The Interferon-induced Hyporesponsive State in Variant and Parental L Cells: A Requirement for Cell Division

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SUMMARY

L cells grown for 18 to 22 months in the presence of interferon (IFN) retained their sensitivity to the antiviral effect of IFN but were less sensitive to cell growth inhibition by IFN. Moreover, the parental and variant lines became hyporesponsive (i.e. less responsive to virus induction of IFN) with different kinetics after IFN treatment. Experiments using mitomycin C to inhibit cell division suggested that cell division is required to enter the hyporesponsive state.

INTRODUCTION

Cells treated with interferon (IFN) respond in a variety of ways: they may enter an antiviral state, alter their reproductive rate (cell growth inhibition, CGI), change their morphology, alter the amount of and/or kinetics of IFN synthesis upon subsequent induction. That is, the cells respond to IFN in a pleiotropic fashion (Stewart, 1979). The specific type of response elicited by IFN is often dependent upon the concentration of IFN and/or the duration of treatment. For example, cells treated with a high concentration of IFN for a short period of time are stimulated or 'primed' for IFN synthesis whereas cells treated for a long period enter a hyporesponsive state and are inhibited or 'blocked' for subsequent virus-induced synthesis of IFN.

One approach to understanding the cellular effects of IFN is to study and characterize cellular variants with alterations in the normal pleiotropic responses to IFN. Numerous investigators have selected cell lines resistant to the CGI activity of IFN (Chany & Vignal, 1970; Gresser et al., 1974; Fuchsberger et al., 1974; Kuwata et al., 1976; Borecky et al., 1977). Investigations of these selected lines and of naturally occurring variants have illustrated the range of cellular responses which can be obtained. For example, while some lines are resistant to the antiviral effect and CGI by the type I IFNs, they remain sensitive to these cellular effects of type II IFN (DeMaeyer-Guignard et al., 1980b; Ankel et al., 1980). Another line selected for resistance to IFN has a high basal level of the enzymes induced by IFN but increased enzymic activities are not induced in this line by IFN (Verhaegen et al., 1980). A naturally occurring variant, which is resistant to the antiviral effect of IFN when challenged by encephalomyocarditis virus and resistant to CGI by IFN, remains sensitive to the antiviral effect when challenged by a murine leukaemia virus (Czarniecki et al., 1981; Epstein et al., 1981).

We have selected and are investigating a line of L cells that is resistant to CGI by IFN. We report here a partial characterization of this line and the results of investigations of the hyporesponsive state(s) in this line and the parental L cells. We have examined the hyporesponsive state which is dependent on (i) prior synthesis of IFN (hereafter called 'refractory state') and (ii) IFN treatment (hereafter called 'blocked state'). Our experiments suggest that cellular division is a prerequisite for expression of the 'blocked state'.

METHODS

Cells. All cell lines were grown in Eagle's minimal essential medium (Gibco) supplemented with 10% foetal bovine serum, 50 International Units/ml penicillin and 50 μg/ml streptomycin. Cultures were maintained in glass bottles and transferred to plastic tissue culture vessels for experiments.
For all experiments in which IFN synthesis was induced, 1.5 x 10^6 cells were plated into tissue culture flasks (25 cm^2 growth area) in 5 ml of normal growth medium and grown overnight before subsequent treatment or IFN induction.

**IFN procedures.** IFN induction by infection with Newcastle disease virus (NDV) (LaSota strain), collection and preparation of cellular fluids and titration of IFN using a modification of an RNA synthesis reduction assay were as previously described (Burke & Veomett, 1977). The specific activities of the IFNs used ranged from 5.8 x 10^4 to 1.4 x 10^5 units/mg protein. A sample of more highly purified mouse IFN (1.1 x 10^7 units/mg protein) was a generous gift of Dr B. Dalton (Dept. of Microbiology, Medical College of Pennsylvania, Philadelphia, Pa., U.S.A.). All IFN units have been converted to International Units using Standard G 002-904-511.

**Cell growth inhibitory effect of IFN.** Equal cell numbers of the line being tested were seeded into flasks containing growth medium supplemented with various concentrations of IFN. Four days later the cells were collected and counted. The number of cells in flasks containing medium without IFN (controls) was taken as 100% and the relative number of cells in the other flasks was determined.

**Mitomycin C treatment.** The growth medium was removed from cultures, the cultures washed once with growth medium and growth medium containing mitomycin C (20 μg/ml) was added. After incubation for 2 h at 37 °C in medium containing mitomycin C, the medium was removed; the cultures were washed twice with normal growth medium and normal growth medium was added for further incubations.

