Selection and Characterization of Interferon Response Mutants from Mouse L929 Fibroblast Cells

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SUMMARY

Interferon (IFN) response mutants were selected from mouse L929 fibroblast cells and their specific resistance to is-1, an IFN-sensitive mutant of mengovirus, was studied. The standard L cell subline used in our laboratory (G3), is resistant to is-1 infection after pretreatment with low levels of IFN. Two clonal sublines that support the growth of is-1 in the presence of IFN (AS-4 and TA-6) were isolated from it, and two revertant lines (AS-4R1 and TA-6R1) were subsequently selected from AS-4 and TA-6. The kinetics of is-1 growth in the presence of IFN were found to vary in each of these sublines. Specific resistance to is-1 cannot be accounted for by enhanced induction of IFN, ability to bind IFN, or increased 2'-5'-oligo(A)-dependent endonuclease activity. AS-4 and TA-6 appear to have arisen through loss of one or more whole chromosomes. The origin of TA-6R1 is unclear.

INTRODUCTION

Two distinct antiviral activities (AVA-1 and AVA-2) have been detected in L cells treated with low levels of interferon (IFN) and infected with an IFN-sensitive mutant of mengovirus (is-1). AVA-1 includes those antiviral activities which affect both wild-type mengovirus (is+) and is-1. AVA-2 consists of those activities which are specific for is-1 (Fout & Simon, 1981, 1983; Simon et al., 1984). Only 20% of randomly tested L cell lines exhibit AVA-2. By definition, these lines contain the is-1 expression gene(s) which we term is-1 ex+. A function analogous to AVA-2 has been found in HeLa cells (Sakuragi & Simon, 1986).

Yields of is-1 and is-1+ in unprotected L cells are comparable and generally reach a maximum at 12 h post-infection. Pretreatment of cells with low levels of IFN delays the replication of is+, with titres increasing steadily between 12 and 24 h post-infection. Significantly, all infected cells die. In contrast, when is-1 is grown in protected L cells, the mutant virus produces 100 to 1000 times fewer infective particles than is+, and most cells survive the infection (Fout & Simon, 1981; Simon et al., 1984). In contrast to is+, however, maximum is-1 titres are usually attained by 12 h post-infection. This implies that about 1% of the cells are simply not protected by IFN (Fout & Simon, 1981, 1983). Cell lines which survive infection with is-1 are said to express the is-1 phenotype.

Mouse L cells readily produce IFN. Although amounts produced by individual cells are highly variable, nearly every cell in a monolayer can produce IFN (Brown et al., 1980). Marcus et al. (1981) reported that in the L(Y) line of cells is-1 can induce IFN in cells that have been primed with IFN, whereas is+ cannot. They suggested that this may provide an explanation for the is-1 phenotype. However, experiments performed by Simon et al. (1984) showed that this was not true in general. When equal or much larger amounts of IFN than is-1 is capable of inducing were added to G3 cells post-infection, no effect on yields of either is-1 or is+ was seen.

Randomly selected clones from a late passage of the G3 cell line were tested for sensitivity to is-1. Approximately 10% of the clones initially tested showed some loss of the is-1 phenotype. Two clones were selected for further study. AS-4 showed a moderate loss of the is-1 phenotype.

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and TA-6 a complete loss (i.e. is-1 and is+ were equally susceptible to IFN). Two revertant lines that fully expressed the is-1 phenotype (TA-6R1) or partially expressed the phenotype (AS-4R1) were later isolated from these sublines. This paper describes a partial characterization of these lines.

METHODS

Cell lines. L929 mouse fibroblast cells used in this study were a subclone (designated G3) from an original stock obtained from Dow Chemical and Pharmaceutical Company (Zionsville, Ind., U.S.A.), or were derived from this subclone. These cells were cultured in Eagle's essential growth medium supplemented with 10% newborn calf serum (K. C. Biological).

IFN. Mouse α/β IFN was obtained from W. R. Fleischmann (University of Texas, Galveston, Tx., U.S.A.) or was prepared and titrated as described by Fleischmann & Simon (1973) and Fout (1980). One unit of these IFNs is approximately equivalent to 2 mouse reference (G-002-904-511) IFN units. Working stocks of IFN were stored at 4 °C and concentrated IFN at −100 °C.

