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Comparison of Interferon Action in Interferon Resistant and Sensitive L1210 Cells

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SUMMARY

Translation inhibition, leu-tRNA aminoacylation and double-stranded RNA and ATP dependent phosphorylation were examined in interferon-treated and control cell-free lysates of leukaemic mouse L 1210 R and L 1210 S cells. No differences were observed between the respective interferon-treated and control cell-free extracts, except for the presence of an enhanced 67K dalton phosphoprotein fraction in interferon-treated L 1210 S cell-free extracts. In non-responding cell-free lysates, the lack of stimulation of a 67K dalton phosphoprotein fraction cannot be explained by the presence of an increased level of some inhibitory activity, such as a phosphatase.

In cell-free extracts of interferon treated cultures, numerous alterations in metabolic activity have been observed, such as translation inhibition (Falcoff *et al.* 1972; Friedman *et al.* 1972; Gupta *et al.* 1974*b*; Samuel & Joklik, 1974), deficiency in aminoacyl tRNA species (Content *et al.* 1974; Gupta *et al.* 1974*a*), inhibition of mRNA methylation (Sen *et al.* 1975), activation of a dsRNA dependent endoribonuclease (Sen *et al.* 1976*a*), an increase in sensitivity of mRNA translation to inhibition by dsRNA (Kerr *et al.* 1974) and an increase in the phosphorylation of discrete protein fractions (Lebleu *et al.* 1976; Roberts *et al.* 1976; Wérenne & Rousseau, 1976; Zilberstein *et al.* 1976). We have examined translation inhibition, leu-tRNA aminoacylation and specific protein phosphorylation in cell free lysates from Ehrlich ascites tumour (EAT) cells and leukaemic mouse L 1210 R and L 1210 S cells (Gresser *et al.* 1970, 1974). L 1210 cells, R and S type, (Gresser *et al.* 1970, 1974) were grown in suspension in RPMI 1640 medium, supplemented with 10% (v/v) foetal calf serum (GIBCO) and gentamycin (50 mg/l). EAT cells were maintained in MEM suspension medium, supplemented with 10% (v/v) foetal calf serum (GIBCO) and gentamycin (50 mg/l). At a cell density of approx. 2×10^6 cells/ml, cultures of L 1210 R and L 1210 S cells were divided and one part was treated with 100 units/ml of partially purified mouse interferon ($2{\cdot}8 \times 10^5$ NIH units/mg of protein) for 18 to 24 h. EAT cell cultures were treated in the same way, but the split was made at a cell density of $0{\cdot}3 \times 10^6$ cells/ml.

Interferon treatment of EAT and L 1210 S cells resulted in a more than 99·9% inhibition of vesicular stomatitis virus growth after infection of the cultures at a multiplicity of 0·1 (Falcoff *et al.* 1972); with L 1210 R cells, no reduction of the yield was observed. The same dose of interferon (100 units/ml) reduced the growth rate of L 1210 S and EAT cells by 25% and 33% respectively, whereas the growth rate of L 1210 R cells was unaffected. These observations are in agreement with the findings of Gresser *et al.* (1970, 1974). Translation inhibition of exogenous added mRNA and endogenous leu-tRNA aminoacylation was followed in pre-incubated cell-free extracts (Falcoff *et al.* 1972) of interferon-treated and control EAT and L 1210 cells. As shown by others (Falcoff *et al.* 1972; Friedman *et al.* 1972; Gupta *et al.* 1974*a*, Samuel & Joklik, 1974), globin mRNA translation is reduced in extracts from interferon-treated EAT cells; in our study up to 55% inhibition was obtained. Our

