-1) primarily delayed virus RNA and protein synthesis and thereby lengthened the virus replication cycle. It did not prevent cell death. The second antiviral activity (AVA-2) allowed the virus-induced inhibition of host macromolecular synthesis but inhibited all other virus functions. By 9 to 12 h post-infection host synthesis resumed and most cells survived. The data suggest that some step in the virus replication cycle activates AVA-2 leading to the destruction of the virus genome 6 to 12 h after infection. In unprotected cells the yields of parental virus (is+) and is-1 were similar. No qualitative or quantitative differences in virus products were observed by several techniques. The is-1 virus seems to have lost a wild-type function which normally blocks the action of AVA-2.

INTRODUCTION

Treatment with low levels of interferon (IFN) has little effect on cellular metabolism. This may be due to the IFN-induced pppA(2'p5'A)n synthetase and protein kinase activities which are latent until activated by double-stranded RNA (for review, see Baglioni & Nilsen, 1981). The pppA(2'p5'A)n synthetase activates an endonuclease which can degrade cytoplasmic RNA (Williams et al., 1978), and the protein kinase inhibits eukaryotic initiation factor eIF-2, leading to an inhibition of initiation of protein synthesis (Chernajovsky et al., 1979; Samuel, 1979). The presence of these activities correlates well with the development of the antiviral state in several cell systems (Baglioni et al., 1979; Ball, 1979; Kimchi et al., 1979).

Although much is known about the pppA(2'p5'A)n synthetase and protein kinase activities from in vitro studies, their role in vivo is unclear. Recently, Nilsen et al. (1980) showed that IFN-treated mouse embryonal carcinoma cells were resistant to infection with encephalomyocarditis (EMC), but not vesicular stomatitis (VSV) or influenza viruses. Since IFN apparently does not induce the protein kinase activity in these cells, it would seem that the pppA(2'p5'A)n synthetase system is sufficient for the inhibition of some viruses (e.g. EMC), while the protein kinase is necessary for others. The situation, however, is more complicated. Hovanessian et al. (1981) demonstrated that mouse NIH/3T3 cells do not produce the protein kinase, yet become resistant to both mengovirus (serologically identical to EMC) and VSV in response to IFN. They also found that mouse K/Balb cells not treated with IFN contain very high levels of pppA(2'p5'A)n synthetase activity, but were sensitive to infection with both viruses. Finally, Meurs et al. (1981) found that human MRC5 cells treated with either α or β IFN became resistant to EMC and VSV, but did not show increased levels of either the pppA(2'p5'A)n synthetase or protein kinase activities. This suggests that additional antiviral activities are involved in IFN action. There are many gene loci for human α and β IFNs (Allen & Fantes, 1980; Nagata et al., 1980; Taniguchi et al., 1980), and these may play different roles in inducing antiviral and anti-tumour activities and in modulating immune functions. If viruses have

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differing sensitivities to a number of complementing activities, variation in the response of viruses in different cell lines could be explained.

Simon et al. (1976) isolated a one-step IFN response mutant of mengovirus (is-1). The is-1 virus was 10- to 30-fold more sensitive to IFN than the parental virus (is+) when measured by a yield reduction assay. Subsequently, we found that some clones of L cells gave a 30- to 100-fold difference in yield in protected cells, whereas others showed no difference at all. The basis for this variation has not yet been determined. In this paper we report the results of studies examining the site of the mutation in is-1 and how, and at what stage, IFN blocks is-1 replication. These studies have allowed us to partially characterize a previously unknown activity which operates in L cells in vivo.

METHODS

Cells and interferon. A subclone of L cells (designated G3) was prepared from an original stock obtained from Dow Chemical and Pharmaceutical Co. (Zionsville, Ind., U.S.A.). These cells were cultured in Eagle's essential growth medium supplemented with 10% newborn calf serum (KC Biological) or 5% foetal calf serum (Gibco).

IFN was prepared and titrated as described by Fleischmann & Simon (1973), but expressed in terms of mouse reference (G-002-904-51 l) units. One unit of crude IFN (sp. act. 1400 reference units/mg protein) by our assay was equivalent to 2 reference units. Titres obtained when using is+ or is-1 as the challenge virus were essentially the same. Partially purified mouse IFN (obtained from Dr P. Lengyel, Yale University; sp. act. 6.3 × 10^6 u/mg protein) was indistinguishable in its effects on is-1 and is+ from the crude preparations routinely used.

