Selection and Characterization of Interferon-sensitive Cells Derived from an Interferon-resistant NIH 3T3 Line

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(Accepted 7 July 1987)

SUMMARY

We have developed a selection protocol to isolate interferon (IFN)-sensitive subclones directly from an IFN-resistant cell population. The protocol uses encephalomyocarditis virus (EMCV) as a selection agent in combination with pretreatment with low doses of IFN and subsequent growth in the presence of virus-neutralizing antiserum. We have applied this protocol to the partially IFN-resistant NIH 3T3 clone 1 line and have obtained a number of IFN-sensitive subclones. Sensitivity to IFN was restricted to protection against EMCV. Replication of vesicular stomatitis virus as well as cell growth were resistant to IFN treatment as in the original clone 1 line. We have compared levels of 2',5'-oligoadenylate (2-5A) synthetase, dsRNA-activated protein kinase and 2-5A-dependent RNase in some IFN-sensitive subclones and found no difference from the resistant clone 1 cells. Markedly decreased levels of 2-5A-dependent RNase and thus a defective 2-5A pathway have been implicated as a possible cause for the partial resistance of clone 1 cells to IFN. Since the selected IFN-sensitive subclones are of the same phenotype in this respect as the clone 1 line we suggest that inhibition of EMCV in these lines occurs through a mechanism independent of the 2-5A system.

Two dsRNA-activated pathways have been implicated in the interferon (IFN)-induced antiviral state: the 2',5'-oligoadenylate (2-5A) system [pppA2'(p)nA, n = 2 to >4] and the dsRNA-activated protein kinase. Activation of both pathways leads to inhibition of protein synthesis either through an enhanced RNA breakdown by 2-5A-dependent endoribonuclease or a reduced rate of initiation of protein synthesis via phosphorylation of initiation factor e-IF2 (Johnston & Torrence, 1984). The role of each of these pathways in the antiviral activity is based on circumstantial evidence, e.g. correlating induction and decay of the antiviral state and 2-5A-synthetase or protein kinase activity, accumulation of 2-5A in IFN-treated, virus-infected cells in vivo and characterization of cell lines deficient in antiviral activity (Baglioni, 1979; Epstein et al., 1981; Williams et al., 1979; Samuel & Knutson, 1982a,b). The NIH 3T3 clone 1 line has been shown to be unprotected by IFNs against encephalomyocarditis virus (EMCV) and vesicular stomatitis virus (VSV). Likewise, cell growth was not affected by IFN (Czarniecki et al., 1981; Epstein et al., 1981). There was, however, inhibition of the replication of a chronically infecting murine leukaemia virus and of infecting reovirus (Gupta et al., 1982). Although the original finding that this line lacks 2-5A-dependent endoribonuclease has been modified to some extent by later reports, the observation that EMCV and VSV replication are resistant to IFN still holds (Krause et al., 1985a,b; Nilsen et al., 1982).

To extend our understanding of IFN-induced antiviral mechanisms, it would be of interest to determine the relation between deficiency in 2-5A-dependent endoribonuclease and the defective antiviral state. One approach could be the selection of IFN-sensitive clones from the unprotected NIH 3T3 line. To this end, we have applied a positive selection protocol to isolate
Short communication

IFN-sensitive (IFN<sup>s</sup>) subclones from the resistant (IFN<sup>r</sup>) parental line using EMCV as a selection agent. These subclones have been characterized with respect to the antiviral and anticellular effects of IFN and biochemical parameters affected by IFN.

An NIH 3T3 clone 1 line at a high passage number (>30) was used as the starting IFN<sup>r</sup> cell population. Cells (10<sup>6</sup>) were seeded in 100 mm dishes and treated with 20 IU/ml murine IFN-α/β (sp. act. 5 × 10<sup>7</sup> IU/mg protein) for 20 h followed by infection with EMCV at an m.o.i. of 1. About 12 h post-infection, when complete c.p.e. was observed, medium and cell debris were removed and fresh medium containing EMCV-neutralizing rabbit serum (α-EMCV) was added. Clones which appeared after 7 to 10 days were subjected to a second selection cycle. Surviving ones were individually picked and subcloned by single cell dilution in 96-well microtitre plates. These subclones were used for further experiments. They were maintained in medium containing α-EMCV except for virus assays, for which they were passaged twice in α-EMCV-free medium prior to IFN treatment and virus infection.

Out of a starting population of 2 × 10<sup>6</sup> cells we obtained on average 10 surviving colonies after two cycles of IFN treatment and EMCV selection. Considering the passage number and average number of cell divisions per passage we calculate a reversion frequency to the IFN<sup>s</sup> phenotype of 10⁻⁷ to 10⁻⁸ per generation. A reversion frequency of less than 10⁻⁷ was confirmed by control selection where freshly subcloned NIH 3T3 clone 1 cells of the IFN<sup>r</sup> phenotype were used as the starting population.