Viruses. Wild-type mengovirus (is+), was from a stock originally obtained from Dr Richard Franklin and selected for resistance at 37 °C. The origin of is-1, an IFN-sensitive mutant of mengovirus, has been described by Simon et al. (1976). All virus stocks were stored at −100 °C.

Isolation of is-1 non-expressive and revertant subclones. Subclones of G3 were obtained by distributing an average of two cells per well in a 96-well microtitre plate. The medium was supplemented with 20 µg/ml serum which enhances the survival of cells plated at very low concentrations. About 10 days later, cells in wells containing single clones were transferred to three others. Several days later, two of the wells were treated with 4 units/ml IFN and infected with 10,000 p.f.u. of either is-1 or is+. Clones that failed to express the is-1 phenotype were killed by both viruses in either the presence or absence of IFN. Two clones which failed to express the phenotype fully (AS-4 and TA-6) were expanded for further testing.

Revertant lines which appeared to regain the is-1 phenotype were isolated as follows. 1 × 10⁵ G3, AS-4 or TA-6 cells were placed in 1 cm Nunc multidish wells containing a dilution series of mouse α/β IFN. Wells containing 1 × 10⁵ AS-4 or TA-6 cells supplemented with about 10 G3 cells were included as controls. After 24 h incubation, medium was removed, wells were washed once with phosphate-buffered saline (PBS, 0.137 M-NaCl, 0.003 M-KCl, 0.008 M-Na₂HPO₄, 0.0015 M-KH₂PO₄, 0.001 M-CaCl₂, 0.003 M-MgCl₂, pH 7.2), and monolayers were infected with either is-1 or is+ at an m.o.i. of 4 p.f.u./cell. The surviving cells were rescued 48 h later. Virus was removed, monolayers were washed once with PBS and enough anti-mengovirus antibody to reduce virus titre by 1000-fold in 1 h at 37 °C was added to each well. Five days later, the number of clones surviving in each well were counted.

Antibody was then removed and fresh medium containing 10 units/ml IFN was added to each well; 24 h later the clones were reinjected with 4 × 10⁵ p.f.u. of is-1 (no cells ever survived is+ infection). The number of surviving clones was determined 48 h later. AS-4R1 and TA-6R1 are clones which survived the two rounds of infection.

Mengovirus inactivation. Mengovirus was inactivated in supernatants of infected cell monolayers by lowering the pH to 2.0 with 1.0 M-HCl, and then incubating at 37 °C for 4 h. Before assaying for IFN, the pH was adjusted to 7.0 with 1.0 M-NaOH.

IFN induction assay. Monolayers of 1 × 10⁶ cells were prepared in 1 cm Nunc multidish wells in the presence or absence of IFN and incubated for 24 h. The medium was then removed, wells were washed with PBS, and infected with is-1 or is+ at an m.o.i. of 4 p.f.u./cell. Forty-eight h later, supernatants were collected and virus was inactivated as described above.

To assay IFN, twofold dilutions of the supernatants were dispensed into microtitre trays. IFN of known concentration was included as a control. Approximately 2 × 10⁶ G3 cells were added to each well, incubated for 24 h and infected with is-1 at an m.o.i. of 10 p.f.u./cell. Cytopathic effect was recorded 24 h later.

IFN binding assay. The assay was a modification of the technique described by Stewart et al. (1972). Monolayers of 3 × 10⁶ cells in 6 cm Nunc Petri plates were incubated with 1000 units of IFN in 1.0 ml for 2 h at 4 °C. Medium was removed and cells were washed twice with cold PBS. Cells were scraped into 1.0 ml of cold medium and centrifuged. The supernatant was decanted, the cells were resuspended in 0.2 ml cold PBS and sonicated for 3 min in a Heat Systems Ultrasonics sonic bath. Twofold dilutions were made of each sonicate and IFN was assayed as described above, except that an m.o.i. of 0.1 p.f.u./cell of is-1 was used.