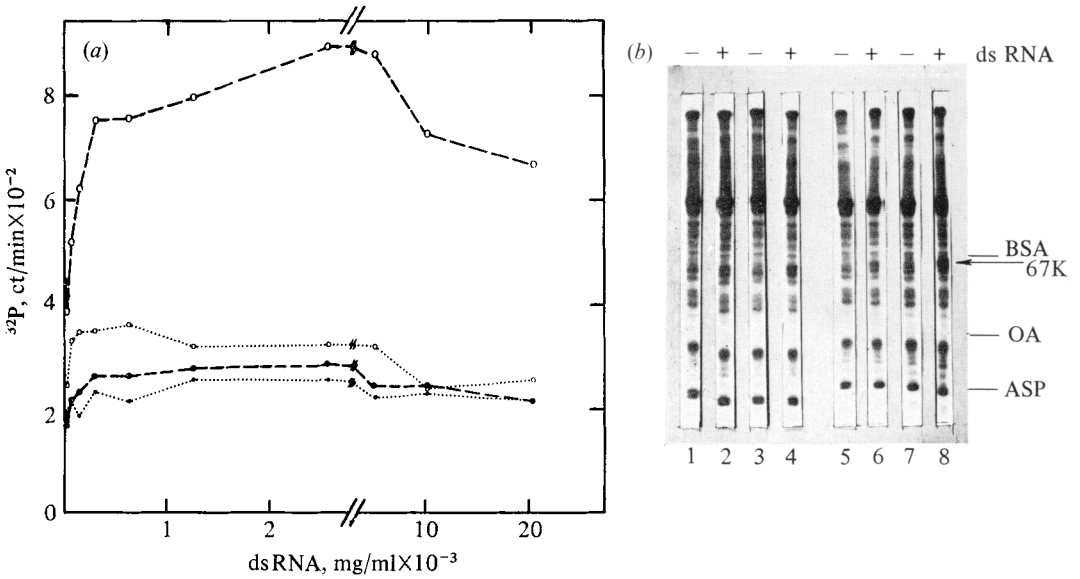

Fig. 1. (*a*) Effects of dsRNA concentration on the phosphorylation of a 67K dalton protein in interferon-treated and control cell-free lysates of L 1210 R and L 1210 S cells. Different lysates were tested for protein phosphorylation activity. Reactions were carried out in a vol. of 0·015 ml; dsRNA was added in different amounts (from 0 to 20480 ng/ml) together with 0·5 mM-(γ-^{32}P)-ATP (800 mCi/mmol) and 0·25 A_{260} unit of each lysate. Incubation was for 15 min at 30 °C. Ten μl of the *in vitro* reaction products were subjected to PAGE on 10% (w/v) polyacrylamide gels for 6 h at 80 V, stained with Coomassie brilliant blue, dried and autoradiographed. Radioactive fractions of 67K daltons were cut out from the dried gel and the radioactivity determined in a liquid scintillation counter after addition of 5 ml toluene based scintillation fluid. The radioactivity obtained in each 67K dalton fraction was plotted against the tested dsRNA concentration. Interferon treatment was as described in the text. ○---○, Cell-free extract of interferon-treated L 1210 S cells; ●---●, cell-free extract of control L 1210 S cells; ○ ····· ○, cell-free extract of interferon-treated L 1210 R cells; ● ····· ●, cell-free extract of control L 1210 R cells. (*b*) Autoradiograms of protein phosphorylation patterns obtained in the different cell-free lysates. Ten μl samples of the *in vitro* reaction products were loaded on 10% (w/v) polyacrylamide slab gels, run for 6 h at 80 V, stained, dried and submitted to autoradiography. Slot 1, 2: cell-free lysate of control L 1210 R cells; slot 3, 4: cell-free lysate of interferon-treated L 1210 R cells; slot 5, 6: cell-free lysate of control L 1210 S cells; slot 7, 8: cell-free lysate of interferon-treated L 1210 S cells. In slots 1, 3, 5, 7, no dsRNA was added to the reaction mixture; in slots 2, 4, 6, 8, dsRNA concentration in the reaction mixture was 2560 ng/ml. The arrow indicates the position of the 67K dalton phosphoprotein fraction synthesized in the different cell-free lysates. Mol. wt. markers, bovine serum albumin (BSA) mol. wt. 68K, ovalbumin (OA) mol. wt. 45K and asparaginase (ASP) mol. wt. 33K, are indicated at their respective positions in the gel.

