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THE EFFECTS OF SOME DRUGS WHICH CAUSE AGRANULOCYTOSIS ON PROTEIN SYNTHESIS IN HUMAN GRANULOCYTES

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Abstract—The rate of protein synthesis by human granulocytes *in vitro* has been measured by the incorporation of [U-^{14}C]phenylalanine into the cell protein. The total phenylalanine concentration in the cells was measured in each experiment so that changes in the precursor pool specific radioactivity during the experiment could be allowed for. Cells were obtained from normal subjects, patients who had recovered from an episode of idiosyncratic drug-induced neutropenia, and from a case of idiopathic neutropenia. The effects of the following drugs which sometimes cause idiosyncratic neutropenia, on the rate of protein synthesis in human granulocytes *in vitro* has been studied: sodium aurothiomalate, phenylbutazone, carbimazole, methimazole, methylthiouracil, chlorpromazine, chloramphenicol, sulphamethoxazole and trimethoprim. The last two drugs were studied separately, and in combination in the proportions in which they are found in plasma during co-trimoxazole therapy. When used at the concentrations which are believed to represent the maximum *in vivo* concentrations in serum, none of the following affected the protein synthetic activity of normal human granulocytes *in vitro*: sodium aurothiomalate, phenylbutazone, methimazole, methylthiouracil, sulphamethoxazole and trimethoprim (the latter pair either separately or together as in co-trimoxazole). Chlorpromazine (1×10^{-4} M) markedly inhibited protein synthesis by the cells of one normal subject out of the 10 who were studied. Higher concentrations (1×10^{-3} M) of chlorpromazine virtually abolished protein synthesis in the cells of the two normal subjects studied. Chloramphenicol reduced the protein synthetic activity of normal human granulocytes. We obtained no evidence that protein synthesis by the granulocytes from patients who had experienced neutropenia due to sodium aurothiomalate (4 cases), chloramphenicol (2 cases), carbimazole (1 case), co-trimoxazole (1 case), methylthiouracil (1 case) were abnormally sensitive to inhibition by the drug which had caused the neutropenia.

A number of drugs in common use may cause agranulocytosis, severe neutropenia and other forms of bone marrow depression. The idiosyncratic nature of some of these reactions, excluding those thought to be due to immune mechanisms, suggests that the fundamental abnormality which causes them may reside in the patient's haemopoeitic tissue. Such an abnormality might be expressed in the end cells of the system, the neutrophil granulocytes.

Protein synthesis is inhibited in certain mammalian systems by antibiotics such as puromycin. Chloramphenicol, a drug which is frequently associated with reversible marrow depression, has been shown to inhibit protein synthesis by mammalian mitochondria [1–3]. However, this does not explain the fatal aplastic anaemia which more rarely complicates chloramphenicol treatment.

The present work was undertaken to explore the possibility that potentially myelotoxic drugs might affect protein synthesis directly, and that protein synthesis in granulocytes from patients with a history of drug-induced neutropenia might be peculiarly sensitive to inhibition by the relevant drug. Peripheral blood granulocytes are capable of synthesising protein at a measurable rate which, if modified by drugs *in vitro*, may reflect a sensitivity of myeloid precursors to those drugs *in vivo*.

The effects of sodium aurothiomalate, trimethoprim, sulphamethoxazole, co-trimoxazole (a formulation of trimethoprim with sulphamethoxazole), chlorpromazine, methylthiouracil, methimazole, phenylbutazone and chloramphenicol, on protein synthesis *in vitro* were examined using peripheral blood granulocytes from normal individuals, and where possible, from patients known to be sensitive to the relevant drug. Idiosyncratic drug-induced neutropenia is however, a relatively rare occurrence so that, for certain of the drugs, patients with a history of myelotoxic reactions were not available for study.

MATERIALS AND METHODS

Analytical grade reagents were used throughout and solutions were prepared from glass-distilled water. Cycloheximide was purchased from Koch–Light Laboratories; NCS tissue solubiliser from Nuclear Chicago Corp. (G. D. Searle & Co., High Wycombe, Bucks.); [U^{14}C]-L-3-phenylalanine (513 mCi/m-mole) from the Radiochemical Centre, Amersham, Bucks. The composition of the isotonic buffer used for the granulocyte incubations was: Tris–HCl (20 mM, pH 7·4 at 37°), KCl (4·5 mM), $MgCl_2$ (0·8 mM), NaCl (125 mM), Na_2HPO_4 (1 mM), D-glucose (5·5 mM). Granulocytes were separated from human blood as described previously [4], and phenylalanine was determined spectrophotofluorimetrically [5].