Viruses. Mengovirus growth and plaque assay procedures have been described by Fleischmann & Simon (1973). Unless otherwise indicated, all infections were carried out at an m.o.i. of 3 to 5. The origin of is-1, an IFN-sensitive mutant of mengovirus, was described by Simon et al. (1976). In the absence of IFN, virus titres for is+ and is-1 averaged 1.5 × 10^8 and 1 × 10^8 p.f.u./ml respectively. Virus stocks were stored at −60 °C. Vaccinia virus stocks (obtained from J. A. Holowczak) were prepared similarly and stored at −20 °C.

Irradiation of virus. Undiluted mengovirus stocks in growth medium were irradiated with a 15 W GE germicidal lamp at 3.9 J/m²/s.

Infectious centre assay. Unprotected or protected monolayers were infected with mengovirus or mengovirus RNA (see below). The cells were removed at various times from the plates by trypsinization, suitably diluted in growth medium and added to uninfected monolayers in 35 mm plates. After 45 min incubation at 37 °C, 5 drops of starch were added to each monolayer, allowed to harden, and then 2 additional ml added. Non-eclipsed virus was detected by plating the virus released following three freeze–thaw cycles, and infectious centre data were corrected accordingly.

Inhibitors of cellular RNA and protein synthesis. Inhibitors were added directly to growth medium from stock solutions stored at 4 °C. The stock solutions were: 200 μg/ml actinomycin D (act. D; Merck, Sharp & Dohme) in phosphate-buffered saline (PBS) or growth medium, and cycloheximide (Upjohn), fluorophenylalanine (Sigma) and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; Calbiochem), all at 1 mg/ml in PBS. DRB was dissolved by adding 1 M-HCl dropwise. Growth medium without phenylalanine and supplemented with dialysed calf serum was used with fluorophenylalanine.

RNA analysis. Intracellular RNA was examined by pulse-labelling for 30 min with 15 μCi/ml [3H]uridine. At the end of the labelling period the plates were placed on ice and washed with cold PBS. The washed cells were resuspended in 0.05 M-Tris pH 9, 0.1 M-NaCl, 0.005 M-EDTA, 0.5% SDS, phenol-extracted with water-saturated, redistilled phenol. The aqueous layer/protein interface was then re-extracted with phenol–chloroform (1:1). DNA was removed after the addition of one-fifth vol. 3 M-NaCl and 1 vol. ethanol by spooling on a Pasteur pipette. RNA was precipitated at −20 °C overnight by the addition of a second volume of ethanol and analysed on linear-log sucrose gradients as described by Fout & Simon (1981).

The same technique was used to extract RNA from mengovirus. The RNA was precipitated from the sucrose gradient fractions and then resuspended in 0.12 M-potassium acetate, and stored at −60 °C. Infectious RNA assays were performed by plating 0.2 ml of viral RNA diluted in PBS or Earle's salts containing 300 μg/ml DEAE-dextran (Pharmacia; 2 × 10⁶ mol. wt.) onto 35 mm Petri dishes as described by McCutchan & Pagano (1968). The Petri dishes were washed with PBS before plating and also 5 to 10 min after plating. Additionally, 2 ml of starch were added to each plate after the last wash. The infections were carried out at room temperature in a semi-darkened hood. Specific infectivities were in the range of 10⁶ p.f.u./μg RNA.

Protein analysis. Intracellular proteins from uninfected cells were radiolabelled with [3H]leucine or [15]methionine at various concentrations. The labelling medium was Eagle's essential medium without the appropriate unlabelled amino acid and supplemented with 10% dialysed calf serum. Cellular proteins were solubilized directly in electrophoresis sample buffer (0.025 M-Tris pH 8.3, 0.0022 M-glycine, 1% SDS, 5% 2-mercaptoethanol), or were fractionated as described by Fout & Simon (1981). Analysis was by 10 to 20% polyacrylamide gradient gel electrophoresis (Maizel, 1971).
### RESULTS

#### The is phenotype

The maximum extracellular yields of is+ (wild-type mengovirus) and is-1 occurred by 12 h post-infection in control cells. Pretreatment of the cells with low levels of IFN delayed the replication of is+, with the titre increasing until cell lysis occurred about 24 h after infection. In contrast IFN greatly reduced the yield of is-1 but the maximum yield was usually attained by 12 h post-infection. Typically, in the presence of IFN, is+ gave 30- to 100-fold higher yields than the mutant. These points were previously discussed in detail by Simon et al. (1976) (see also Table 1).