Cells were initially selected on the basis of their survival after EMCV infection. Thus, it was important to assay whether this property was indeed due to a newly acquired IFN sensitivity or to an inability of the selected cells to replicate EMCV. For this purpose subclones were treated with different doses of IFN, infected with EMCV and virus yield was determined (Fig. 1a). Control infected cultures yielded virus titres comparable to the original clone 1 cells. Interferon pretreatment led to a dose-dependent reduction in virus titre, confirming that the selected subclones replicated virus efficiently and that IFN treatment caused inhibition of virus growth. The interferon sensitivity of EMCV replication exceeded that in standard L929 cells at all IFN doses tested. Immunofluorescence studies where cells were analysed 6 h post-infection for expression of viral antigen confirmed the IFN-dependent inhibition of EMCV replication at the single cell level. In control infected IFN<sup>r</sup> clone 1 cells as well as in IFN<sup>s</sup> subclones the same proportion (>90%) of cells showed positive fluorescence, which demonstrates that there was no difference in susceptibility to virus infection. Following IFN treatment, the number of virus antigen-positive cells decreased in a dose-dependent manner in IFN<sup>s</sup> subclones whereas in clone 1 cells only a marginal effect was observed (Fig. 2).

In addition to EMCV, NIH 3T3 clone 1 cells are markedly resistant to the anti-VSV effects of IFN. We assayed growth of VSV in our IFN<sup>s</sup> subclones after pretreatment with various doses of IFN (Fig. 1b). We found some reduction in virus titre in the IFN<sup>r</sup> clone 1 line at higher IFN concentrations (>100 IU/ml). However, more importantly, the selected subclones that displayed an IFN<sup>s</sup> phenotype with regard to EMCV had the IFN<sup>r</sup> phenotype of the parental line with regard to VSV. Thus, the anti-EMCV response to IFN was dissociated from the anti-VSV response, indicating the presence of a specific antiviral mechanism underlying the reversion to the IFN<sup>s</sup> phenotype.

As an additional biological parameter the effect of IFN on cell growth was examined in clone 1 cells and some selected subclones (Fig. 1c). As reported earlier growth in clone 1 cells was only marginally affected by IFN even after treatment with high doses for extended periods. As in the case of the anti-VSV effect, all subclones followed the IFN<sup>r</sup> phenotype of the parental line showing little reduction in cell number and thereby making it unlikely that IFN-mediated inhibition of EMCV replication and cell growth are related activities in NIH 3T3 cells.

Several IFN-induced or constitutively expressed enzymes have been implicated in the antiviral activities, foremost the 2-5A synthetase, the dsRNA-activated protein kinase and the 2-5A-dependent endoribonuclease. Whereas levels of the former enzymes have been reported to be similar to those in other IFN-sensitive lines, a marked reduction in the nuclease level has been repeatedly demonstrated in clone 1 cells. We assayed these three activities in IFN<sup>r</sup> clone 1 cells and IFN<sup>s</sup> subclones. The 2-5A synthetase and dsRNA protein kinase were expressed in all
Short communication

Fig. 1. Effect of IFN on viral replication and cell growth in NIH 3T3 clone 1 (●) and IFN-sensitive subclones B7 (■), A3 (□), A5 (▲) and L929 cells (○) treated with various doses of IFN-α/β. For determination of the antiviral effect cells were washed 18 h after treatment and infected with EMCV (a) or VSV (b) at an m.o.i. of 0.05. Twelve to 15 h later, when complete c.p.e. in control cultures was observed, virus titre was determined on L929 cells. Cell growth inhibition (c) was monitored after 4 days of IFN treatment. Cell number was determined using a haemocytometer.

Fig. 2. Inhibition of EMCV replication as revealed by indirect immunofluorescence. (a, b, c) NIH 3T3 clone 1; (d, e, f) IFN-α subclone B10. Cells seeded on glass coverslips were incubated without (a, d) with 50 IU/ml (b, e) or 400 IU/ml IFN α/β (c, f) for 20 h and subsequently infected with EMCV at an m.o.i. of 50. Immunostaining was performed 6 h post-infection as described elsewhere (Ankel et al., 1985). Bar marker represents 50 µm.
Fig. 3. DsRNA-dependent protein kinase levels in control and IFN-treated NIH 3T3 clone 1 and IFN* subclones assayed in cell extracts equivalent to 200 μg of protein following poly(I):poly(C)-cellulose adsorption and autophosphorylation of the 68K protein product (P1) in the presence of [γ-32P]ATP (1 μCi/assay) as described (Hovanessian & Kerr, 1979). Radioactive products were analysed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. Cells were treated with 200 IU of IFN/ml where indicated. Numbers mark positions of molecular weight markers.

