Inhibition of cell proliferation by interferons

Relative contributions of changes in protein synthesis and breakdown to growth control of human lymphoblastoid cells

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Treatment of the Daudi line of human lymphoblastoid cells with concentrations of human interferons within the physiological range progressively inhibits cell proliferation over 1–4 days. Rigorous measurement of the overall rate of protein synthesis during this period, using a concentration of [³H]phenylalanine sufficient to equalize the specific radioactivity of intracellular and extracellular precursor pools, shows that protein synthesis becomes progressively inhibited as the growth inhibition develops. There is a strong correlation between inhibition of amino acid incorporation and inhibition of cell proliferation. In contrast, we find no evidence for any increase in protein degradation rate under these conditions. These results suggest that interferon treatment of susceptible cells can inhibit protein synthesis even in the absence of virus infection and that this inhibition is of a sufficient magnitude to account for the anti-proliferative effect.

INTRODUCTION

The ability of interferons to inhibit viral replication in infected cells through effects on protein synthesis has been well established (reviewed in [1] and [2]). Suppression of growth and proliferation in some cell types has also been demonstrated, in the absence of virus infection, indicating that purified interferons can act as negative growth factors [3]. However, the relationship between these effects and the rate of protein synthesis in uninfected cells is less clear. A number of reports have suggested that the overall rate of protein synthesis is not altered by treatment of cultured cells with interferons in the absence of viral infection [4–6]. On the other hand, inhibition of incorporation of labelled amino acids has been observed in some systems in which cell proliferation is inhibited [7–9]. The human lymphoblastoid Daudi cell line is extremely sensitive in exhibiting a marked suppression of growth when human interferons are added in culture [10–12]. We have investigated, using this system, the extent to which the changes in growth rate can be correlated with changes in protein synthesis. In order to do this it is necessary to quantify both processes rigorously. Such an approach also allows indirect estimates to be made of the rate of protein breakdown, and of the relative contributions of changes in synthesis versus breakdown to the inhibition of cell growth caused by interferon treatment.

The rate of incorporation of a radioactive amino acid into protein reflects both the actual rate at which protein is synthesized and the specific radioactivity of the precursor which is being incorporated. If the values of the latter are different between two populations of cells then spurious conclusions can be drawn. In an attempt to overcome any such problems we have adapted procedures developed for measurement of protein synthesis rates in perfused organs [13] and in tissues of

intact animals [14]. The method uses a relatively high concentration of low specific activity radioactive amino acid so that any differences in rates of uptake or intracellular pool sizes of this precursor between two cell populations are minimized by the flooding dose of label added to the medium. This paper reports the results of these measurements. In the accompanying paper [15] we describe an analysis of the mechanisms by which protein synthesis may be regulated in this system.

MATERIALS AND METHODS

Cell culture

Daudi cells were grown in stationary suspension culture as described previously [11,12,16]. The cells were diluted with 80% (v/v) fresh medium plus 20% (v/v) conditioned medium (i.e. medium in which cells had been grown) and maintained at $(2-10) \times 10^5$ cells/ml. Cell viability was determined by Trypan Blue exclusion. Human interferons (7.1×10^7 units/mg of protein, a mixture of α species) were kindly provided by Drs. K. H. Fantes and M. D. Johnston (Wellcome Research Laboratories, Beckenham, Kent, U.K.) and were used at 100 units/ml, except where otherwise indicated.

Measurement of rates of protein synthesis

Cells were labelled by the addition of a small volume of complete medium containing L-[4-³H]phenylalanine (Amersham International) to yield a concentration of 2.5 μ Ci and 2.5 μ mol of phenylalanine/ml. After the appropriate times of incubation the specific radioactivity of [³H]phenylalanine in the intracellular acid-soluble pool was measured by pouring 20 ml of cell suspension over crushed frozen PBS (0.14 M-NaCl/2.7 mM-KCl/6.5 mM-Na₂HPO₄/1.5 mM-KH₂PO₄), pH 7.2, and centrifuging at 500 g for 10 min. The cells were washed twice

Abbreviation used: PBS, phosphate-buffered saline.