The absence of visible lysis in protected is-1-infected cultures, and the kinetics of virus growth suggested that only 1% or so of the cells might be productively infected. An infectious centre assay was used to test this idea. Nearly every unprotected cell infected with mengovirus produced an infectious centre. In contrast, only 51% of cells protected with 20 units/ml IFN for 19 h and infected with is+, and 1.2% of those infected with is-1 induced plaques. These data suggest that IFN-treated cultures consist of two classes of cell: protected cells which produce no is-1 at all, and cells which were not protected by IFN. This idea was supported by microdrop experiments in which the yield of virus from individual protected and unprotected cells was calculated. Those few is-1-infected cells that yielded virus did so at nearly normal levels (Fout, 1980).

Simon et al. (1976) suggested that is-1 responds to a different IFN-mediated host function than is+. First, they demonstrated that act. D added at the time of virus infection phenotypically changes is-1 to is+, but has no effect on the growth of is+. To confirm that act. D was acting at the level of RNA transcription, we replaced act. D with DRB. Table 1 shows that DRB, like act. D, reversed the is-1 phenotype without affecting is+.

Secondly, Simon et al. (1976) showed that the is phenotype is recessive in mixed infection with is+. Since vaccinia virus can rescue VSV from IFN-mediated inhibition (Thacore & Youngner, 1973), we tested the possibility that vaccinia virus can rescue is-1 by co-infecting L cells with two viruses. To avoid complications caused by multiple infection, an m.o.i. of 0.1 of each virus was used. Cells producing is-1 were identified by an infectious centre assay on test monolayers that were pretreated with act. D prior to infection. This treatment eliminated vaccinia plaques (Table 2, plates 1 and 2) but did not affect the plating efficiency of is+ (Table 2, plates 3 and 4).

(Act. D did not reverse the is-1 phenotype because only the test monolayers were treated with the drug.) When the cells were pretreated with IFN, only 1-9/1000 is-1-infected cells gave rise to infectious centres (Table 2, plate 5). In mixed infection (Table 2, plate 6) this number increased to 7-5 cells/1000 is-1-infected cells. However, only 10% of these cells were infected with both viruses. This means that 56/1000 cells infected with both mengovirus and vaccinia produced mengovirus. Hence, co-infection increased the total number of is-1-producing cells by 30-fold.
Table 2. Ability of vaccinia virus to rescue is-1*

<table>
<thead>
<tr>
<th>Plate no.</th>
<th>is-1</th>
<th>IFN</th>
<th>Act.D†</th>
<th>Vaccinia efficiency‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* L cells were pretreated with or without 20 units/ml IFN for 36 h and then infected with the indicated virus at an m.o.i. of 0·1. At 3 h post-infection the cells were removed from the plates by trypsinization and plated for infectious centres as described in Methods.
† Assay plates were pretreated with 1 µg/ml act. D for 3 h and then washed prior to adding the infectious centres. This treatment prevented the development of vaccinia virus plaques.
‡ Plating efficiency = the number of plaques produced/the number of infected cells plated.

Structural differences between is + and is-1

Strains is + and is-1 appear to be identical as regards density, sedimentation coefficient and the molecular weight of the four structural proteins (Fout, 1980). The viruses are also antigenically indistinguishable and their RNAs have identical sedimentation coefficients. Hence, the is-1 lesion does not involve a large deletion (greater than 5%), and it is unlikely that it affects a structural protein. In addition, more than 90% of the viral RNA of both viruses bind to oligo(dT)-cellulose (Fout & Simon, 1981), indicating that both possess poly(A) 3' ends. Attempts to examine the 5' terminal protein, VPg, were unsuccessful due to insufficient material.

The site of action of the anti-is-1 activity

We assume that our line of L cells has two classes of antiviral activity: one which acts on both is + and is-1 (AVA-1) and one which acts only on is-1 (AVA-2). AVA-2 could block at any of the following levels: (i) adsorption, penetration and uncoating; (ii) early events involved in taking command of the cell machinery; (iii) viral protein synthesis; (iv) viral RNA synthesis; (v) virus maturation and release.

Adsorption, penetration and uncoating

If AVA-2 blocked is-1 at these steps, the virus yields following treatment of protected cells with infectious RNA from either virus would be identical. When protected and control monolayers were infected with 2 µg is + or is-1 RNA, the expected 100-fold difference in yield between the two viruses was seen (data not shown). Table 3 shows a more detailed experiment in which plaque formation by is-1 and is + are compared under three conditions. The first is a normal plaque assay using intact virus. In the second, cell monolayers were infected with infectious RNA. In this case only the initial round of infection was caused by RNA; subsequent rounds were the result of infection of protected cells with whole virions. In the third condition, cells were infected with infectious RNA, and then trypsinized and plated on unprotected monolayers. Hence, only the RNA molecules were subjected to the antiviral state. These results confirm that infectious is-1 RNA is sensitive to AVA-2 action and, thus, that AVA-2 acts after uncoating.