Table 1. 2-5A synthetase activity in clone 1 cells and IFN* subclones*

<table>
<thead>
<tr>
<th>Cell</th>
<th>Activity (pmol 2-5A/mg protein/h)</th>
<th>Control</th>
<th>20 IU IFN</th>
<th>200 IU IFN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone 1</td>
<td></td>
<td>8.4 x 10^2</td>
<td>7.8 x 10^3</td>
<td>5.4 x 10^4</td>
</tr>
<tr>
<td>Subclone A3</td>
<td></td>
<td>6.6 x 10^1</td>
<td>7.5 x 10^3</td>
<td>2.6 x 10^4</td>
</tr>
<tr>
<td>Subclone A4</td>
<td></td>
<td>5.1 x 10^1</td>
<td>7.6 x 10^3</td>
<td>7.8 x 10^4</td>
</tr>
<tr>
<td>Subclone B10</td>
<td></td>
<td>1.4 x 10^2</td>
<td>1.1 x 10^4</td>
<td>6.3 x 10^4</td>
</tr>
<tr>
<td>L929</td>
<td></td>
<td>NA†</td>
<td>NA</td>
<td>8.7 x 10^4</td>
</tr>
</tbody>
</table>

*2-5A synthetase activity was assayed by binding of postmitochondrial extracts to poly(I):poly(C)-cellulose. 2-5A was determined by a radiobinding competition assay using [32P]Pc-labelled 2-5A as a radioactive ligand and L929 extracts as a source of 2-5A binding activity (Knight et al., 1980; Silverman et al., 1981).
†NA, Not assayed.

lines at comparable levels either constitutively or following IFN treatment (Fig. 3, Table 1). In addition, however, no consistent differences in 2-5A-dependent nuclease between these cells were observed. This was demonstrated both by an assay which measures the binding activity for 2-5A in cell extracts (and thus the presumptive ribonuclease) or by a functional assay for 2-5A-dependent nuclease in vivo using the characteristic cleavage pattern of rRNA following activation of the 2-5A pathway (Table 2, Fig. 4). Thus, no difference in any of these biochemical markers for the IFN-mediated antiviral state could be demonstrated between lines displaying the IFN* or the IFN† phenotype.
Short communication

Fig. 4. 2-5A-mediated cleavage of rRNA in intact cells. Cells were incubated for 20 h with 500 IU/ml IFN where indicated. 2-5A tetramer [ppp(5'A2')2p5'A; P-L Biochemicals] at 1 μM was introduced into intact cell monolayers by the calcium–phosphate coprecipitation procedure (Hovanessian et al., 1979). Total cellular RNA was isolated by phenol extraction and rRNA cleavage was analysed by denaturing glyoxal–agarose gel electrophoresis (Wreschner et al., 1981). Control transfections were performed with precipitates containing H2O instead of 2-5A. Arrowheads indicate positions of rRNA cleavage products in L929 cells. Neither NIH 3T3 clone 1 cells nor IFNε subclones show 2-5A-enhanced cleavage of rRNA.

Table 2. 2-5A binding activity in clone 1 cells and IFNε subclones*

<table>
<thead>
<tr>
<th>Cells</th>
<th>Control</th>
<th>100 IU IFN</th>
<th>200 IU IFN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clone 1</td>
<td>NA†</td>
<td>5-1</td>
<td>4-7</td>
</tr>
<tr>
<td>Subclone A3</td>
<td>1-6</td>
<td>7-8</td>
<td>9-0</td>
</tr>
<tr>
<td>Subclone A4</td>
<td>1-1</td>
<td>6-7</td>
<td>5-8</td>
</tr>
<tr>
<td>Subclone B10</td>
<td>1-1</td>
<td>4-2</td>
<td>3-7</td>
</tr>
<tr>
<td>L929</td>
<td>37-1</td>
<td>25-3</td>
<td>NA</td>
</tr>
</tbody>
</table>

* 2-5A-dependent RNase levels were determined by a radiobinding assay using postmitochondrial extracts equivalent to 200 μg protein and [32P]pCp-labelled 2-5A as a probe (Silverman et al., 1981).
† NA, not assayed.

Evidence exists that IFN treatment leads to the induction of a number of antiviral mechanisms which may affect replication of different viruses to various degrees (Friedman, 1977; Lengyel, 1982). This concept is supported by our finding that the reversion to IFN sensitivity in our clones is limited to EMCV and does not extend as a general response to e.g. other lytic RNA viruses since VSV is not inhibited. Thus, in our system IFN action on these two viruses can clearly be dissociated as can inhibition of cell growth from the anti-EMCV activity. Gupta et al. (1982) reported earlier that reovirus replication is inhibited by IFN in the original clone 1 line, separating the effects of IFN treatment on this virus from those on EMCV and VSV. Thus it appears that IFN induces defined cellular responses with considerable specificity for different viruses.