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with cold PBS and the cell pellets stored at -20°C . Subsequently, cells were thawed by the addition of 1.8 ml of water followed immediately by 0.2 ml of 20% (w/v) HClO_4 . Centrifugation at $3000g$ for 10 min separated acid-soluble amino acids from precipitated protein. Samples of culture medium were likewise precipitated with HClO_4 at a final concentration of 2% (w/v) for analysis of extracellular amino acid specific radioactivity. Samples (2 ml) of the acid-soluble fractions were neutralized with 1 ml of saturated potassium citrate and the KClO_4 was removed by centrifugation.

The protein-containing pellets from the above acid precipitations were washed twice with cold 2% HClO_4 , resuspended in 2 ml of 0.3 M-NaOH and incubated at 37°C for 30 min. Protein was reprecipitated by the addition of 0.8 ml of 20% HClO_4 , washed twice with cold 2% HClO_4 and hydrolysed in 1 ml of 6 M-HCl for 24 h at 110°C .

The method for analysing the specific radioactivity of phenylalanine was as described previously [14]. This involved the enzymic conversion of phenylalanine to β -phenylethylamine, which was quantified fluorimetrically and analysed for radioactivity by scintillation counting.

Cellular protein content was determined by a modification of the Lowry method [17], with bovine serum albumin (fraction V) (Sigma) as a standard. The rate of protein synthesis, expressed as a fraction of the protein pool synthesized/h, was calculated from the ratio of the specific radioactivity of phenylalanine in protein to the specific radioactivity of acid-soluble phenylalanine, divided by the length of the labelling time [14].

In some experiments incorporation of L-[^{35}S]methionine, L-[1- ^{14}C]leucine or L-[4,5- ^3H]leucine (New England Nuclear) was measured by trichloroacetic acid precipitation of protein as described previously [11,12,16,18]. The concentrations and specific radioactivities of these precursors are given in the legends.

Determination of specific radioactivity of phenylalanyl-tRNA

Cells were incubated at 5×10^6 cells/ml in complete medium for 60 min, followed by incubation for 15 min with 1 mCi and $1 \mu\text{mol}$ of L-[4- ^3H]phenylalanine/ml at 37°C . The cells were then lysed with 50 mM-sodium acetate, pH 5, containing 1% SDS and extracted with 4 ml of redistilled phenol saturated with the same buffer. After centrifugation to separate the phases, RNA was precipitated from the aqueous phase by the addition of 0.6 ml of 20% potassium acetate, pH 6.8, plus 16.5 ml of cold ethanol overnight at -20°C . The RNA-containing precipitates were then washed four times by centrifugation and resuspension in cold ethanol. The analysis of the specific radioactivity of tRNA-bound phenylalanine was kindly performed by Drs. R. Low and W. Stirewalt (University of Vermont, Burlington, VT, U.S.A.) using a procedure involving dansylation of the amino acid followed by t.l.c. [19].

RESULTS AND DISCUSSION

Effect of interferon treatment on cell proliferation and the accumulation of protein

The ability of human α interferons to suppress the proliferation of Daudi cells is shown in Fig. 1. Under

Fig. 1. Effect of human interferons (100 units/ml) on the growth of Daudi cells

Daudi cells were grown in unstirred suspension culture as described in the Materials and methods section. At the times indicated quadruplicate estimates of cell number/ml were made with a haemocytometer. Control cells (●) were diluted on day 3 and the cell number on day 4 has been calculated allowing for this dilution. Interferon-treated cells (○) were not diluted further. Estimates of cell number refer to viable cells, as assessed by Trypan Blue exclusion. The proportion of non-viable interferon-treated cells was less than 10% on days 1–3 and 12% on day 4.