Early events

Host RNA and protein syntheses are strongly inhibited by 3 h post-infection. Both viruses inhibited protein synthesis by 75 to 85% at 4 to 5 h post-infection in protected cells, while u.v.-inactivated viruses did not. Since the virus was irradiated to give only a 3 log reduction in titre (7 to 8 lethal hits per genome), functional viral RNA (rather than coat protein) was probably responsible for the inhibition.
Basis for IFN-sensitive phenotype

Fig. 1. RNA synthesis in IFN-treated cells. L cells were incubated with (open symbols) or without (closed symbols) 20 units/ml IFN for 36 h at 37 °C. (a) Mock-infected cells; (b to d) cells infected with is+ (○, ◀) or is-1 (▲, ▲). Appropriate plates were labelled with 15 μCi/ml [3H]uridine for 30 min ending at 5 h (c), 7.5 h (b) and 12 h (d) post-infection. After the labelling period the RNA was extracted and analysed on linear-log gradients as described in Methods. The positions of 4S tRNA, 18S rRNA and 28S rRNA shown in (a) were based on absorbance at 254 nm. The region greater than 28S consists of ribosomal precursors.

Table 3. Sensitivity of mengovirus infectious RNA to IFN*

<table>
<thead>
<tr>
<th>Virus</th>
<th>IFN (units/ml)</th>
<th>Whole virus†</th>
<th>Plaques/μg§</th>
<th>Infectious centres|</th>
</tr>
</thead>
<tbody>
<tr>
<td>is+</td>
<td>0</td>
<td>1</td>
<td>0.06</td>
<td>0.26</td>
</tr>
<tr>
<td>is+</td>
<td>10</td>
<td>0.03</td>
<td>0.014</td>
<td>0.025</td>
</tr>
<tr>
<td>is+</td>
<td>20</td>
<td>1</td>
<td>1</td>
<td>0.038</td>
</tr>
<tr>
<td>is-1</td>
<td>0</td>
<td>0.024</td>
<td>0.016</td>
<td>0.0032</td>
</tr>
<tr>
<td>is-1</td>
<td>10</td>
<td>0.0008</td>
<td>0.0016</td>
<td>0.0032</td>
</tr>
<tr>
<td>is-1</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Confluent monolayers were pretreated with the indicated dilutions of IFN for 24 h. Cells were then infected with appropriate levels of infectious RNA as described in Methods.
† Relative efficiency = the plaque number in the presence of IFN/the number in its absence.
‡ Virus was assayed on protected monolayers in the standard manner.
§ The specific infectivity on control cells was 5 × 10⁶ plaques/μg for is+ and 1.3 × 10⁶ plaques/μg for is-1.
\| The plating efficiency for infectious centres was 45% for is+ and 80% for is-1. Protected cells were infected with 0.01 infectious RNA molecules/cell. After 30 min adsorption at room temperature, the cells were washed, trypsinized and an appropriate number of cells added to unprotected monolayers.

Viral and cellular RNA syntheses

Total RNA from cells labelled with [3H]uridine was analysed on sucrose gradients to determine the effects of IFN on viral RNA synthesis. Fig. 1 (a) shows the distribution of RNA from uninfected cells. IFN treatment had little or no effect on this RNA profile. Both viruses caused a slight inhibition of transfer RNA synthesis and a strong inhibition of 18S rRNA synthesis by 5 h after infection of control cells (data not shown). Viral RNA was detectable by
5 h and migrated at about 32S. Oligo(dT) chromatography confirmed that the 32S peak from uninfected cells did not contain poly(A) while the is+ and is-1 RNA peaks did (data not shown). At 7.5 h post-infection of control cells (Fig. 1b) almost all newly synthesized RNA was viral. In contrast to unprotected cells, no viral RNA was observed at 5 h after infection of protected cells (Fig. 1c), but the synthesis of high mol. wt. RNA was strongly inhibited. At 7.5 h (not shown) a small amount of is+ RNA was observed. By 12 h post-infection (Fig. 1d) substantial amounts of is+ RNA were being synthesized (compare Fig. 1b, d). However, no is-1 RNA was detected and host rRNA synthesis had resumed as shown by the presence of a peak of 18S rRNA. In agreement with this interpretation, total RNA synthesis in is-1-infected cells was inhibited 59% at 5 h but only 28% at 12 h.