We have found no difference in the activity of IFN-inducible enzymes between the IFNε clone 1 line and the IFNε subclones. This was to be expected for the 2-5A synthetase and dsRNA-activated kinase since the inducible levels and dose–responses for these enzymes in
clone 1 cells are comparable to other IFN\textsuperscript{s} cell lines, e.g. L929 (Epstein \textit{et al.}, 1981). Surprisingly, 2-5A binding activity as an indicator of 2-5A-dependent RNase levels were similar in all clones tested and markedly lower than in L929 cell-free extracts. The 2-5A binding data as measures of 2-5A-dependent RNase levels are in agreement with the 2-5A transfection data which fail to show any rRNA cleavage pattern characteristic of the RNase in the IFN\textsuperscript{s} subclones. Deficiency in 2-5A-dependent RNase has been discussed as a possible cause of partial resistance to IFN of clone 1 cells. Krause \textit{et al.} (1985a,b) recently reported that confluence and treatment with high doses of IFN leads to some 2-5A-dependent RNase induction as previously described for JLS-V9 cells. However, these authors reported that even under conditions of maximal nuclease induction growth of EMCV was unaffected. This lack of correlation between 2-5A-dependent RNase induction and protection against EMCV in their study is supported by our findings based on a completely different approach which yielded clones of low 2-5A binding activity but which were highly protected by IFN against EMCV.

There is substantial evidence that an active 2-5A system may play a role in IFN-mediated inhibition of EMCV, e.g. demonstration of 2-5A and 2-5A-dependent RNase activation in IFN-treated, EMCV-infected cells and partial reversal of EMCV inhibition by analogues of 2-5A (Watling \textit{et al.}, 1985; Williams \textit{et al.}, 1979). It is, however, conceivable that inhibition of a virus may occur by more than one mechanism and our IFN\textsuperscript{s} subclones may have been selected for expression of such a 2-5A-independent inhibitory pathway. Thus, they may provide valuable tools to elucidate further the individual components that cause the antiviral state.

It has been reported that in HeLa cells and L cells infected with EMCV without prior IFN treatment the 2-5A-dependent RNase is inactivated (Caley \textit{et al.}, 1982). Since in our study EMCV was used as a selection agent it is possible that in our selected clones a chronic infection would obscure restoration of a functional 2-5A system. Two observations argue against a chronic EMCV infection in the IFN-sensitive subclones. Uninfected cell controls prior to IFN treatment failed to show positive fluorescence when probed with \textalpha-EMCV. Thus, no major replication of residual virus carried over from the selection procedure took place. Secondly, selected clones were grown for a number of passages (> 10) in the presence of \textalpha-EMCV. When such cultures were propagated in the absence of serum, virus growth was only observed after prolonged passaging. These findings would indicate that some carrier cells existed which could give rise to new virus outgrowth but are not compatible with a model where most cells of the population are chronically infected.

Cell lines with partial or complete defects in the IFN-induced antiviral state have often been used to evaluate the various components of this activity and their underlying mechanisms. In most cases these lines have been found by a general screening procedure rather than by direct selection (Krause \textit{et al.}, 1985a,b; Nilsen \textit{et al.}, 1980; Salzberg \textit{et al.}, 1983; Wood & Hovanessian, 1979). The latter approach has been used successfully to isolate IFN receptor-negative cells from a positive population of L1210 cells (Gresser \textit{et al.}, 1974) and in vivo by the generation of \textit{Mx}\textsuperscript{positive} mouse strains in an \textit{Mx}\textsuperscript{negative} genetic background via repeated inbreeding or most recently by gene transfer techniques (Horisberger \textit{et al.}, 1983; Staeheli \textit{et al.}, 1986). Our approach is different in that we have used IFN treatment plus virus directly as selecting agents to isolate IFN-sensitive clones from an IFN-resistant parental background. Thus, both IFN\textsuperscript{s} and IFN\textsuperscript{r} phenotypes are available to analyse the basis of the reversion to IFN sensitivity with respect to EMCV growth.

The approach described here is not restricted to a specific virus or cell line, i.e. EMCV and NIH 3T3 clone 1, but should be applicable to any other line which is defective in its antiviral response to a lytic virus. Indeed, we have applied this protocol to clone 1 cells using VSV as selection agent and have obtained a number of clones showing increased protection against VSV by IFN, which are currently under characterization. Thus, the selection protocol may yield cell clones with some virus specificity in their antiviral response and thereby may be a useful tool in analysing the complexity of the antiviral mechanisms.

The authors thank B. Klein for excellent technical assistance, H. Kirchner and H. Ankel for critical reading of the manuscript and M. Kasamasch for expert secretarial help.
Short communication


(Received 1 June 1987)