Study of the incorporation of [*U-*^{14}*C*]*phenylalanine into human granulocyte total protein* in vitro. Samples (100 μl) of granulocyte suspension (1×10^7 cells/ml in

isotonic buffer) were preincubated at 37° for 30 min in the presence and absence of the drug dissolved in isotonic buffer (0·45 ml). The reaction was started by adding [U-^{14}C]-L-3-phenylalanine [50 μl, (2·0 μCi/ml; 513 mCi/m-mole)] and continued at 37° for 30 min. The reaction was stopped by exposing the cells to ultrasonic vibrations (8 μm amplitude and 20 kHz) for 30 sec, and the immediate addition of trichloroacetic acid [5 ml, 5% (w/v) solution (TCA)]. The resulting precipitates were washed once with TCA (cold) once with TCA (75–80°), twice with ethanol–ether [3:1 (v/v)] dried *in vacuo* over P_2O_5 and the radioactivity measured. Blank values were obtained by adding cycloheximide (final concn 1×10^{-2} M) to the incubation mixture, measuring the radioactivity of the protein precipitate and subtracting this value from the sample value.

Study of the changes in the human granulocyte phenylalanine specific activity during incubation in vitro *with [U-^{14}C]phenylalanine.* The intracellular phenylalanine content of granulocytes could not be measured in 1×10^6 cells, so whenever experiments were carried out to measure the intracellular phenylalanine specific radioactivity as well as the incorporation of [U-^{14}C]phenylalanine into the trichloroacetic acid insoluble material, parallel incubations were performed using 5×10^6 cells and increasing the volumes of reagents proportionately. Samples (0·5 ml) of the granulocyte suspension (1×10^7 cells/ml in isotonic buffer) were preincubated for 30 min at 37° in the presence and absence of drugs dissolved in 2·25 ml of isotonic buffer. The reaction was started by adding [U-^{14}C]-L-3-phenylalanine [250 μl (2 μCi/ml, 513 mCi/m-mole)] the samples were incubated for 30 min at 37° and the reaction stopped by cooling in ice. It had previously been established that the transmembrane flux of amino acid is reduced to a negligible level when leukocytes are cooled to 4° [6]. The samples were centrifuged at 75 g_{av} for 5 min at 4° and the supernatants discarded. The pellets of cells were resuspended in isotonic buffer (1 ml) and centrifuged at 75 g_{av} for 5 min at 4°, the supernatants again discarded and the pellets of cells dried *in vacuo* over P_2O_5. Virtually all of the extracellular [^{14}C]phenylalanine was removed by this single wash. Distilled water (100 μl) was added to the dessicated cell preparations which were allowed to stand in ice for 10–15 min. The cells were then disrupted by ultrasonic vibrations (6 μm, 20 kHz) for 30 sec while the sample was cooled in ice. Trichloroacetic acid (100 μl of 0·6 M) was added to each sample and the precipitate allowed to form for 15 min before the samples were centrifuged at 1000 g_{av} for 10 min at 4°. Aliquots (20 μl) of the supernatant were either assayed for phenylalanine or added to glass counting vials and dried *in vacuo* over P_2O_5, and the radioactivity measured. The radioactivity (dis/min per 20 μl) of the trichloroacetic acid soluble material divided by the phenylalanine content (nmoles/20 μl), gives the specific radioactivity of intracellular free phenylalanine. The average intracellular specific radioactivity during the 30-min incubation was calculated by multiplying this value by the factor 0·82, derived as follows:

The specific radioactivity of intracellular free phenylalanine was measured in duplicate at various periods of incubation up to 60 min, as described above. The results of three separate experiments showed a large increase in the specific radioactivity of intracellular free phenylalanine with time during the first 30 min of incubation. After this initial rise, the specific activity levelled off to a very slow rate of increase. The shape of the plot of intracellular phenylalanine specific radioactivity against time is a rectangular hyperbola which is described by the equation $s = S \times t/(K + t)$ where s = intracellular phenylalanine specific radioactivity, S = maximum intracellular specific radioactivity, t = time, and K = a constant. This equation can be transformed into the linear form: $1/s = 1/S + K/St$ and the experimental data fitted to the equation by the method of least squares. The values of S and K calculated by this method were: $S = 2940$ dis/min per nmole and $K = 3{\cdot}73$. The average intracellular specific radioactivity in the first 30 min of incubation can then be calculated by integrating the first equation between the limits of 0 and 30. This results in the value of 2135 dis/min per nmole which equals 82 per cent of the phenylalanine specific radioactivity at 30 min.