An infectious RNA assay was performed to determine the level of intracellular is-1 RNA more accurately. This assay had a sensitivity of $1.5 \times 10^6$ p.f.u./µg RNA. Fig. 2 shows that the level of infectious RNA extracted from is+-infected cells increased until 26 h post-infection. The level then decreased in correlation with cell lysis. In contrast, infectious is-1 RNA reached a maximum 10 h after infection and then fell for the remainder of the test period. The initial accumulation most likely reflected normal virus synthesis in the small fraction of cells which escaped IFN protection. Between 12 and 26 h post-infection there was a 20- to 60-fold difference in the amounts of is+ and is-1 infectious RNA. Thus, the inhibition of RNA synthesis in is-1-infected cells parallels the reduction in virus yield.

**Viral protein synthesis**

Fig. 3 demonstrates that the proteins made by is+ and is-1 in unprotected cells are qualitatively and quantitatively identical throughout the replication cycle. Viral proteins were demonstrable at 4 h post-infection and peak syntheses occurred 2 h later. By 9 h post-infection (not shown) very little viral or host protein synthesis was observed.

In cells pretreated with IFN, is+ proteins were not in evidence until 6 h post-infection (Fig. 4a, lane 4). Maximum synthesis occurred at 9 to 12 h (lanes 5 and 6), and was completed by 24 h (lane 7). In contrast, no is-1 proteins were observed in protected cells at any time post-infection (Fig. 4b). This is particularly clear in the case of the γ protein, although initially both viruses
Basis for IFN-sensitive phenotype

Fig. 3. Protein synthesis in unprotected cells. L cells were mock-infected (lane 1) or infected with is+ (lanes 2, 4 and 6) or is-1 (lanes 3, 5 and 7). The cells were labelled with 27 μCi/ml [35S]methionine for 1 h ending at: 2 h (lanes 2 and 3), 4 h (lanes 4 and 5) and 6 h (lanes 6 and 7) post-infection. After the labelling period intracellular proteins were analysed on a 10 to 20% polyacrylamide gel. A sample containing the same number of cts/min was added to each lane. The positions of several viral proteins and coat components α, β, γ and ε (Ziola & Scraba, 1976) are indicated.

greatly reduced host protein synthesis (Fig. 4a, b, lanes 3 and 4). By 9 h post-infection synthesis of host proteins had resumed in is-1-infected cells.

Table 4 shows that in unprotected cells at 4 to 6 h post-infection total protein synthesis was inhibited by 40 to 60% (note, however, that both viral and host proteins were being made at these times). The total inhibition in IFN-treated cells at 4 to 6 h was 80 to 90%. Fig. 4(a) shows that the slight decrease in inhibition by is+ at 9 to 12 h post-infection was due to viral protein synthesis. The increase at 24 h was due to cell lysis. In is-1-infected cells, the amount of inhibition decreased to only 25 to 35% by 24 h. This reflects the resumption of host protein synthesis seen in Fig. 4(b).

What is the mechanism of AVA-2 action?

In some systems IFN induces the synthesis of a membrane-associated ribonuclease (Marcus et al., 1975). The possibility that AVA-2 is such a nuclease was tested by blocking mengovirus replication for 12 h with concentrations of cycloheximide or fluorophenylalanine that did not block virus adsorption, penetration or uncoating (Penman & Summers, 1965). Table 5 shows that there was about a 40-fold difference in the yields of is+ and is-1 from cells treated with IFN only. Act. D added at the time of the infection reversed the is phenotype (Simon et al., 1976). In contrast, act. D added at 12 h had absolutely no effect on the phenotype as measured by either yield or plaque reduction assays. Cycloheximide consistently reduced the yields of both viruses
while fluorophenylalanine increased the yields of is-1 by 10- to 20-fold. The mechanisms of these inhibitor effects are unknown. Although the effects of cycloheximide on is-1 yields might suggest the presence of an IFN-induced nuclease activity, cycloheximide typically decreased is+ yields more than is-1. This makes it unlikely that AVA-2 is involved. Furthermore, the effects of fluorophenylalanine do not agree with the nuclease hypothesis. Final proof that an active
Basis for IFN-sensitive phenotype