Ribosomal RNA assay for 2'-5'-oligo(A)-dependent endonuclease. The procedure was a modification of the standard technique of Wreschner et al. (1981) as developed by J. G. Calvert in our laboratory. Briefly, 2 × 10⁶ cells were grown on 35 mm tissue culture dishes and then treated with 10 units/ml IFN for 24 h and infected with is-1 or is+ at an m.o.i. of 10 p.f.u./cell. Pellets were suspended in 0.3 ml saturation buffer (0.1 M-Tris–HCl pH 8.0, 1.0 M-EDTA, 0.2% v/v 2-mercaptoethanol) and 0.044 ml 3 M-sodium acetate (pH 5.2) was added to them. After gentle shaking, 0.6 ml of a 1:1 mixture of phenol : chloroform saturated with aqueous (saturation) buffer, and containing 0.1% 8-hydroxyquinoline (an antioxidant) was added. Samples were kept at room temperature for 10 min with periodic vortex-mixing. They were then centrifuged for 10 min in a microfuge, and most of the lower organic phase
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Table 1. Isolation of revertant TA-6 cells*

<table>
<thead>
<tr>
<th>Time of rescue (h)†</th>
<th>Cells added‡</th>
<th>Round of infection§</th>
<th>Clones surviving at indicated IFN concentration (units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>52</td>
<td>-</td>
<td>1</td>
<td>35 9 4 1 2 0 1 0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2</td>
<td>1 1 0 1 0 1 0 0</td>
</tr>
</tbody>
</table>

* Cells were treated with IFN and then infected with is-1.
† Cells were rescued by washing and adding anti-mengovirus antibody. Cells rescued at 72 h produced similar results, but lower overall survival rates.
‡ Ten G3 cells were added to 10⁵ TA-6 cells in indicated wells.
§ Surviving clones were counted 8 days after adding antibody. Cells were then treated with 10 units/ml IFN, and reinfected the next day with is-1. The second count was 2 days later.
1 TA-6R1 was derived from this clone.

was removed. Samples were centrifuged again for 1 min, and the aqueous phase was collected. RNA was precipitated with 100% ethanol, denatured with formamide and subjected to constant voltage electrophoresis on 1.8% agarose gels containing 2 M-formaldehyde. The gels were stained (1 μg/ml ethidium bromide), and visualized according to standard procedures.

Chromosome analysis. Cells were blocked just before S phase by incubating monolayers at 4 °C for 15 to 18 h. The S phase block was released by returning the cultures to 37 °C. Ten to 15 min later bromodeoxyuridine in 0.9% NaCl was added to a final concentration of 30 μg/ml. After 3 to 3.5 h incubation at 37 °C, Hoechst 33258 dye in saline was added to a final concentration of 60 μg/ml (Ronne, 1984). Cells were incubated for an additional 3.5 to 4 h. Colcemid (Gibco) was added to each plate 15 to 30 min prior to harvesting to give a final concentration of 5 × 10⁻⁷ M (Ronne, 1984). The procedure for hypotonic treatment, fixation and slide preparation for high resolution R-banding was that described by Ronne (1985).

To prepare chromosome spreads, cell suspensions were dropped from a height of approximately 1-2 m onto clean microscope slides (placed flat on the floor) which had been briefly steamed over a boiling water bath. As soon as the cell suspension started to dry, slides were again steamed for a few seconds and briefly flooded with fixative (3:1 methanol : glacial acetic acid). Slides were allowed to dry completely in air and were then stored overnight on a 60 °C slide warmer. The staining procedure used to obtain R-banding was that of Ronne (1983). Photographs of chromosome spreads were obtained on a Zeiss photomicroscope using Kodak 35 mm Panatomic X film and a 546 MM PIL filter (Zeiss). Negatives were developed in Microdol (Kodak) and prints were made on Kodak Polycontrast rapid II RC paper using a Beseler enlarger (Model 23 C) and no. 3-4 Beseler filters.

