Isolation of Daudi Cells with Reduced Sensitivity to Interferon. III. Interferon-induced Proteins in Relation to the Phenotype of Interferon Resistance

By MICHEL DRON, MICHAEL G. TOVEY* AND PIERRE EID

LaborATORY OF VIRAL ONCOLOGY, INSTITUT DE RECHERCHES SCIENTIFIQUES SUR LE CANCER,
7, RUE GUY MOCQUET, 94802 VILLEJUIF, FRANCE

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SUMMARY

The pattern of both constitutive and interferon-induced proteins was determined by two-dimensional gel electrophoresis in parental and interferon-resistant clones of Daudi cells in relation to the phenotype of interferon resistance. The complement of constitutive proteins present in clones DIF3, DIF5, DIF4, and DIF10 appeared to be identical to that of parental Daudi cells even though these cells were resistant to both the antiviral and anti-proliferative actions of interferon. Treatment of Daudi cells for 20 h with 10^3 reference units/ml of electrophoretically pure human interferon-α resulted in the induction of 15 proteins of molecular weights ranging from 15000 to 62000 detected after a 4 h labelling period with L-[35S]methionine. A number of these proteins were also induced in interferon-resistant clones of Daudi cells although some of these proteins appeared later and in smaller amounts than in the interferon-treated parental cells. However, seven proteins with molecular weights ranging from 18000 to 58000 which were induced by interferon in parental Daudi cells were not induced in any of the four interferon-resistant clones, suggesting that the phenotype of interferon resistance of these cells may be related to a reduction or absence of certain interferon-induced protein(s).

INTRODUCTION

Interferons constitute a group of closely related proteins which exert multiple effects on cells, including the establishment of an antiviral state, inhibition of cell multiplication, modifications of the cell surface, and modulation of specialized cellular functions (Gresser, 1977; Gresser & Tovey, 1978). The expression of certain cellular gene(s) appears to be required for the development of interferon action (Taylor, 1964; Lockart, 1964) and the levels of a number of enzymes are markedly enhanced in interferon-treated cells (Kerr & Brown, 1978; Roberts et al., 1976; St Laurent et al., 1983). In addition, interferon appears to induce several proteins of unknown function (Ball, 1979; Colonnio, 1983; Cooper et al., 1982; Farell et al., 1979; Gupta et al., 1979; Horisberger et al., 1983; Knight & Korant, 1979; Leanderson et al., 1982; Rubin & Gupta, 1980; Sundström et al., 1983; Weil et al., 1983). Cellular mutants resistant to interferon provide a valuable means of studying the role of induced proteins in interferon action. We have recently described the isolation and characterization of two clones of Daudi cells (DIF2 and DIF3) with markedly reduced sensitivity to both the antiviral and anti-cellular actions of human interferon-α and -β (Dron & Tovey, 1983; Tovey et al., 1983). These cells possess functional high affinity interferon receptors similar to those of parental Daudi cells, and respond to interferon treatment by the increased production of 2′-5′-oligo-isoadenylate synthetase but not guanosine 3′-5′-cyclic monophosphate (Dron & Tovey, 1983; Tovey et al., 1983). We describe here the pattern of constitutive and interferon-induced proteins in parental and interferon-resistant clones of Daudi cells in relation to the phenotype of interferon resistance.
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METHODS

Cell and cell culture. The isolation and characterization of the interferon-resistant clone of Daudi cells, DIF3, have been described previously (Dron & Tovey, 1983; Tovey et al., 1983). The interferon-resistant clones DIF8, DIF9 and DIF10 were isolated by a similar procedure with the exception that these cells were cloned and then maintained in the continuous presence of 10^4 reference units/ml of human interferon-\(\alpha\). These cells were cultivated for 15 days in the absence of interferon prior to use. All cells were cultivated in RPMI 1640 medium with 15% foetal calf serum (Flow Laboratories) in static suspension culture.

Interferon preparations. Human interferon-\(\alpha\) was prepared from the human lymphoid cell line Namalwa as previously described (Mogensen & Cantell, 1977) and purified by affinity chromatography (Mogensen & Cantell, 1979) to a specific activity of 2 \times 10^8 reference units/mg protein. This material was electrophoretically pure when subjected to electrophoresis in polyacrylamide gels containing SDS (Mogensen et al., 1981).