optimal growth conditions control cells doubled in number approximately every 24–30 h. Following treatment with interferons cell growth and proliferation were slowed within 24–36 h and declined progressively over the subsequent 2 days. After 3 days of treatment the cells ceased to proliferate altogether. Trypan Blue exclusion data (not shown here) indicated that the majority of the cells were still viable, at least up to 3 days of interferon treatment. Protein content per cell was assessed by successive sampling of both control and treated cultures over 4 days. There was no indication of any difference between control cells [$97 \pm 4 \mu\text{g}$ of protein/ 10^6 cells ($\pm$ S.E.M., $n = 18$)] and cells treated with interferons [$95 \pm 5 \mu\text{g}$ of protein/ 10^6 cells ($\pm$ S.E.M., $n = 10$)]. Because the accumulation of protein in rapidly growing cells is exponential, the data have been expressed as $\ln(\text{cell number/ml})$ or $\ln(\text{protein content/ml})$. The rate of accumulation of protein at any one time is the gradient of the curve of the latter value plotted against time, and is expressed here as a fractional rate (i.e. as a proportion of the amount of protein present in the culture at that time). Table 1 shows the rates of accumulation of protein (k_g), determined from the changes in protein content of the cultures. In the interferon-treated cultures, where the rate of accumulation was not constant, the growth rates in Table 1 are averages of sequential estimates of the rate of accumulation. It can be seen that interferon treatment lowered the value of k_g from 2.2 mg of protein/h per 100 mg of protein (a doubling time of 31.5 h) to 0.8 by 2 days, and completely prevented further growth by 3 days.

Table 1. Effects of human interferons on rates of protein accumulation, synthesis and degradation

Daudi cells were diluted to approximately 3×10^5 cells/ml to maintain exponential growth in the controls and incubated in the presence or absence of human interferons (100 units/ml) for 1–4 days. The rate of change in protein content (k_g) was assessed from the slope of the plot of $\ln(\text{protein content/ml})$ versus time. The rate of protein synthesis (k_s) was calculated from the rate of incorporation of L-[4- ^3H]phenylalanine into protein and the specific radioactivity of intracellular phenylalanine, between 15 and 90 min after addition of label. Protein degradation (k_d) was calculated as the difference between k_g and k_s (see the text for details). The results are the means $\pm$ S.E.M. for the number of determinations shown in parentheses.

Cells	Time of treatment (days)	Rate (mg of protein/h per 100 mg of protein)		
		k_g	k_s	k_d
Controls	—	2.2(6)	$3.18 \pm 0.08(26)$	1.0
Interferon-treated	1	2.0(3)	$2.46 \pm 0.08(5)$	0.5
	2	0.8(3)	$1.18 \pm 0.14(10)$	0.4
	3	−0.1(3)	$0.87 \pm 0.05(12)$	1.0
	4	−0.4(3)	$0.78 \pm 0.11(12)$	1.2

Incorporation of [^3H]phenylalanine into protein

Estimates of the rate of protein synthesis were made under conditions which were as close to the normal conditions of culture as possible, except that a concentration of [^3H]phenylalanine of 2.5 mM was used in order to flood to the same specific radioactivity all pools which might provide phenylalanine for protein synthesis. Under these conditions the ratio of the specific radioactivity of phenylalanine in the intracellular pool to that in the medium was 0.99 ± 0.02 ($n = 30$). As expected, incorporation of label into protein was linear with time (Fig. 2). The presence of a high concentration of phenylalanine in the medium did not perturb the rate of protein synthesis, as judged by the lack of effect on incorporation of other amino acids into protein (Table 2).

Measurements of the specific radioactivity of phenylalanine bound to tRNA were also undertaken because of reports that elevated concentrations of amino acids are insufficient, in some instances, to ensure that the amino acid bound to tRNA has the same specific radioactivity as the intracellular or extracellular free amino acid [20–22]. The measurement of phenylalanyl-tRNA specific radioactivity required cell densities and amounts of isotope which were higher than those routinely used (see the Materials and methods section). However, at least under these conditions, all three pools of the amino acid had essentially identical specific radioactivities in both control and interferon-treated cells (Table 3). These results suggested that an accurate measure of the specific radioactivity of the precursor for protein synthesis could be obtained from values for either the medium or the intracellular phenylalanine pool.