**Chemicals.** [5 H]Uridine was obtained from either New England Nuclear or Amersham Corp.; actinomycin D, poly(rI).poly(rC), DEAE-dextran and mitomycin C were obtained from Sigma.

**Statistical analysis.** For analysis, the amount of IFN produced by treated cells was converted to a percentage of the amount produced by untreated, control cells and the effects of various cellular treatments were analysed using Student's t-test on the population means. We take P < 0.05 to be significant.

**RESULTS**

**Derivation and growth characteristics of an IFN-selected L cell line**

The WDIFN line (IFN-selected, withdrawn from IFN) was developed by growth of L cells for 18 to 22 months in the presence of IFN. Cultures were initiated with IFN present at a concentration of 100 units/ml, with the concentrations being increased approximately every 2 months as follows: 100, 200, 400, 800, 1000 and 2000 units/ml of culture medium. This regimen is similar to that reported recently by Kuwata (1981). Before being used for experiments, the WDIFN line was subcultured four times in the absence of IFN over a period of 2 weeks. The WDIFN line grew more slowly than the parental L line, generation times in the absence of IFN being approx. 24 to 26 h and 18 to 20 h respectively (data not shown).

The results of experiments examining CGI by IFN on L cells and WDIFN cells are shown in Fig. 1. The degree of CGI in cultures of WDIFN cells grown in the presence of 100000 units/ml crude L cell IFN (about 15%) was approximately the same as that in cultures of parental L cells grown in the presence of 10 units/ml crude IFN (about 20%). Thus, the WDIFN line was apparently 10^4-fold less sensitive to CGI by IFN. Moreover, the difference between the cell lines in CGI by IFN was evident with both crude and more highly purified IFN.

**Further characterization of the IFN system in WDIFN cells**

**Ability to enter an antiviral state**

Stock preparations of crude L cell IFN were titrated using either parental L cells or WDIFN cells. The results showed that the titre of the IFN preparations was only slightly greater on the parental L cells (3.3 log_{10} units) as compared to the WDIFN cells (3.1 log_{10} units). Moreover, Semliki Forest virus had the same titre when plaque-assayed on the parental and WDIFN lines and the virus yield 24 h after infection was only slightly different, being threefold less on the WDIFN line (data not shown). This showed that the WDIFN line was not in a constitutive antiviral state, but entered an antiviral state when treated with IFN.

**Production of IFN**

 Cultures of WDIFN or L cells were induced to synthesize IFN by infection with u.v.-irradiated NDV (uvNDV) or treatment with double-stranded RNA [poly(rI)-poly(rC)]. Additional cultures were induced either with the double-stranded RNA after pretreatment with IFN, or with double-stranded RNA and DEAE-dextran added simultaneously. The results
Cell division and IFN-induced hyporesponsive state

Fig. 1. Effect of IFN on reproduction of L and WDIFN cells. Parental L (O, □) and WDIFN (■, ■) cells were seeded at a density of $2 \times 10^5$ cells/25 cm$^2$ tissue culture flask containing medium unsupplemented with IFN or supplemented with the indicated concentration ($\log_{10}$) of crude (O, ■) or partially purified (□, □) IFN. Four days later the cells were collected and counted on a Coulter counter or with a haemocytometer. The number of cells in the flasks containing medium lacking IFN was taken as 100%. The relative number of cells in flasks containing IFN-supplemented media was determined. The points shown are the averages of three independent experiments.

showed that the WDIFN line produced as much IFN as L cells after induction either by virus or by double-stranded RNA in the presence of DEAE-dextran. Moreover, the induction of IFN synthesis by double-stranded RNA was 'primed' by a preliminary cellular IFN treatment to the same extent in the WDIFN and parental L lines (data not shown).

The 'refractory state'

Cultures of L and WDIFN cells were induced by treatment with double-stranded RNA and DEAE-dextran, allowed to synthesize IFN for 24 h, the culture fluids collected, the amount of IFN formed determined and the cultures washed twice with growth medium. The cultures were then re-induced with UVNDV, the culture fluids collected 24 h later, and again the amount of IFN formed was determined. The results (Table 1) showed that previously induced L cells produced $14(\pm 8)\%$, and previously induced WDIFN cells produced $48(\pm 8)\%$ of the amount of IFN produced by cells which had not previously synthesized IFN. For both cell lines, the amount of IFN produced by previously induced cells was significantly less than that produced by the controls, i.e. both cell lines were 'refractory'.