The calculations of protein synthetic rates were made as follows: If x = radioactivity in precipitate of sample (dis/min) y = radioactivity in precipitate of cycloheximide blank (dis/min) and a = [^{14}C]phenylalanine incorporation into protein (dis/min), then $a = x - y$. If b = specific radioactivity of intracellular free phenylalanine measured after 30-min incubation (dis/min per nmole) and c = average specific radioactivity of intracellular free phenylalanine throughout the 30-min incubation (dis/min per nmole), then $c = b \times 0{\cdot}82$. If d = phenylalanine incorporation into protein (nmoles) then $d = a/c$.

Measurement of radioactivity. Samples, dissolved in NCS tissue solubiliser solution (0·5 ml, 0·3 M in toluene), were mixed with 10 ml of scintillator solution [0·05 g/l. *p-bis*(2-[5-phenyloxazolyl]-benzene) and 4 g/l. 2,5-diphenyloxazole in toluene] in glass counting vials, and the radioactivity measured using a Nuclear Chicago Mark 1 liquid scintillation spectrometer (G. D. Searle & Co., High Wycombe, Bucks. cooled to 4°. The efficiency of counting was 85 per cent.

RESULTS

Rates of protein synthesis by isolated granulocytes from normal subjects, patients who have experienced an episode of drug induced neutropenia, and from one case of idiopathic neutropenia are shown in Table 1.

The effect of the drugs on protein synthesis in isolated human granulocytes are summarised in Table 2. Only chloramphenicol consistently inhibited protein synthesis in this system, and the effect was only marked at concentrations which are considerably greater than the plasma concentrations during therapy (Fig. 1). This effect was no more marked in granulocytes from two patients who had recovered from chloramphenicol induced granulocytopenia than in the cells from the eight control subjects.

Chlorpromazine inhibited protein synthesis in granulocytes from one of the ten control subjects studied, to about 34 per cent of the control value when the concentration of the drug was 1×10^{-4} M. In a separate experiment, in which granulocytes from two of the control subjects were studied, 5×10^{-4} M and 1×10^{-3} M chlorpromazine reduced protein synthesis to 9 and 11 per cent of the control values respectively in one subject's cells and to 2 and 3 per cent of the control

Table 1. Protein synthesis by isolated granulocytes from normal subjects, patients with a history of drug induced neutropenia and one case of idiopathic neutropenia

Subject	Interval between episode and test	Protein synthetic rate (pmoles/30 min/1 × 10^6 cells)
Normals	—	Mean = 580 (49 subjects) 95% confidence limits 85–1996.
Drug-induced neutropenia		
Co-trimoxazole	3 months	225
Carbimazole	6 months	333
*Chloramphenicol	9 months	1222
*Chloramphenicol	11 yr	1110
Methylthiouracil	7 yr	584
†Sodium aurothiomalate	1 yr	255
†Sodium aurothiomalate	13 days	290
†Sodium aurothiomalate	37 days	606
†Sodium aurothiomalate	9 days	812
Idiopathic neutropenia	—	532

* Two separate cases.
† Four separate cases.

in the other. It was also shown that 1×10^{-4} M chlorpromazine markedly reduced the viability of the cells as judged by the trypan blue dye exclusion test. In this test, duplicate cell suspensions were incubated for 60 min at 37°, both in the presence and in the absence of the drug and the cell viability was then assessed with the results shown in Table 3.

Protein synthesis in the granulocytes of patients who had recovered from idiosyncratic neutropenia was not abnormally sensitive to inhibition by the relevant drugs (Table 2). Methimazole (1×10^{-3} M) appeared to increase the rate of protein synthesis in the cells although lower concentrations had no effect.

DISCUSSION

The suggestion that drugs which cause neutropenia do so by interfering with macromolecule synthesis was explored in the case of the pyrimidine precursors of the nucleic acids by Westwick and her colleagues [13–15]. These workers obtained evidence that certain specific steps in the pathway for *de novo* synthesis of pyrimidines are potentially susceptible to particular drugs. They subsequently identified two patients in whom an episode of sodium aurothiomalate-induced granulocytopenia was associated with reduced activity of the enzymes which were specifically susceptible to inhibition by this drug [16]. The present studies were differently designed in that we have examined overall protein synthesis and have not considered the individual biochemical reactions separately. We have aimed wherever possible to examine the effects of drugs at concentrations *in vitro* which approximate to the best available estimates of the plasma concentrations during therapy. Since plasma levels may be unrelated to the local concentration of a drug at the active site of an enzyme, or in a critical cell organelle, we have also studied the effect of higher drug concentrations.