Comparison of rates of protein synthesis and protein accumulation

Rates of protein synthesis in control and interferon-treated cells were calculated from the specific radioactivity of phenylalanine in protein and the specific radioactivity of acid-soluble phenylalanine in washed cells and in the medium, as described in the Materials and methods section. The values (k_s) are expressed as the amount of protein synthesized/h relative to the amount of protein present in the culture (Table 1). It can be seen that

Fig. 2. Time-course of incorporation of [^3H]phenylalanine into protein

Daudi cells at approx. 2×10^5 cells/ml were incubated with 2.5 μCi and 2.5 μmol of L-[4- ^3H]phenylalanine/ml. At the times indicated samples were taken and the specific radioactivity of phenylalanine in protein was determined as described in the Materials and methods section. Each point is the mean $\pm$ S.D. for four determinations.

protein synthesis in Daudi cells is progressively affected by treatment of the cells with interferons. One day of treatment with 100 units/ml depressed synthesis by about 23%. After 2 days of treatment with this dose the rate of protein synthesis was less than half the rate in control, exponentially growing cells. Further treatment, however, resulted in relatively little additional decrease in protein synthesis. Similar results were obtained when less rigorous methods of assessing protein synthesis were

Table 2. Incorporation of labelled precursors into protein in the presence of increasing concentrations of phenylalanine

Control and 2 day interferon-treated cells were incubated for 1 h at 37 °C with either [³H]leucine (10 µCi/ml, 0.38 mM) or [³⁵S]methionine (15 µCi/ml, 0.1 mM) in complete medium containing additional unlabelled phenylalanine as indicated. Samples (0.6 ml) were diluted with 10 ml of cold PBS, centrifuged at 400 *g* for 10 min and the cell pellets were suspended in 10 ml of 5% (w/v) trichloroacetic acid. Radioactivity incorporated into protein was determined as described previously [12].

Phenylalanine added (mM)	10 ⁻³ × Incorporation (c.p.m.) of:			
	[³ H]Leucine		[³⁵ S]Methionine	
	Controls	Interferon-treated	Controls	Interferon-treated
0	4.94	1.75	20.53	6.62
0.5	4.74	1.58	23.80	8.40
1.0	4.49	1.54	21.72	7.05
1.5	4.32	1.53	22.72	8.45
2.5	4.21	1.20	22.16	8.36

Table 3. Specific radioactivity of [³H]phenylalanine in potential precursor pools for protein synthesis in control and interferon-treated Daudi cells

Control and 2 day interferon-treated cells were incubated at 5 × 10⁶ cells/ml in complete medium for 1 h at 37 °C followed by the addition of 1 µmol and 1 mCi of L-[4-³H]phenylalanine/ml for 15 min. Phenylalanyl-tRNA was prepared as described in the Materials and methods section. The specific radioactivity of phenylalanine in medium, intracellular and tRNA-bound pools was determined by dansylation of the amino acid followed by t.l.c. as described in [19].

Cells	Specific radioactivity of phenylalanine (d.p.m./pmol)		
	Medium	Intracellular	Phe-tRNA
Controls	1590	1610	1540
Interferon-treated	1580	1620	1660

employed (measurement of incorporation of labelled leucine or methionine into protein). Fig. 3 illustrates the composite results from several separate experiments in which various interferon concentrations and times of treatment were used. Taken together with the data in Table 1, these results most probably reflect a true inhibition of protein synthesis rather than decreased transport or lower specific radioactivities of the precursor amino acids in the interferon-treated cells. It can be seen that relatively high doses of interferons are required to produce a greater than 50% inhibition of incorporation, particularly at early times of treatment.

Fig. 4 presents a correlation of the extent of the inhibition of amino acid incorporation into protein with the extent of cell growth inhibition, based on several experiments employing different interferon concentrations and times of treatment (as in Fig. 3). There is a high probability of a correlation between the two parameters ($P < 0.001$, $r = 0.895$), except when the growth inhibition is small and thus difficult to measure accurately.

A comparison of the data on changes in protein synthesis with changes in the rate of protein accumulation

provides some insight into the role of overall protein degradation in control and interferon-treated cells. Since protein accumulation reflects the balance between the rates at which protein is synthesized and degraded the latter can be calculated and expressed as a fractional rate (k_d). Table 1 shows that in exponentially growing control cells the rate of degradation is low (about one-third of the rate of protein synthesis), as observed with other cells in culture [23]. Following 1–2 days of interferon treatment the rate of protein degradation apparently falls somewhat, but then increases again to the original rate as the cells cease to proliferate altogether.