RESULTS

Isolation of TA-6R1 and AS-4R1

The procedures for isolating TA-6R1 and AS-4R1 are given in Methods. Details of the experiment in which TA-6R1 was isolated are given in Table 1. After the second infection, infected (dead) and surviving clones were often seen in close proximity, proving that enough virus was present to infect all cells and that every cell in a clone was equally susceptible to the virus. The efficient recovery of the added G3 cells (compare infection 2 with and without added G3 cells) showed that this method can be used to select cells transfected with the is-1 ex+ gene at a frequency as low as 1 in 10⁵. AS-4R1 was isolated in a subsequent experiment.

Growth of mengovirus

The data of Fig. 1 show that G3 cells pretreated with 5 units/ml IFN yielded about 100-fold fewer is-1 p.f.u. than is+. In repeated experiments, the relative yields were reproducible within ± 20%. The non-responsive TA-6 and AS-4 cell lines showed much less difference; is-1 and is+ grew equally well in the TA-6 line whether or not these cells were pretreated with IFN. In pretreated AS-4 cells, is-1 yields were about 10-fold lower at 12 h post-infection and about fivefold lower at 24 h post-infection, but eventually reached control levels. The increase of is-1 yield in these cells between 12 and 48 h was in sharp contrast to what was observed in G3 cells. Is-1 yields from the TA-6R1 revertant cell lines were markedly lower than is+ yields even in the
absence of IFN (Fig. 1). In the presence of IFN, the is-1 yields were comparable to those found in G3. The AS-4R1 line appeared to be only a partial revertant. Is-1 yields were strongly depressed early in infection, but rapidly increased to control levels (Fig. 1).

**IFN induction**

The pH inactivation procedure described in Methods effectively destroyed mengovirus in cell supernatant fluids with little effect on any IFN which was present (Table 2). Low levels of IFN were induced in all three lines by is-1, but only if they had been first pretreated with IFN (data not shown). The TA-6 line produced five to 10 times less IFN than the G3 and TA-6R1 lines. Is+ did not induce IFN under any circumstances.

The role of IFN in expression of the is-1 phenotype

TA-6R1 and G3 cells both produced more IFN than TA-6 cells but it was unclear whether this played a significant role in the expression of the is-1 phenotype. To study this further, either IFN or anti-IFN antibody was added immediately after infection. Neither of these treatments had a marked effect on virus yields until 36 h post-infection (Fig. 2). In three separate experiments, yields of is-1 from protected TA-6 cells infected in the presence of 40 units/ml IFN, were four- to fivefold lower at 24 and 36 h post-infection than in similarly treated TA-6 cells without added IFN. However, virus yields were always much higher than those observed from protected G3 or TA-6R1 cells.

**IFN binding in the G3, TA-6 and AS-4 cell lines**

In four identical experiments, no difference in the amount of IFN bound to G3, TA-6 or AS-4 cells was observed (data not shown).
Fig. 2. Effect of treatment before and after infection with is-1 of G3, TA-6 and TA-6R1 cells with IFN and/or anti-IFN antibody. Cells (1 x 10^5) in Nunc multidish wells were pretreated with 0 or 10 units/ml IFN and infected with is-1 at an m.o.i. of 4 p.f.u./cell. (a) Standard control experiment using (●, ○) G3, (△, ▲) TA-6 and (■, □) TA-6R1; closed symbols, no IFN pretreatment, open, 10 units/ml IFN pretreatment. (b) Is-1 yields from (●) G3 cells pretreated simultaneously with 40 units/ml IFN and 10 arbitrary units/ml anti-IFN antibody. (■) TA-6R1 cells pretreated with 40 units/ml IFN and 10 units/ml antibody simultaneously. G3 cells (○) or TA-6R1 cells (□) pretreated with 10 units/ml IFN and infected in the presence of 10 units/ml antibody. G3 cells (■) or TA-6 cells (△) pretreated with 10 units/ml IFN and infected in the presence of antibody. In (b) the 24 h virus titre in G3 cells infected in the presence of antibody is high compared to the results of three other experiments.