Table 4. Inhibition of protein synthesis by mengovirus

<table>
<thead>
<tr>
<th>IFN Time (h)</th>
<th>Percentage inhibition*</th>
</tr>
</thead>
<tbody>
<tr>
<td>- 4</td>
<td>Expt. 1† 54</td>
</tr>
<tr>
<td>- 6</td>
<td>Expt. 2‡ 62</td>
</tr>
<tr>
<td>- 9</td>
<td>Expt. 1 91</td>
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<tr>
<td>+ 4</td>
<td>79</td>
</tr>
<tr>
<td>+ 6</td>
<td>90</td>
</tr>
<tr>
<td>+ 9</td>
<td>86</td>
</tr>
<tr>
<td>+ 12</td>
<td>87</td>
</tr>
<tr>
<td>+ 18</td>
<td>87</td>
</tr>
<tr>
<td>+ 24</td>
<td>97</td>
</tr>
</tbody>
</table>

* Percentage inhibition = ct/min of the appropriate mock-infected control - ct/min from the virus-infected plate/the ct/min from control × 100.
† Cells were treated with growth medium alone or growth medium containing 20 units/ml IFN for 36 h at 37 °C. The cells were either mock-infected or infected with mengovirus and labelled with 27 μCi/ml [35S]methionine for 1 h ending at the times indicated. Cells were scraped from the plates and analysed for acid-insoluble radioactivity. The total ct/min for the mock-infected controls were: unprotected 1.8 x 10⁷; IFN-protected 1.4 x 10⁷.
‡ The experimental conditions were the same as above, except that 20 μCi/ml [3H]leucine was used to label proteins. The total ct/min for the mock-infected control was 1.3 x 10⁷.

Table 5. Effect of metabolic inhibitors on virus growth

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>Conc. (μg/ml)</th>
<th>Controls</th>
<th>IFN only</th>
<th>Cycloheximide</th>
<th>Fluorophenylalanine</th>
<th>Act. D added after reversal of</th>
<th>Cycloheximide</th>
<th>Fluorophenylalanine</th>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
| * L cells were treated with 20 units/ml IFN for 36 h. The cells were then infected in the absence (IFN only control) or presence of the indicated inhibitor. Plates with cycloheximide and fluorophenylalanine were washed at 12 h post-infection and growth medium or growth medium containing act. D was added to them. These plates were sampled for virus 24 h later. The IFN only control was sampled at 24 h post-infection.
† These data are expressed relative to the maximum yields on plates without IFN or inhibitors. These yields were 1.8 x 10⁸ and 1.5 x 10⁸ p.f.u./ml for is+ and is-1 respectively.

nuclease is not present at the time of infection is provided in the second part of Table 5 which shows that act. D added after the removal of cycloheximide and fluorophenylalanine completely reversed the is phenotype. Under 'normal' growth conditions, is-1 is destroyed by 12 h as shown by the inability of act. D to rescue the virus at that time.

IFN induction by is-1

Marcus et al. (1981) reported that is-1 can induce IFN in cells pretreated with IFN whereas is+ cannot, and suggested that this may provide an explanation for the is-1 phenotype. To test this idea, virus was grown in control and protected cells and the amount of IFN induced was measured. Furthermore, virus was grown in cells to which large amounts of IFN were added immediately following infection. These experiments showed that while is+ did not induce IFN under any circumstances, is-1 induced from 20 to 60 units of IFN, but only in pretreated cells. (In simultaneous control experiments, cells infected with Newcastle disease virus produced about 1000 units/ml IFN.) Table 6 shows that much larger quantities of IFN added after infection had
Table 6. Production of IFN by is-1 and is+, and the effect of post-infection treatment with IFN*

<table>
<thead>
<tr>
<th>IFN concn</th>
<th>Pretreated</th>
<th>Post-treated</th>
<th>24 h yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>9.5 × 10^7</td>
<td>4.8 × 10^7</td>
</tr>
<tr>
<td>20</td>
<td>0</td>
<td>2 × 10^7</td>
<td>2.8 × 10^5</td>
</tr>
<tr>
<td>20</td>
<td>20</td>
<td>1.5 × 10^7</td>
<td>4.9 × 10^5</td>
</tr>
<tr>
<td>20</td>
<td>200</td>
<td>9 × 10^6</td>
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</tr>
<tr>
<td>0</td>
<td>200</td>
<td>1 × 10^6</td>
<td>7 × 10^5</td>
</tr>
</tbody>
</table>

* Cells were pretreated with IFN for 24 h. IFN was added to post-treated cells immediately following a 45 min adsorption period.

no effect on the yield of either is-1 or is+. Hence, the ability to induce IFN does not appear to play a major role in determining the is-1 phenotype. More extensive studies, involving different cell lines and the use of anti-IFN antibody, lead to the same conclusion (Simon & Isono, 1982; E. H. Simon & N. Isono, unpublished results).