attempts to reproduce this phenomenon in pre-incubated extracts from L 1210 R and L 1210 S cells have been unsuccessful: less than 1% inhibition was obtained in interferon-treated cell-free extracts. It should be noticed, however, that these extracts are less efficient in their capacity to translate added globin mRNA: L 1210 R and L 1210 S cell-free extracts incorporate only 40% and 33% of the radioactivity obtained with cell-free extracts of control EAT cells.

Sen *et al.* (1976*b*) showed that tRNA charging capacity was impaired in interferon-treated pre-incubated cell-free extracts of EAT cells, especially for endogenous leu-, lys- and ser-tRNA species. Taking advantage of this study, we decided to examine the effect of interferon treatment on aminoacylation of endogenous leu-tRNA in the L 1210 S cell-free lysate compared with the EAT cell-free extract. In the latter cell-free lysate, interferon treatment

Table 1. *dsRNA and ATP-dependent phosphorylation of a 67K dalton protein fraction in cell-free extracts of interferon-treated and control L 1210 R and L 1210 S cells and in mixtures of varying proportions*

Cell-free lysate tested	Amount of cell-free lysate in test sample (μl)	Amount of active lysate L $1210^{S}S_{30}$ added (μl)		
		None	2·5	7·5
(*a*)*				
L $1210^{R}S_{30}$co†	10	42‡	—	—
	7·5	—	68	—
	2·5	—	—	270
L $1210^{R}S_{30}$int	10	67	—	—
	7·5	—	85	—
	2·5	—	—	297
L $1210^{S}S_{30}$co	10	67	—	—
	7·5	—	94	—
	2·5	—	—	295
L $1210^{S}S_{30}$int	10	358	—	—
(*b*)§				
L $1210^{R}S_{30}$co	2‖	44	114	237
	6	88	—	—
L $1210^{R}S_{30}$int	2	52	122	235
	6	117	—	—
L $1210^{S}S_{30}$co	2	51	106	269
	6	108	—	—
L $1210^{S}S_{30}$int	2	116	—	—
	6	250	—	—

* 2·5 μl and 7·5 μl cell-free lysate of interferon-treated L 1210 S cells were mixed with 7·5 μl and 2·5 μl respectively of the other lysates and assayed for ATP and dsRNA dependent protein phosphorylation; 10 μl of each lysate was assayed separately: in one series no dsRNA was added, in a second series dsRNA concentration was 160 ng/ml. The reaction products were subjected to PAGE and further analysed as outlined in Fig. 1 (*a*).

† L $1210^{R}S_{30}$co: cell-free extract of control L 1210 R cells; L $1210^{R}S_{30}$int: cell-free extract of interferon-treated L 1210 R cells; L $1210^{S}S_{30}$co: cell-free extract of control L 1210 S cells; L $1210^{S}S_{30}$int: cell-free extract of interferon-treated L 1210 S cells.

‡ Figures given in the table are net counts: ct/min (67K) = ct/min $(67K)_{+dsRNA}$ − ct/min $(67K)_{-dsRNA}$.

§ Samples of each lysate and mixtures of varying proportions were tested under conditions of phosphorylation at a saturating amount of dsRNA (1280 ng/ml) see (Figure 1 *a*). Ten μl samples of the *in vitro* reaction were subjected to PAGE and further analysed as described in Fgire 1 (*a*).

‖ Counts (^{32}P ct/min) are not corrected for background activity.

provoked a 45% reduction of the leu-tRNA charging capacity, whereas no change was noted in the interferon-treated pre-incubated cell-free extract of L 1210 S cells, compared with its control cell-free lysate.