These results suggest that the determining factor in the control of Daudi cell growth by interferons is the rate of protein synthesis rather than the rate of degradation. The calculation of the latter is, however, complicated by methodological difficulties. Changes in rates of protein synthesis can be detected much earlier than changes in rates of protein accumulation or degradation, since determination of the latter requires that measurements be made over long times [24,25]. Furthermore, since the rate at which protein is accumulating is not constant, the determination of accurate rates of breakdown is difficult. The values obtained must therefore be interpreted with some caution. Nevertheless, the calculated changes in protein degradation in Table 1 are quite large and the overall trend is probably correct.

Regulation of protein synthesis by interferons

Although changes in overall rates of protein synthesis have not always been observed following interferon treatment of cells not infected with viruses [4–6], direct comparisons with the present data are difficult to make. Not only are different cell lines differentially sensitive to growth inhibitory effects of interferons [8,26] but methods for measuring rates of synthesis have not always been rigorous. If cells are resuspended in medium lacking one amino acid (except for a tracer dose of the radiolabelled form) it is likely that amino acid starvation will depress synthesis in both controls and treated populations [27,28]. It would then be possible that any interferon-induced inhibition would be obscured by this further depression of translation. Even in studies where amino acids have not been omitted from the medium the concentrations present may not always have been large

Fig. 3. Inhibition of amino acid incorporation into protein as a function of interferon concentration and time of treatment

Daudi cells were cultured in the absence or presence of human interferons at the concentrations indicated, as described in the Materials and methods section. At daily intervals the cells were counted and small aliquots were removed for determination of incorporation of a labelled amino acid into protein. The precursors employed in the experiments from which this composite figure has been prepared were: [35 S]methionine (10 μ Ci/ml, 0.1 mM); [14 C]leucine (2 μ Ci/ml, 0.38 mM); [3 H]phenylalanine (8 μ Ci/ml, 0.09 mM). In order to normalize the different values for incorporation of the various precursors the data are expressed as a percentage rate of incorporation relative to control cells on day 1 of each experiment (when the untreated cells were growing exponentially).

enough to ensure that the precursor pool for protein synthesis has the same specific radioactivity in both control and treated cells, a condition which is essential if conclusions about protein synthesis are to be made from rates of incorporation of radioactivity into protein. In our experiments an additional problem was revealed during the measurement of the specific radioactivity of phenylalanyl-tRNA. We found that when cells were centrifuged and resuspended at high densities (greater than 10^6 cells/ml), rates of amino acid incorporation were initially only 20% of normal. The ability of the cells to incorporate label recovered with time so that by 2 h it was about 80% of normal. Moreover, when the rate of incorporation was low, the difference between control

Fig. 4. Relationship between inhibition of amino acid incorporation and inhibition of cell growth rate in interferon-treated cells

The data are derived from a series of experiments similar to those described in Fig. 3, employing various interferon concentrations and times of treatment. The percentage inhibition of amino acid incorporation, relative to control cells on day 1 of each experiment, was determined as described in Fig. 3. In the same cell populations the cell growth rate was calculated by measuring the number of cell doublings in the 24 h preceding and succeeding the time of protein synthesis measurement and averaging these two values. The percentage inhibition of growth was then determined by relating this value to the number of cell doublings per 24 h in exponentially growing control cells. The parameters derived from a regression analysis of the data, and represented by the straight line, are shown on the Figure. The broken line indicates the uncertainty concerning the relationship between inhibition of protein synthesis and cell growth at the lower end of the plot.

and interferon-treated cells was no longer observed. This difference gradually returned as the rates of incorporation were restored to higher levels. The methods used for measuring protein synthesis are therefore crucial to the interpretation of data on the ability of interferons to affect translation in uninfected cells.

The results reported in this paper are reminiscent of the effects of a number of other conditions which regulate cell growth, such as serum or growth factor deprivation [23,29-31], amino acid starvation [27,28,32,33] and growth of cells to confluence [34]. In all

of these cases protein synthesis is closely controlled, largely through mechanisms operating at the level of polypeptide chain initiation. Changes in protein degradation are less consistent since both decreased rates [23] and increases rates [30,35,36] have been observed in other systems where the growth of cells in culture is inhibited. It is clear that regulation of the rate of protein synthesis is a common mode of controlling the rate at which cells can grow and proliferate. In the case of interferon-induced growth inhibition the molecular basis of this regulation has not previously been analysed. In the accompanying paper [15] we present our results of such an analysis.

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