Isotopic labelling and preparation of cell extracts. Cells (0.5 \times 10^6/ml) were labelled for 4 h (from either 0 to 4 h, 4 to 8 h, or 20 to 24 h) with 100 \muCi/ml L-[\textsuperscript{35}S]methionine (1200 Ci/mmol, Amersham), in the presence or absence of interferon in RPMI 1640 medium without L-methionine. In some experiments cells were treated with interferon in the presence of 2 \mug/ml actinomycin D.

Cell extracts were prepared by the method of Garrels (1979) with the exception that the cells were disrupted by three successive rapid freezing and thawing cycles. Aliquots of each sample were tested for their radioactive content following precipitation with TCA.

Two-dimensional gel electrophoresis. Isoelectric focusing and SDS-polyacrylamide gel electrophoresis were performed as described by O’Farrell (1975). The first-dimension separation was by isoelectric focusing using 1.6\% pH 5 to 8 and 0.4\% pH 3.5 to 9.5 Ampholines (LKB). The second-dimension separation was in a 12.5\% polyacrylamide slab gel. Samples were standardized by applying to the first dimension the amount of cell lysate corresponding to 10^6 c.p.m. of TCA-precipitable material. The second-dimension polyacrylamide gels were dried and then exposed for a standard exposure time of 15 days to Kodak DEF-5 X-ray film.

RESULTS

Sensitivity of cloned Daudi cell variants to the antiviral and anti-proliferative actions of interferon

A number of clones of Daudi cells with a common phenotype of interferon resistance similar to that previously described for clone DIF3 (Dron & Tovey, 1983; Tovey et al., 1983) were used in this study. These cells exhibited marked resistance to the antiviral action of 10^3 reference units/ml of human interferon-\(\alpha\) when challenged with vesicular stomatitis virus (VSV) (Table 1). These cells also showed a pronounced resistance to the anti-proliferative action of interferon (Table 1). The phenotype of interferon resistance of these clones contrasted sharply with the phenotype of parental Daudi cells which were highly sensitive to both the antiviral and anti-proliferative actions of human interferon-\(\alpha\) (Table 1).

Comparison of constitutive proteins in parental and interferon-resistant Daudi cells

Analysis of labelled proteins by two-dimensional gel electrophoresis following a 4 h exposure of cells to L-[\textsuperscript{35}S]methionine revealed a large number of newly synthesized proteins in both parental (Fig. 1a) and interferon-resistant Daudi cells (Fig. 1c). Under the experimental conditions used, numerous proteins were detected with isoelectric points ranging from 5 to 8, with an accumulation of proteins at the basic end of the range. Proteins were detected with molecular weights ranging from 14000 to 94000 (Fig. 1). Under these conditions no consistent differences were observed between the complement of proteins present in parental Daudi cells and interferon-resistant DIF8 cells (Fig. 1) or three other clones of Daudi cells with a similar phenotype of interferon resistance (data not shown).

Interferon-induced proteins in parental Daudi cells

Treatment of Daudi cells for 20 h with 10^3 reference units/ml of electrophoretically pure human interferon-\(\alpha\) resulted in a 70\% reduction in the incorporation of [\textsuperscript{35}S]methionine into cellular proteins following a 4 h labelling period irrespective of the presence or absence of cold methionine. However, in spite of the apparent inhibition of protein synthesis in interferon-treated Daudi cells, a number of specific proteins were induced by interferon in these cells (Fig. 1b). In three separate experiments 15 proteins of molecular weights ranging from 15000 to 62000 were consistently induced by interferon treatment (Table 2). Five of these proteins were
Table 1. Sensitivity of cloned Daudi cells to the antiviral and anti-proliferative actions of interferon*

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Treatment</th>
<th>VSV titre (log_{10}, TCID_{50}/0.2 ml)</th>
<th>Cell number (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Daudi</td>
<td>Untreated</td>
<td>5.50 ± 0.21</td>
<td>P &lt; 0.001†</td>
</tr>
<tr>
<td></td>
<td>IFN</td>
<td>5.27 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>DIF₃</td>
<td>Untreated</td>
<td>6.00 ± 0.45</td>
<td>NS‡</td>
</tr>
<tr>
<td></td>
<td>IFN</td>
<td>5.50 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>DIF₈</td>
<td>Untreated</td>
<td>6.30 ± 0.30</td>
<td>NS‡</td>
</tr>
<tr>
<td></td>
<td>IFN</td>
<td>6.00 ± 0.20</td>
<td></td>
</tr>
<tr>
<td>DIF₀</td>
<td>Untreated</td>
<td>5.30 ± 0.30</td>
<td>NS‡</td>
</tr>
<tr>
<td></td>
<td>IFN</td>
<td>5.00 ± 0.40</td>
<td></td>
</tr>
<tr>
<td>DIF₁₀</td>
<td>Untreated</td>
<td>5.30 ± 0.30</td>
<td>NS‡</td>
</tr>
<tr>
<td></td>
<td>IFN</td>
<td>5.00 ± 0.40</td>
<td></td>
</tr>
</tbody>
</table>