**DISCUSSION**

The IFN response mutant of mengovirus, is-1, has an enhanced sensitivity to IFN (Simon et al., 1976). The final yields of is-1 in untreated cells range from 50 to 100% of its wild-type parent (is+), but are typically 40- to 100-fold less than is+ in cells treated with low levels of IFN. Moreover, is-1 differs from is+ in two other characteristics: firstly, IFN does not affect the time-course of is-1 growth and, secondly, the vast majority of treated cells survive the infection (Simon et al., 1976). This latter characteristic is very striking because IFN treatment does not normally prevent cell death (Haase et al., 1969; Stitz & Schellekens, 1980). Although Vaquero et al. (1981) report that mengovirus-infected L cells can survive infection, they require both pre- and post-treatment with 250 units/ml IFN. These are far more stringent conditions than the 20 units/ml pretreatment required for protection against an is-1 infection. These facts suggest that while is-1 replication was completely blocked in most cells, a small fraction gives a normal yield. This was confirmed by an infectious centre assay which showed that only about 1% of the is-1-infected cells released progeny virus. The phenotypic differences between the two viruses disappeared when is-1-infected cells were treated at the time of the infection with either act. D or DRB (Table 1), or when the cells were co-infected with either is+ (Simon et al., 1976) or vaccinia virus (Table 2).

The above data (summarized in Table 7) suggest that there are at least two distinct types of antiviral activities operating in IFN-treated L cells. The first antiviral activity (AVA-1), which may be the net effect of several antiviral activities such as pppA(2'p5'A)n synthetase and protein kinase (Baglioni & Nilsen, 1981) acts to delay mengovirus yield, while the second antiviral activity (AVA-2) specifically blocks the replication of is-1. Recently, Marcus et al. (1981) have observed that is-1, but not is+, will induce IFN and suggest that this ability is the basis for the is phenotype. According to these authors, addition of act. D and co-infection with is+ rescue is-1 by preventing IFN induction. E. H. Simon & N. Isono (unpublished results) have confirmed that this occurs in some cell lines, but find no consistent correlation between expression of the phenotype and production of IFN. Furthermore, Table 6 shows that addition of IFN following infection has no effect on the yield of either virus. This demonstrates that IFN induction cannot explain the is-1 phenotype.

Act. D and DRB inhibit RNA synthesis in quite different ways (Tamm & Sehgal, 1979) but both eliminate AVA-2 activity. This implies that both the mRNA(s) and protein(s) for this activity have short half-lives. No direct measurement of these half-lives has been made, but a rough estimate of the overall half-life (which is equal to that of the longest-lived component) is possible. When act. D is added 1 h after infection, it must totally eliminate AVA-2 activity because the final is+ and is-1 yields are identical. On the other hand, Fig. 4 shows that is+ proteins were being made in protected cells by 6 h post-infection, whereas is-1 proteins were
Basis for IFN-sensitive phenotype

Table 7. Summary of is+ and is-1 phenotypes from IFN-treated cells

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>is+</th>
<th>is-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Virus yield</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of yielding cells*</td>
<td>50% (Table 1)</td>
<td>1%</td>
</tr>
<tr>
<td>Time of yield*</td>
<td>Delayed</td>
<td>Not delayed</td>
</tr>
<tr>
<td>Yield/yielding cell†</td>
<td>Reduced</td>
<td>Not reduced</td>
</tr>
<tr>
<td>2. Cytopathic effect</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nearly 100% by 24 h</td>
<td>is-1 became phenotypically</td>
<td></td>
</tr>
<tr>
<td></td>
<td>identical to is+</td>
<td></td>
</tr>
<tr>
<td>3. Effect of inhibitors of RNA synthesis (act. D and DRB)*†</td>
<td>No effect (Table 2)</td>
<td>is-1 producing cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>increased 30-fold</td>
</tr>
<tr>
<td>4. Mixed infection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>is*/is-1†</td>
<td>50% of yield is+</td>
<td>50% of yield is-1</td>
</tr>
<tr>
<td>Vaccinia/mengovirus*</td>
<td>No effect on growth</td>
<td>is-1 producing cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>increased 30-fold</td>
</tr>
<tr>
<td>5. IFN induction*§</td>
<td>None</td>
<td>Variable amounts depending on system</td>
</tr>
</tbody>
</table>

* This paper.
† Simon et al. (1976).
‡ Fout (1980).
§ Marcus et al. (1981).

not. This means that AVA-2 had probably inactivated most of the is-1 genomes by this time (discussed in more detail below). Since act. D and DRB prevent this inactivation, we suggest that three half-lives of decay have occurred between 1 and 6 h post-infection implying that the half-life of AVA-2 is about 1.5 h. Although most proteins from eukaryotic cells have much longer half-lives, those of the rat liver enzymes ornithine decarboxylase, 5-aminolevulinate synthetase, tyrosine aminotransferase, tryptophan oxygenase, deoxythymidine kinase and serine dehydratase range from 11 min to 4 h (Rechcigl, 1971). Therefore, the inferred half-life for AVA-2 is well within a normal range for short-lived proteins.