Table 2. Induction of IFN by mengovirus

<table>
<thead>
<tr>
<th>Dilution†</th>
<th>Titre</th>
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<tbody>
<tr>
<td></td>
<td>2</td>
</tr>
<tr>
<td>IFN control (20 units/ml)</td>
<td>-</td>
</tr>
<tr>
<td>pH-Treated, IFN (10 units/ml)</td>
<td>-</td>
</tr>
<tr>
<td>pH-Treated IFN (10 units/ml) + 4 × 10^5 is-1</td>
<td>-</td>
</tr>
<tr>
<td>G3†</td>
<td>4.0</td>
</tr>
<tr>
<td>TA-6†</td>
<td>4.0</td>
</tr>
<tr>
<td>TA-6R1†</td>
<td>4.0</td>
</tr>
</tbody>
</table>

* G3 cells in microtitre trays were pretreated for 24 h with inactivated supernatant fluid diluted as shown above from infected cells and then challenged with is-1 at an m.o.i. of 10. See Methods.
† Symbols are −, 0% c.p.e.; ±, 50% c.p.e.; +, 100% c.p.e.
‡ Cells pretreated with 10 units/ml IFN prior to infection.

2'-5'-Oligo(A)-dependent endonuclease activity

Evidence of endonuclease activity was found in IFN-treated cells of the G3, TA-6 and AS-4 lines. Fig. 3 shows the rRNA cleavage products obtained in these three lines after 5 h infection with either is-1 or is+. The cellular 2'-5'-oligo(A)-dependent endonuclease appears to be activated equally and under the same conditions in all three lines, since no consistent differences were observed in either the nature or quantity of the induced rRNA cleavage products.
Fig. 3. Ribosomal RNA cleavage products found 5 h after infection with mengovirus. Ribosomal RNA cleavage products were observed in \(i\&-1\) or \(i\&+\)-infected TA-6 cells (lanes 1 to 5), AS-4 cells (lanes 6 to 10) and G3 cells (lanes 11 to 15). Samples in lanes 1, 2, 6, 7, 11 and 12 were pretreated with 10 units/ml mouse \(\alpha/\beta\) IFN for 24 h prior to infection and those in lanes 3, 4, 8, 9, 13 and 14 had no IFN pretreatment. Samples in lanes 2, 4, 7, 9, 12 and 14 were infected with \(i\&-1\) and those in lanes 1, 3, 6, 8, 11 and 13 were infected with \(i\&+\). Lanes 5, 10 and 15 were uninfected controls. Products I, II and III are indicated.

Treatment of cells with IFN alone did not appear to activate the endonuclease in any of the cell lines. In the absence of IFN pretreatment, cleavage products were induced at very low levels or were absent at all times investigated (3, 5 and 7 h) post-infection. Three primary rRNA degradation products were observed consistently in all three lines, following IFN pretreatment and infection with \(i\&-1\) or \(i\&+\). These appear as bands (products I, II and III) migrating ahead of the 18S ribosomal subunit (Fig. 3). It is interesting to note that in all three lines, \(i\&-1\) appears consistently to induce a greater quantity of these three cleavage products than \(i\&+\).
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Fig. 4. Karyotype of G3 cells. The preparation technique is given in Methods.

Karyotype analysis

Based on an analysis of 28 spreads, the G3 subline was found to have an average of 55 chromosomes. Analyses of 24 TA-6 spreads and 20 TA-6R1 spreads showed both of these to have an average of 52 chromosomes. More recently, it has been found that the number of chromosomes counted in spreads of G3, TA-6 and TA-6R1 cells varied too much to establish a modal number reliably. However, the chromosome number generally did not vary more than ± 5 from the average observed in all three lines. Fig. 4 shows a representative karyotype of the G3 line. All three L cell sublines were found to contain a large unpaired dicentric chromosome (one centromere is non-functional) which is characteristic of mouse L929 cells. The additional chromosomes found in the G3 line appear to be one or two large metacentric chromosomes and/or one or two intermediate sized submetacentric chromosomes.

DISCUSSION

Cells which express the is-1 ex+ phenotype show two distinct antiviral activities after exposure to IFN, AVA-1 which affects both is-1 and is+, and AVA-2 which is specific for is-1. The five
mouse fibroblast cell sublines included in this study each demonstrated unique responses to IFN. The data of Fig. 1 show that although the G3, and TA-6R1 (and possibly AS-4R1) sublines display the is-I phenotype, their sensitivity to IFN is not identical. Yields of both is-I and is* appeared to be generally lower in TA-6R1 cells as compared to G3 cells, especially after IFN pretreatment. IFN binding assays indicated that there was no difference in numbers of receptors among these L cell sublines.