* Cells were cultivated either in RPMI 1640 medium with 15% foetal calf serum alone or in medium containing 10⁴ reference units/ml of electrophoretically pure human interferon-α. Antiviral activity was determined after 24 h treatment with interferon. Cells were resuspended in medium without interferon but containing VSV (Indiana strain) at a m.o.i. of 0.1 for 1 h adsorption at 37 °C. Then centrifuged, washed three times and resuspended in nutrient medium. Virus replication was determined 18 h later. Cultures of Daudi cells were then frozen and thawed three times, centrifuged (2000 r.p.m., 10 min) and the supernatant was titrated on L929 cells. Virus titres are expressed as TCID_{50} per 200 μl. Results are expressed as the mean of three replicates. Cell concentration was determined after 96 h treatment with interferon using a model ZB-1 Coulter counter. Results are the mean of three replicates expressed as a percentage of the control.
† Determined by Student's t-test.
‡ NS, Not statistically significant.

Table 2. Characteristics of interferon-induced proteins*

<table>
<thead>
<tr>
<th>Molecular weight (× 10⁻³)</th>
<th>Isoelectric point</th>
<th>Daudi 4 h</th>
<th>8 h</th>
<th>24 h</th>
<th>DIF₃ 4 h</th>
<th>24 h</th>
<th>DIF₈ 24 h</th>
<th>DIF₀ 24 h</th>
<th>DIF₁₀ 24 h</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>15-b</td>
<td>7.4</td>
<td>+++++</td>
<td>++++</td>
<td>++++</td>
<td>±</td>
<td>+</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>18</td>
<td>7.9</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>±</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>7.4</td>
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<td>−</td>
<td>+</td>
<td>−</td>
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<td>++++</td>
<td>++++</td>
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<td>+</td>
<td>+</td>
<td>+</td>
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<td>++</td>
<td>++++</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−</td>
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</tr>
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</table>

* Cells were treated with either 100 μCi/ml L-[³⁵S]methionine alone or with L-[³⁵S]methionine and 10³ reference units/ml of electrophoretically pure human interferon-α from either 0 to 4 h, 4 to 8 h, or from 20 to 24 h. At the times indicated, the intensity of induction of proteins in interferon-treated cells relative to untreated control cells was estimated on an arbitrary scale of 1 to 4+. ± denotes a level which is just detectable.

strongly induced after 20 h treatment with interferon (Table 2) and indeed two of them (P15-b and P33) were strongly induced after only 4 h treatment with 10⁻² units/ml interferon-α (Fig. 2). These two proteins were also clearly detectable after 4 h treatment with only 1 unit/ml interferon-α (data not shown). The three other strongly induced proteins appeared between 4 and 8 h (P15-a and P56-a) and between 8 and 20 h (P62) after interferon treatment (Table 2). Interferon treatment also appeared to increase the synthesis, albeit to a lesser extent, of ten other
Fig. 1. Analysis of interferon-induced polypeptides in parental and interferon-resistant Daudi cells. Cellular proteins from cells treated for 24 h with or without $10^{3}$ units/ml of electrophoretically pure human interferon-α were labelled with L-[35S]methionine, extracted, and analysed by two-dimensional electrophoresis as described in Methods. One-million c.p.m. of each sample were loaded on the gel for the first dimension separation. The arrows in the body of the figure indicate the presence or absence of a particular protein identified by a number corresponding to its molecular weight $\times 10^{-3}$. The molecular weight markers used were phosphorylase b (94), albumin (67), catalase (60), ovalbumin (43), lactate dehydrogenase (33), and carbonic anhydrase (30).
dehydrogenase (36), carbonic anhydrase (30), trypsin inhibitor (20.1), ferritin (18.5) and $\alpha$-lactalbumin (14.4). Isoelectric points were determined by measuring the pH of 5 mm slices of the first-dimension gel as described by O'Farrell (1975). (a) Untreated parental Daudi cells; (b) interferon-treated Daudi cells; (c) untreated interferon-resistant DIF$_8$ cells; (d) interferon-treated DIF$_8$ cells.
Fig. 2. Kinetics of interferon-induced polypeptides in parental and interferon-resistant Daudi cells. Cells were treated with electrophoretically pure human interferon-\(\alpha\) for the times indicated in the figure, labelled with L-\([\text{\textsuperscript{35}}\text{S}]\)methionine during the last 4 h of interferon treatment, extracted and analysed by two-dimensional electrophoresis as described in Methods. The area surrounding the protein of interest was cut from each autoradiogram and assembled as presented in the figure so as to facilitate comparison of interferon-induced proteins in parental and interferon-resistant Daudi cells.