Phosphorylation of specific protein fractions in interferon-treated cell-free extracts due to the activation of an ATP and dsRNA dependent protein kinase has been well documented (Lebleu *et al.* 1976; Roberts *et al.* 1976; Wérenne & Rousseau, 1976; Zilberstein *et al.* 1976). It was of interest to know whether a correlation existed between the dsRNA dependent phosphorylation of a 67K dalton protein and the sensitivity of L 1210 cells to interferon.

Cell free extracts were prepared from interferon-treated and control L 1210 R and L 1210S cells. Two × 10^{7} cells were spun down, washed in buffer A (35 mM-Hepes, pH 7·5; 146 mM-NaCl; 12 mM-glucose), suspended in 0·5 ml buffer B (25 mM-Hepes, pH 7·5: 80 mM-

KCl; 4 mM-$Mg(OAc)_2$; 6 mM-2-mercaptoethanol) containing 0·4% (v/v) of Nonidet P40 (Shell Co.) and lysed during 15 min at 0 °C. The cell-free lysate was spun down (2 × 2 min) at 9600 *g* in an Eppendorf minicentrifuge and the supernatant fraction passed over a Sephadex G25 column, equilibrated in buffer B. Samples of the eluent were tested for protein phosphorylation or stored in liquid nitrogen until used.

Protein phosphorylation in crude extracts was performed as described previously (Lebleu *et al.* 1976), except that (γ-^{32}P)-ATP concentration was adjusted to 0·5 mM and 800 mCi/mmol. The dsRNA added was that of *P. chrysogenum* mycophage; incubation was for 15 min at 30 °C. Samples of the *in vitro* reaction product were subjected to PAGE described by Zilberstein *et al.* 1976, through a slab gel containing 10% (w/v) polyacrylamide for 6 h at 80 V. Radioactive fractions, corresponding to 67K dalton, were cut out of the dried gel using the autoradiogram as a guide and counted in a liquid scintillation counter.

Preliminary experiments with different concentrations of dsRNA indicated that in cell free extracts of interferon-treated L 1210 S cells, a 67K dalton phosphoprotein fraction was increased and the amount produced depended on the concentration of dsRNA added. This test was repeated with the four different cell free lysates: increasing concentrations of dsRNA (from 0 to 20480 ng/ml) were added to the reaction mixtures and the extent of phosphorylation measured (Fig. 1*a*). Page analysis indicates that in all cell-free lysates, a 67K dalton phosphoprotein is already present without addition of dsRNA, but a good response to dsRNA is only observed in the cell-free extract of interferon-treated L 1210 S cells. A maximal amount of 67K dalton phosphoprotein is formed for 2500 ng/ml of dsRNA added (Fig. 1*a*, *b*). Stimulation by dsRNA of a 67K dalton phosphoprotein in the other lysates remains low, even at high concentrations of dsRNA (Fig. 1*a*). Low levels of 67K dalton phosphoprotein formed in non-responding cell-free lysates, could be a result of enhanced phosphatase activity antagonizing the protein kinase activity. Such a mechanism was proposed by Revel *et al.* (1977) to explain the role of dsRNA, stimulating overall phosphorylation by inhibition of a phosphatase action that would otherwise degrade newly synthesized phosphoprotein. This hypothesis was tested indirectly (no phosphatase activity measurements were made) by mixing active and non active cell-free lysates in different amounts under conditions of protein phosphorylation. In one series no dsRNA was added, in a second series dsRNA concentration was 160 ng/ml, which means a non saturating level (see Fig. 1*a*). Table 1(*a*) illustrates the net amount of 67K dalton phosphoprotein formed under different conditions tested: net ct/min (67K) = ct/min $(67K)_{+dsRNA}$—$(67K)_{-dsRNA}$. The extent of 67K dalton protein phosphorylation is proportional to the amount of cell free lysate of interferon-treated L 1210 S cells present in any reaction mixture.

Addition of any other cell free lysate leads to a decrease in the level of 67K dalton phosphoprotein produced, as expected by the dilution factor only, but does not inhibit its formation. With mixtures of varying proportions, similar results are obtained.