Is-I has been shown to induce IFN in infected L cells (Marcus et al., 1981, Simon et al., 1984). Since significantly more IFN was induced in TA-6R1 cells than in TA-6 cells (Table 2), it was desirable to see whether this could account for the recovery of the is-I phenotype. The data of Fig. 2 show that this is not the case. Addition of anti-IFN antibody to TA-6R1 and G3 monolayers following infection had little effect on virus yields for the first 24 h. The marked increase in is-I yield at 36 h post-infection was probably due to loss of the short-lived AVA-2 (Fout & Simon, 1983). The IFN induced in G3 or TA-6R1 cells would ordinarily continue to stimulate AVA-2 indefinitely. However, when anti-IFN antibody was added at the time of infection, the protective effect of AVA-2 was reduced after 24 h. Typically, protected TA-6 monolayers infected with either is* or is-I produced yields comparable to unprotected monolayers by 24 to 36 h post-infection (Fig. 1 and 2). When 40 units/ml IFN was added at the time of infection, is-I yields dropped four- to fivefold (Fig. 2,b). Clearly, the presence of IFN during infection had an effect on the establishment and maintenance of some aspect of the antiviral state in this line, but did not fully restore AVA-2. Two explanations may be considered:

(i) The is-I ex+ 'gene' may actually be two or more genes acting in concert to establish AVA-2. TA-6 may be defective in only one of them and the continued presence of IFN may help restore its action or (ii) TA-6 completely lacks is-I ex+ and thus any aspect of AVA-2. Infection in the presence of IFN simply prolongs the effectiveness of AVA-1, leading to a somewhat lower yield of is-I. Although the second explanation seems plausible, Simon et al. (1984) demonstrated that treatment of is*-infected G3 cells with IFN had virtually no effect on virus yields (i.e. AVA-1 was not affected). In fact, there is evidence that is-I ex+ may actually have two components, one possibly mitochondrial, which act cooperatively in establishing AVA-2 (A. L. Z. Sakuragi, E. A. Bakich & E. H. Simon, unpublished observations). The AS-4 and partially revertant AS-4R1 sublines showed an intermediate degree of expression. This is consistent with the notion that AVA-2 is a multicomponent system.

2'-5'-Oligo(A)-dependent endonuclease is a key enzyme in the classic IFN response (Silverman et al., 1982; Revel, 1984; Whitaker-Dowling & Youngner, 1987). Is-I was found to induce a consistently greater level of rRNA cleavage than is* in protected cell lines (Fig. 3), but this (in itself) does not account for the is-I phenotype since this phenomenon was observed whether or not the cells expressed the is-I phenotype. The G3 cell subline has, on the average, three more chromosomes than the TA-6 or TA-6R1 sublines. Since the TA-6 line was isolated without prior selection from the parental G3 culture, it seems likely that its phenotype is due to loss of one or more whole chromosomes, which is known to occur at high frequency. The TA-6 line was found to revert back to an expressive phenotype at the rate of about 10^-3 to 10^-6 (Table 1). This suggests that TA-6R1 arose via a point mutation, although other explanations are not excluded.

The extra chromosomes in the G3 line could not be identified specifically, but appear to be one or two of the largest metacentrics and/or one or two intermediate sized submetacentrics. The large unpaired marker chromosome, characteristic of mouse L929 cells, was found in all of our tested sublines. It has been described as an elongated metacentric chromosome with heterochromatin segments that are the product of a translocation between chromosomes 6 and 9 (Cherednichenko et al., 1985). With further improvements in chromosome elongation and R-banding, this technique might be useful in establishing a standard karyotype for this widely used cell line.

The molecular basis of AVA-2 remains to be determined, although preliminary studies indicate that there is a direct attack on the viral RNA. The five mouse L cell sublines described in this study display a wide range of is-I phenotype expression and should prove to be valuable
tools in the characterization of AVA-2 and the study of the IFN-induced antiviral response in general.

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REFERENCES


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