The 'blocked state'

Cultures of WDIFN and L cells were grown in the presence of IFN for various periods of time, the IFN-containing medium removed, the cultures washed and IFN synthesis induced with UVNDV. The results showed that when L cells were exposed to high concentrations of IFN for 20 to 24 h they became 'blocked', producing $23(\pm 4)\%$ of the expected amount of IFN (Table 2, culture L-A). This is significantly less than that produced by cells not pretreated with IFN. On the other hand, WDIFN cells similarly treated (Table 2, culture WDIFN-A) produced $95(\pm 18)\%$ of the expected amount of IFN, not significantly less than the control amount and significantly more than L cells similarly treated.

However, WDIFN cells pretreated with IFN for 44 to 48 h (Table 2, culture WDIFN-B) were 'blocked', producing $23(\pm 3)\%$ of the amount of IFN produced by cells not pretreated with IFN. This is significantly less than the amount produced by control cultures. Hence, both L and
Table 1. IFN production after prior IFN synthesis in L and WDIFN cells

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Cell type</th>
<th>Treatment on day 1</th>
<th>IFN (log_{10} units/ml) produced after viral induction on day 2*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>L</td>
<td>Dx†</td>
<td>3.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dx - RNA + Dx‡</td>
<td>2.9</td>
</tr>
<tr>
<td>2</td>
<td>L</td>
<td>Dx</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dx - RNA + Dx</td>
<td>2.3</td>
</tr>
<tr>
<td>3</td>
<td>WDIFN</td>
<td>Dx</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dx - RNA + Dx</td>
<td>2.9</td>
</tr>
<tr>
<td>4</td>
<td>WDIFN</td>
<td>Dx</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dx - RNA + Dx</td>
<td>2.3</td>
</tr>
<tr>
<td>5</td>
<td>WDIFN</td>
<td>Dx</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dx - RNA + Dx</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* Cultures exposed to DEAE-dextran only did not produce IFN on day 1 and all cultures exposed to double-stranded RNA + DEAE-dextran produced over 3.0 log_{10} units/ml of IFN on day 1 (data not shown).
† Dx = DEAE-dextran (100 μg/ml), 1 h treatment.
‡ Ds-RNA + Dx = poly(rI)-poly(rC) + DEAE-dextran (20 μg/ml and 100 μg/ml respectively), 1 h treatment.

Table 2. Effects of pretreatment with IFN and/or mitomycin C on subsequent synthesis of IFN

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Culture</th>
<th>IFN*</th>
<th>Mitomycin C†</th>
<th>Time IFN induced‡</th>
<th>IFN subsequently produced§</th>
</tr>
</thead>
<tbody>
<tr>
<td>L</td>
<td>A</td>
<td>+ (0-24 h)</td>
<td>(−)</td>
<td>24</td>
<td>23 ± 4 (4)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>(−)</td>
<td>+ (−2-0 h)</td>
<td>24</td>
<td>82 ± 19 (2)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>+ (0-24 h)</td>
<td>+ (−2-0 h)</td>
<td>24</td>
<td>82 ± 19 (2)</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>+ (0-5 h)</td>
<td>(−)</td>
<td>5</td>
<td>170 ± 30 (2)</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>+ (0-5 h)</td>
<td>(−)</td>
<td>24</td>
<td>30 ± 2 (2)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>+ (0-5 h)</td>
<td>+ (5-7 h)</td>
<td>24</td>
<td>55 ± 5 (2)</td>
</tr>
<tr>
<td>WDIFN</td>
<td>A</td>
<td>+ (0-24 h)</td>
<td>(−)</td>
<td>24</td>
<td>95 ± 18 (4)</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>+ (0-44 h)</td>
<td>(−)</td>
<td>44</td>
<td>23 ± 3 (4)</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>+ (0-20 h)</td>
<td>(−)</td>
<td>20</td>
<td>126 (1)</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>+ (0-20 h)</td>
<td>(−)</td>
<td>44</td>
<td>16 ± 1 (2)</td>
</tr>
<tr>
<td></td>
<td>E</td>
<td>+ (0-20 h)</td>
<td>+ (20-22 h)</td>
<td>44</td>
<td>67 ± 4 (2)</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>+ (0-42 h)</td>
<td>+ (42-44 h)</td>
<td>44</td>
<td>10 (1)</td>
</tr>
</tbody>
</table>

* IFN was absent (−) or present (+) (1000 units/ml except WDIFN-C,D,E,F where it was present at 2000 units/ml) in the culture medium between the hours shown in parentheses; start of experiment was hour 0.
† Cells were either not treated (−) or treated (+) with mitomycin C as described in Methods; figures in parentheses indicate the hours during which mitomycin C was present; start of experiment was hour 0, e.g. in L-B, mitomycin C was present from −2 h to 0 h.
‡ Time after start of experiment (hour 0) when IFN synthesis was induced with uvNDV.
§ As mean percentage (± standard error of mean) of amount produced by control cultures; numbers in parentheses are number of experiments used. Controls: cultures L-A,B,C,D,E, WDIFN-A,B,C,D,E, cells not treated with IFN; L-F and WDIFN-E, cells not treated with IFN but treated with mitomycin C at the same time as these cultures.