proteins in Daudi cells. These proteins were of molecular weights ranging from 18 000 to 58 000. Two of these proteins (P58-a and P58-b) with the same molecular weight of 58 000, also had very similar isoelectric points, suggesting that the two proteins may be related (Fig. 1).

**Interferon-induced proteins in interferon-resistant Daudi cells**

Treatment of interferon-resistant Daudi cells for 20 h with 10\(^3\) reference units/ml of electrophoretically pure human interferon-\(\alpha\) had no significant effect on the overall incorporation of L-\([\text{\textsuperscript{35}}\text{S}]\)methionine into cellular proteins following a 4 h labelling period. Interferon treatment did, however, induce the synthesis of a number of proteins in DIF\(\gamma\) cells, even though these cells were resistant to both the antiviral and anti-proliferative actions of interferon (Fig. 1d). In particular the two proteins (P15-b and P33) most strongly induced by interferon in parental Daudi cells were also induced by interferon in interferon-resistant Daudi cells although these proteins appeared later, and in the case of protein P33 in smaller amounts, than in the parental cells (Fig. 2; Table 2). Three other proteins (P15-a, P18 and P40) were also induced by interferon treatment in the interferon-resistant clones although to a lesser extent
than in the parental cells (Table 2). However, seven other proteins, including two proteins (P56-a and P62) which were strongly induced by interferon in parental Daudi cells, did not appear to be induced by interferon in any of the four interferon-resistant clones (Table 2). A further protein (P56-b) was slightly increased following interferon treatment in clone DIFs in one of three experiments (Fig. 1), but was not increased at all in clone DIF3 in three separate experiments (Fig. 2). One protein (P62) induced by interferon in parental Daudi cells appeared to be present endogenously in small amounts in clone DIF8 at least in some experiments (Fig. 1). No interferon-induced proteins were detected in the interferon-resistant clones that were not induced in parental Daudi cells.

Enhancement of protein synthesis in interferon-treated cells

Under the experimental conditions used in this study a number of proteins induced by interferon treatment in Daudi cells appeared to be absent from untreated cells, while others appeared to be present at least in trace amounts in untreated control cells (Fig. 1). However, with the exception of protein P35, which was barely detectable after interferon treatment, all these proteins could be detected in control cells following either overexposure of the films, or by loading fivefold more radioactivity on the first dimension. Thus, we have estimated the relative intensity of induction of each protein, with the exception of protein P35, following interferon treatment (Table 2).

Treatment of Daudi cells with actinomycin D, an inhibitor of RNA polymerase, prevented the induction of several proteins in interferon-treated Daudi cells, suggesting that these proteins are formed as the result of de novo protein synthesis. Thus, treatment of Daudi cells for 8 h with interferon in the presence of actinomycin D (2 μg/ml) prevented the induction of eight proteins usually synthesized in interferon-treated Daudi cells between 4 and 8 h.

DISCUSSION

The complement of constitutive proteins present in clones DIF3, DIF8, DIF9 and DIF10 appeared to be identical to that of parental Daudi cells even though the phenotype of interferon resistance of these cells was markedly different from that of the highly interferon-sensitive parental cells. Our results contrast with those of Leanderson et al. (1982) who reported the absence of six proteins in an interferon-resistant P3 HR-1 substrain compared to wild-type cells. Although two-dimensional gel electrophoresis is a powerful analytical tool allowing several hundred individual proteins to be detected, we cannot exclude the possibility that the phenotype of interferon resistance of the Daudi cell variants is related to differences in protein(s) outside the range of molecular weights or isoelectric points resolved under the experimental conditions used in this study.