To rule out a possible redistribution of dsRNA between the reaction components, phosphorylation reactions were performed at a saturating amount of dsRNA (1280 ng/ml) with active and non-active lysates. These confirmed the stated observations: the amount of 67K dalton phosphoprotein fraction obtained is a simple sum of separate activities present in each lysate (Table 1*b*). Consequently, phosphorylation of a 67K dalton protein fraction in non-active lysates does not seem to be stimulated by the addition of protein kinase containing cell-free lysate of interferon-treated L 1210 S cells.

In pre-incubated cell-free extracts of interferon-treated L 1210 S cells, leu-tRNA aminoacylation is unaltered and exogenous mRNA translation shows no inhibition, compared with its control extract. The latter observation agrees with the statement made in studies

with interferon-treated cell-free lysates of mouse cells (Kerr *et al.* 1974) and recently in extracts from HeLa cells treated with human fibroblast interferon (Shaila *et al.* 1977). In these cases, as in that presented here, specific biological and biochemical phenomena ascribed to interferon action are found, but translation inhibition of exogenous added mRNA in interferon-treated cell-free lysates cannot be detected. This would mean that the *in vitro* observed inhibition of mRNA translation is not directly linked to interferon *in vivo* action or, in certain cell types, is only expressed when cells are infected before interferon treatment, or requires addition of dsRNA *in vitro* (Kerr *et al.* 1974).

Alteration in the pattern of phosphorylated proteins in interferon-treated cell-free extracts is a sensitive indicator of interferon action (Roberts *et al.* 1976; Lebleu *et al.* 1976; Zilberstein *et al.* 1976; Wérenne & Rousseau, 1976). In cell-free extracts of interferon-treated L 1210 S cells, a 67K dalton phosphoprotein is enhanced and the extent of its increase depends on the amount of dsRNA added to the reaction mixture. The curve shape that is obtained with the different lysates after phosphorylation as described in Fig. 1 (*a*) and the results from the mixing experiments (Table 1 *a*) do not indicate that the cell free lysates contain different levels of phosphatase activity. Resistance to interferon treatment in L 1210 R cells, is not a consequence of an increased phosphatase content in these lysates. In any case, the reaction mechanism that leads to the formation of a 67K dalton phosphoprotein in cell-free lysates of L 1210 cells, depends on three characteristics: sensitivity to interferon, interferon treatment and addition of dsRNA. In contrast to the findings of several authors (Lebleu *et al.* 1976; Roberts *et al.* 1976; Zilberstein *et al.* 1976) we did not find other increased phosphoprotein fractions in cell-free extracts of interferon-treated L 1210 cells.

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REFERENCES

CONTENT, J., LEBLEU, B., ZILBERSTEIN, A., BERISSI, H. & REVEL, M. (1974). Mechanism of the interferon-induced block of mRNA translation in mouse L cells: reversal of the block by transfer RNA. *F.E.B.S. Letters* **41**, 125–130.

FALCOFF, E., FALCOFF, R., LEBLEU, B. & REVEL, M. (1972). Interferon treatment inhibits mengo RNA and haemoglobin mRNA translation in in cell-free extracts of L cells. *Nature New Biology* **240**, 145–147.

FRIEDMAN, R. M., METZ, D. H., ESTEBAN, R. M., TOVELL, D. R., BALL, L. A. & KERR, I. M. (1972). Mechanism of interferon action: inhibition of viral messenger ribonucleic acid translation in L cell extracts. *Journal of Virology* **10**, 1184–1189.

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GRESSER, I., BROUTY-BOYÉ, D., THOMAS, M. R. & MACIEIRA-COELHO, A. (1970). Interferon and cell division. I. Inhibition of the multiplication of mouse leukemia L 1210 cells *in vitro* by interferon preparations. *Proceedings of the National Academy of Sciences of the United States of America* **66**, 1052–1058.