WDIFN cells entered a ‘blocked state’, but the kinetics of development of this state were different; WDIFN cells were delayed in the development of the ‘blocked state’.

Effects of mitomycin C on the IFN-induced hyporesponsive state

We used the DNA-crosslinking agent mitomycin C to inhibit cell division. Although mitomycin C-treated cells do not divide after the first few hours of treatment, they remain metabolically active and viable for several days (Shatkin et al., 1962). Control experiments were performed to determine whether mitomycin C-treated cells could enter the antiviral state after IFN treatment and whether they could produce IFN after induction by virus infection or treatment with double-stranded RNA. A stock preparation of IFN, which gave a titre of 3.8 (log_{10} units/ml) with untreated L cells, had a titre of 3.9 (log_{10} units/ml) with mitomycin C-treated L cells. Therefore, mitomycin C-treated cells responded to subsequent treatment with
Cell division and IFN-induced hyporesponsive state

IFN and entered an antiviral state. Table 2 also shows the results of experiments examining the ability of mitomycin C-treated cells to synthesize IFN. L cells pretreated with mitomycin C produced IFN after induction with virus (Table 2, culture L-B) or double-stranded RNA (data not shown). Thus, mitomycin C treatment did not inhibit the induction and synthesis of IFN. However, as shown in Table 2 (culture L-C), mitomycin C treatment prevented the development of the ‘blocked state’. Mitomycin C-treated cells exposed to IFN (1000 units/ml) for 24 h produced 82(± 19)% of the amount of IFN produced by cells not exposed to IFN. This is not significantly different from cells not pretreated with IFN but is significantly different from cells pretreated with IFN and not treated with mitomycin C.

We next determined the effects of mitomycin C on the development of the hyporesponsive state in cells previously treated with IFN. Cultures were pretreated with IFN for several hours (5 h for L cells, 20 h for WDIFN cells) but an insufficient time for them to become hyporesponsive (Table 2, cultures L-D, WDIFN-A, WDIFN-C). Several pretreated cultures were then washed and incubated in normal growth medium (cultures L-E and WDIFN-D) or washed, treated with mitomycin C and incubated in normal growth medium (cultures L-F and WDIFN-E). Subsequently, the cells were induced when the cultures would normally be blocked (24 h for L cells, 44 h for WDIFN cells). As shown in Table 2, cultures pretreated with IFN and subsequently treated with mitomycin C produced significantly more IFN than similarly treated cultures not treated with mitomycin C. In addition, cells that had been treated with IFN for a period of time sufficient for them to be hyporesponsive and then subsequently had been treated with mitomycin C were hyporesponsive (culture WDIFN-F). Therefore, mitomycin C treatment inhibited the development but not the detection of the IFN-induced hyporesponsive state in cells treated with IFN prior to their treatment with mitomycin C.

DISCUSSION

We have shown that the WDIFN line described here is less sensitive than the parental L cell line to CGI by IFN, enters the antiviral state when treated with IFN, produces IFN when induced by a virus or double-stranded RNA, and can be primed for IFN synthesis. Thus, our WDIFN line shares several characteristics with other IFN-resistant lines described in the literature (Fuchsberger et al., 1974; Kuwata et al., 1976; Borecky et al., 1977).

In addition to the lowered CGI by IFN, the WDIFN line differed from the parental line in two respects. First, the generation time of the line was longer than that of the parental L cells (24 to 26 h compared to 18 to 20 h respectively). Second, the two lines differed in their ability to synthesize IFN after a 24 h pretreatment with IFN. The parental L cells were ‘blocked’ after 24 h treatment with IFN (1000 units/ml) whereas WDIFN cells similarly treated were not.

Are both CGI and the ‘IFN-induced’ hyporesponsive states caused by IFN? The WDIFN line was selected and is maintained by growing the cells in medium supplemented with crude L cell IFN. Although IFN itself causes CGI (Paucker et al., 1962; Stewart et al., 1973; DeMaeyer-Guignard et al., 1980b; Evinger et al., 1980; Masucci et al., 1980) it is possible that other factors or impurities in crude preparations of L cell IFN also cause CGI. However, similar results were obtained with partially purified IFN and, since DeMaeyer-Guignard et al. (1980a) showed that mouse cells pretreated with electrophoretically pure IFN also became hyporesponsive, it is likely that all the effects studied were due to IFN.