AVA-1 and AVA-2 act after adsorption, penetration and uncoating (Table 3), and after the shut-off of host protein and RNA syntheses (Fig. 1 and 4). Poliovirus probably mediates shut-off directly by inactivating initiation factors needed to translate host, but not viral, mRNA (Helentjaris et al., 1979; Jen et al., 1980; Trachsel et al., 1980). EMC virus (which is closely related to mengovirus) seems to inhibit host translation by a different mechanism (Jen et al., 1980; Ramabhadran & Thach, 1981). Alonso & Carrasco (1981) suggested that it acts indirectly through intracellular ionic changes and competition between viral and cellular mRNA. All agree, however, that viral RNA synthesis is required for the shut-off to occur. Since the development of the inhibition was as fast in protected cells as in control cells (Table 4, Fig. 3 and 4), it appears that early viral protein and mRNA syntheses occurred at similar rates in the presence and absence of IFN. However, AVA-1 delayed the production of detectable levels of virus protein for about 2 h (compare Fig. 1, 3 and 4), with maximum production occurring about 4 to 6 h after that in untreated cells. We suggest that the eventual loss of antiviral activity may be due to the ability of wild-type virus to overcome all or some aspects of AVA-1 (Chang et al., 1973).

The effects of IFN seem to be the same for both viruses throughout the first 4 to 5 h of the replication cycle. Thus, as pointed out by Collins & Roberts (1972), very little virus protein synthesis is required to maintain the inhibited state, and this amount should occur in is-1-infected cells if the virus RNA remained intact.

Table 5 shows that when protected cells were treated with cycloheximide or fluorophenylalanine for 12 h following infection, is-1 could still be rescued by the addition of act. D after the protein inhibitors were removed. An IFN-induced nuclease present during treatment with the protein inhibitor would have destroyed the virus genome. Yet, some mechanism normally leads to the functional destruction of the genome in less than 12 h, since act. D added at that time failed to rescue the virus. The protein kinase of the pppA(2'p5'A)n synthetase/endonuclease systems cannot be responsible for this. These activities develop normally in the presence of act. D added several hours after IFN (Kimchi et al., 1979). In contrast, AVA-2 rapidly decays.
Moreover, this functional loss of the genome strongly implies that AVA-2 does not simply block is-1 protein or RNA synthesis. If it did, an additional mechanism(s) would have to be involved in actually eliminating the virus genome and this should also work in the presence of the inhibitors.

The simplest interpretation of the data is that AVA-2 is a nuclease activated at an early stage of virus development. Mengovirus replicative form, which is made in the presence of IFN (for review, see Vaquero et al., 1981), would be a prime candidate for the activating structure. Both is+ and vaccinia virus can rescue is-1. Since Fout & Simon (1981) have shown that is-1 and is+ are equally effective in shutting off host macromolecular synthesis, it appears most likely that these viruses directly inhibit AVA-2. Thacore & Youngner (1973) have shown that vaccinia virus can similarly rescue VSV. In addition, Ito et al. (1978) showed directly that haemagglutinating virus of Japan possesses anti-IFN activities. It is also of interest that several phage produce nuclease inhibitors (Sakaki, 1974; Williams & Radding, 1981).

In summary, we propose that the early events of is-1 and is+ replication in protected cells are identical. Later in the infection, however, AVA-2 is activated and destroys the is-1 genome. The evidence for this is as follows: (i) the majority of the is-1-infected cells survive the infection; (ii) surviving cells can be cloned after eliminating residual virus with anti-mengovirus antibody and IFN, (iii) there is transient inhibition of host protein and RNA syntheses. Thus, AVA-2 is a separate, previously uncharacterized, IFN-mediated activity. The presence of AVA-2 in some lines of L cells and in whole mice (Stebbing, 1979) suggests that it may be effective on other viruses as well. Finally, the resistance of is+ to AVA-2 provides evidence that viruses possess anti-IFN activities.

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Basis for IFN-sensitive phenotype


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