GRESSER, I., BANDU, M. T. & BROUTY-BOYÉ, D. (1974). Interferon and cell division. IX. Interferon-resistant L 1210 cells: characteristics and origin. *Journal of the National Cancer Institute* **52**, 553–559.

GUPTA, S. L., GRAZIADEI, W. D. III, WEIDELI, H., SOPORI, M. L. & LENGYEL, P. (1974*a*). Selective inhibition of viral protein accumulation in interferon-treated cells; nondiscriminate inhibition of the translation of added viral and messenger RNAs in their extracts. *Virology* **57**, 49–63.

GUPTA, S. L., SOPORI, M. L. & LENGYEL, P. (1974*b*). Release of the inhibition of messenger RNA translation in extracts of interferon-treated Ehrlich ascites tumor cells. *Biochemical and Biophysical Research Communications* **57**, 763–770.

KERR, I. M., BROWN, R. E. & BALL, L. A. (1974). Increased sensitivity to double-stranded RNA of protein synthesis in cell-free systems from interferon-treated cells. *Nature, London* **250**, 57–59.

LEBLEU, B., SEN, G. L., SHAILA, S., CABRER, B. & LENGYEL, P. (1976). Interferon, double-stranded RNA and protein phosphorylation. *Proceedings of the National Academy of Sciences of the United States of America* **73**, 3107–3111.

REVEL, M., KIMCHI, A., SCHMIDT, A., ZILBERSTEIN, A., GILBOA, E., SHULMAN, L. & FEDERMAN, P. (1977). Regulation of the interferon-induced specific protein phosphorylation and translational inhibition in mouse L cells. 11th F.E.B.S. Meeting, Copenhagen, Abstracts, A2–5, L5, 7.

ROBERTS, W. K., HOVANESSIAN, A., BROWN, R. E., CLEMENS, H. J. & KERR, I. M. (1976). Interferon-mediated protein kinase and low-molecular weight inhibition of protein synthesis. *Nature, London* **264**, 477–480.

SAMUEL, C. E. & JOKLIK, W. K. (1974). A protein synthesizing system from interferon-treated cells that discriminates between cellular and viral messenger RNAs. *Virology* **58**, 471–491.

SEN, G. C., LEBLEU, B., BROWN, G. E., REBELLO, M. A., FURUICHI, Y., MORGAN, M., SHATKIN, A. J. & LENGYEL, P. (1975). Inhibition of reovirus messenger RNA methylation in extracts of interferon-treated Ehrlich ascites tumor cells. *Biochemical and Biophysical Research Communications* **65**, 427–434.

SEN, G. C., LEBLEU, B., BROWN, G. E., KAWAKITA, M., SLATTERY, E. & LENGYEL, P. (1976*a*). Interferon, double-stranded RNA and mRNA degradation. *Nature, London* **264**, 370–373.

SEN, G. C., GUPTA, S. L., BROWN, G. E., LEBLEU, B., REBELLO, M. & LENGYEL, P. (1976*b*). Interferon treatment of Ehrlich ascites tumor cells: effect on exogeneous mRNA translation and tRNA inactivation in the cell extract. *Journal of Virology* **17**, 191–213.

SHAILA, S., LEBLEU, B., BROWN, G. E., SEN, G. C. & LENGYEL, P. (1977). Characteristics of extracts from interferon-treated HeLa cells: presence of a protein kinase activated by double-stranded RNA and of an inhibitor of mRNA methylation. *Journal of General Virology* **37**, 535–546.

WÉRENNE, J. & ROUSSEAU, G. (1976). Double-stranded RNA-dependent protein phosphorylation in interferon-treated cells. *Archives Internationales de Physiologie et de Biochimie* **84**, 1123–1124.

ZILBERSTEIN, A., FEDERMAN, P., SHULMAN, L. & REVEL, M. (1976). Specific phosphorylation *in vitro* of a protein associated with ribosomes of interferon-treated mouse L cells. *F.E.B.S. Letters* **68**, 119–124.

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