Are the two types of hyporesponsive state, the ‘refractory state’ and the ‘blocked state’, the same? While the ability of cells to synthesize IFN is reduced in each case, there are significant differences between them. Cells in the ‘refractory state’ are hyporesponsive regardless of the type of IFN inducer used, whereas cells in the ‘blocked state’ are hyporesponsive only when virus is the inducer (DeMaeyer-Guignard et al., 1980a). Our results also indicate that the two states are different. Both L and WDIFN cells enter a ‘refractory state’ within 24 h of the first induction. On the other hand, only L cells enter a ‘blocked state’ after a 24 h treatment with IFN.

The ‘blocked state’ is known to be dependent upon the concentration of IFN used to pretreat the cells (see e.g. Paucker & Boxaca, 1967; DeMaeyer-Guignard et al., 1980a) and upon the duration of cellular exposure to IFN (e.g. Paucker & Boxaca, 1967). Our comparative studies of the induction of the ‘blocked state’ in L cells and WDIFN cells suggest an additional
requirement. Since the WDIFN line had a longer generation time and since it also required a longer exposure to IFN in order to become 'blocked', it was possible that the two properties are related. For example, it is possible that cells pretreated with IFN must undergo mitosis in order to express the 'blocked state'. Similar hypotheses for special quantal cell cycles during differentiation have been proposed by others (e.g. Holtzer & Holtzer, 1976).

If this hypothesis were true, then cells that cannot divide should not enter the 'blocked state'. We tested this deduction. Mitomycin C-treated cells are inhibited in cell division (Shatkin et al., 1962); moreover, they entered an antiviral state when treated with IFN and did produce IFN when subsequently treated with viral or double-stranded RNA inducers. However, when mitomycin C-treated L-cells were treated with IFN (1000 IU/ml) for 24 h and IFN synthesis was subsequently induced by virus infection, the cells were not 'blocked'. Furthermore, in both the L and WDIFN cell lines, the development of the 'blocked state' in cells previously exposed to IFN was inhibited by treatment with mitomycin C. Mitomycin C-treated cultures produced significantly more IFN than untreated cultures.

Both the inhibitory effects of mitomycin C, on the development of the 'blocked state' and the delayed development of the 'blocked state' in WDIFN cells, are consistent with the hypothesis that cell division is required for development of the 'blocked state'.

One explanation for the 'blocked state' is that the IFN-treated cells are in the antiviral state and cannot support the synthesis of the in vivo 'inducer' of IFN synthesis when infected by a virus (DeMaeyer-Guignard et al., 1980a). The results of the experiments reported here are not entirely consistent with this hypothesis since, firstly, the WDIFN line entered the antiviral state but was not 'blocked' after a 24 h pretreatment with IFN, and, secondly, mitomycin C-treated L cells entered the antiviral state but were not 'blocked' after a 24 h treatment with IFN. In addition, previous investigations using cellular enucleation and reconstitution techniques indicated that the cellular factor(s) responsible for the IFN-induced hyporesponsive state were associated with the nucleated cell fragments rather than the cytoplasmic fragments (Lahm & Veomett, 1980). In contrast, other work from our laboratory and others (Veomett & Veomett, 1979; Radke et al., 1974) has shown that at least a portion of the factor(s) responsible for the antiviral state is associated with the cytoplasm. Thus, the factor(s) responsible for the 'blocked state' and the antiviral state appear(s) to be separable. This is consistent with the separation of the antiviral and 'blocked' states.

However, it is still possible that factor(s) involved in the antiviral state may be involved in the IFN-induced 'blocked state'. IFN treatment of cells induces a protein kinase and an oligoadenylate synthetase, enzymes which have been implicated in the antiviral state (e.g. Baglioni, 1979; Kimchi et al., 1979; Revel et al., 1980). Although these enzymes are localized primarily in the cytoplasm of cells, the oligoadenylate synthetase has recently been detected in the nuclei of HeLa cells (Nilsen et al., 1982). The nuclear enzyme activity is increased after IFN treatment. It is possible that the increase in nuclear activity depends on mitosis and that this nuclear activity is involved in the 'blocked state'. We are currently investigating this possibility.

In summary, the development of the IFN-induced hyporesponsive ('blocked') state appears to depend on three factors: the concentration of IFN, time and mitosis.

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