Our results show that 15 proteins are consistently induced in Daudi cells treated with electrophoretically pure human interferon-α. In some experiments trace amounts of certain of these proteins could be detected in untreated control cells. Indeed, with one exception all the interferon-induced proteins could be detected in untreated control cells following either extended exposure of the films or by loading more radioactivity on the first dimension, raising the question of whether these are truly induced proteins. It is of interest that low levels of 2'-5'-oligo-isoadenylate synthetase and other interferon-induced enzymes can be detected in a number of cells in the absence of interferon treatment (Jacobsen et al., 1983; Tovey et al., 1983), suggesting that interferon may modulate the synthesis of certain cellular proteins rather than inducing novel ones. Although some minor variation in the intensity of induction of individual proteins was observed from one experiment to another, it is important to emphasize that interferon treatment markedly enhanced the synthesis of all 15 proteins in three independent experiments. Furthermore, treatment of cells with actinomycin D blocked the induction of these proteins, indicating a requirement for the synthesis of new mRNA. There is now considerable evidence to suggest that a number of interferon-induced polypeptides are the products of de novo transcription and translation (Gupta et al., 1979; Knight & Korant, 1979; Rubin & Gupta, 1980). Indeed, Colonno (1983) has recently demonstrated the appearance of new mRNAs in human fibroblasts as early as 2 h after interferon treatment.
Among the five interferon-induced proteins which we have described in Daudi cells, the proteins P33 and P62 appear to correspond to the 33000 and 62000 mol. wt. proteins previously reported to occur in both interferon-treated human peripheral blood lymphocytes (Gustafsson et al., 1982) and the Burkitt lymphoma-derived cell line P3,HR-1 (Leanderson et al., 1982). A P62 protein is also induced by interferon in cell lines of non-lymphoid origin (Colonna, 1983). A 56000 mol. wt. protein has been described in interferon-treatment mouse (Farell et al., 1979) and chick fibroblasts (Ball, 1979) which may correspond to the 56000 interferon-induced protein which we see in Daudi cells. A 15000 interferon-induced protein has been found in interferon-treated human, mouse and bovine cells (B. D. Korant, D. C. Blomstrom, G. J. Jonak & E. Knight, Jr, personal communication). However, we have detected two distinct interferon-induced proteins in Daudi cells with the same molecular weight, 15000, but with differing isoelectric points of 7.1 and 7.4 which appear to correspond to the two proteins described by Guardini et al. (1984). The failure of some authors to detect two distinct proteins in some previous studies may be related to the different cells used or due to technical differences such as the range of Ampholines employed for the first-dimension separation (O’Farrell, 1975; O’Farrell et al., 1977).

Our results show that interferon induces a number of proteins in DIF3 cells and the other interferon-resistant clones of Daudi cells, although some of these proteins appear later and in smaller amounts than in the interferon-treated parental cells. These results are in agreement with our previous work showing that even though DIF3 cells are resistant to both the antiviral and anti-proliferative actions of interferon these cells do possess specific high-affinity interferon receptors and produce elevated levels of 2'-5'-oligo-isoadenylate synthetase in response to interferon treatment (Tovey et al., 1983). However, seven proteins induced by interferon in parental Daudi cells were not induced in the interferon-resistant clones. Leanderson et al. (1982) have also reported that several interferon-induced proteins present in P3,HR-1 cells are not induced in an interferon-resistant subline. Horisberger et al. (1983) have also recently described a 72000 interferon-induced protein which is present in cells carrying the gene Mx, which determines the responsiveness to the antiviral action of interferon towards influenza viruses, but which is absent from cells lacking this gene. Although further studies will be necessary to establish whether the lack of induction of all or some of the seven proteins in interferon-resistant Daudi cells is related to the phenotype of interferon resistance, it should be emphasized that the same seven proteins are not induced by interferon treatment in four different clones of Daudi cells each exhibiting the same phenotype of interferon resistance. Although the identity of these seven proteins remains unknown it seems unlikely that these proteins are involved in either of the known interferon-inducible double-stranded RNA-dependent enzyme systems. The induction of 2'-5'-oligo-isoadenylate synthetase appears to the unimpaired in our interferon-resistant Daudi cells. Silverman et al. (1982) have also reported elevated levels of both 2'-5'-oligo-isoadenylate synthetase and dsRNA-dependent protein kinase in an independently isolated line of interferon-resistant Daudi cells following interferon treatment. The widely differing molecular weights and isoelectric points of the seven proteins which are not induced in the resistant clones suggests that the products of a number of genes are involved in the phenotype of interferon resistance.

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IFN-induced proteins in resistant cells


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