Measurements of the rates of incorporation of radioactively labelled precursor compounds into isolated cells and tissues are widely and uncritically used as measures of synthesis rates, no attention being paid to the fact that the specific radioactivity of the precursor pool will determine the extent of labelling observed. Experimental procedures and addition of drugs may alter precursor pool specific activity and affect the total radioactivity incorporated into protein. We therefore measured the specific radioactivity of the intracellular phenylalanine pool, and were able to correct for changes in precursor pool specific radioactivity. Evidence that the precursor pool for protein synthesis in isolated granulocytes is in fact the total intracellular free phenylalanine pool has been presented elsewhere [17]. The value of this approach is illustrated by the results using chlorpromazine at therapeutic plasma concentrations. Large changes in the intracellular specific radioactivity accounted for the observed changes in [U-^{14}C]phenylalanine incorporation in all except one control subject, whose ability to synthesis protein appeared to be abnormally sensitive to inhibition by this drug.

The observation that approximately therapeutic concentrations of chlorpromazine altered the specific radioactivity of intracellular phenylalanine without affecting the rate of protein synthesis suggests that at these concentrations chlorpromazine may damage the plasma membrane and alter its permeability to phenylalanine. This conclusion is supported by the trypan blue dye exclusion test (Table 3) which monitors the integrity of plasma membranes. Garry and Uyeki [18] found that the viability of spleen cells, indicated by dye exclusion, was reduced by incubation with chlorpromazine. Other workers have shown that low concentrations of chlorpromazine stabilise, and higher concentrations labilise, cell membranes [19]. Pisciotta and Santos [20] concluded that chlorpromazine specifically inhibited nucleic acid biosynthesis, but they measured the effects of 2×10^{-4} M chlorpromazine on the incorporation of [^{3}H]thymidine into human bone marrow cells *in vitro* without monitoring the effect on the intracellular thymidine pool. It is clear from our results that in those experiments chlorpromazine may well have led to non-specific alterations in plasma membrane permeability to thymidine rather than direct inhibition of nucleic acid synthesis.

In our system, higher concentrations of chlorpromazine (5×10^{-4} M and above) inhibited protein synthesis as well as damaging cell membranes. This accords with the observations of Goertz, Emmerich

Table 2. Effects of drugs on protein synthesis *in vitro* by granulocytes from control subjects and patients with a history of drug-induced neutropenia

Drug	No. of subjects		Concentration of drug			Effect on rate of protein synthesis	
			Therapeutic in plasma (M)	Used *in vitro*			
	Controls	Patients		Controls (M)	Patients (M)	Controls	Patients
Sodium aurothiomalate	15	4	$1–2 \times 10^{-5}$ (1)	1×10^{-5}	1×10^{-5}	None	None
	2	0		1×10^{-3}		None	—
Trimethoprim	12	1(2)	1×10^{-5} (3)	1×10^{-4}	1×10^{-4}	None	None
	2	0		5×10^{-4}		Reduced	—
Sulphamethoxazole	12	1(2)	4×10^{-4} (4)	1×10^{-4}	1×10^{-4}	None	None
	2	0		1×10^{-3}		None	—
Co-trimoxazole (Sulphamethoxazole) (5) (Trimethoprim)	2	0	$\{(2 \times 10^{-4}$ (3) $(1 \times 10^{-5}$ (3)	1×10^{-3} 5×10^{-5}	— —	None	—
Methylthiouracil	15	1	(6)	1×10^{-4}	1×10^{-4}	None	None
	2	0		1×10^{-3}	—	None	—
Methimazole	11	1(7)	(6)	1×10^{-4}	1×10^{-4}	None	None
	2	0		1×10^{-3}	—	Increased	—
Phenylbutazone	10	0	$2–4 \times 10^{-4}$ (8)	3×10^{-4}	—	None	—
	2	0		1×10^{-3}	—	None	—
Chlorpromazine	9	0		1×10^{-4}	—	None	—
	1	0	1×10^{5} (9)	1×10^{-4}	—	Reduced	—
	2	0		1×10^{-3}	—	Reduced	—
Carbimazole	0	1(7)	(6)	—	1×10^{-4}	—	None
Chloramphenicol	8	2	$0·6–1·2 \times 10^{-4}$ (10)	1×10^{-4}	1×10^{-4}	Slightly reduced	Reduced to same extent as controls.
				1×10^{-2}	1×10^{-2}	Reduced	Reduced to same extent as controls.

(1) Osol, Pratt and Altschule [7].
(2) This patient had experienced neutropenia in association with co-trimoxazole therapy.
(3) Concentration achieved during co-trimoxazole therapy [8].
(4) Concentration achieved when sulphamethoxazole is used alone [9].
(5) Co-trimoxazole is a formulation of trimethoprim with sulphamethoxazole.
(6) Data not available.
(7) This patient had experienced neutropenia in association with carbimazole therapy.
(8) Clark [10].
(9) Concentration achieved after an oral dose of 3·5 mg/kg per day [11], higher doses than this are commonly used therapeutically.
(10) Weinstein [12].

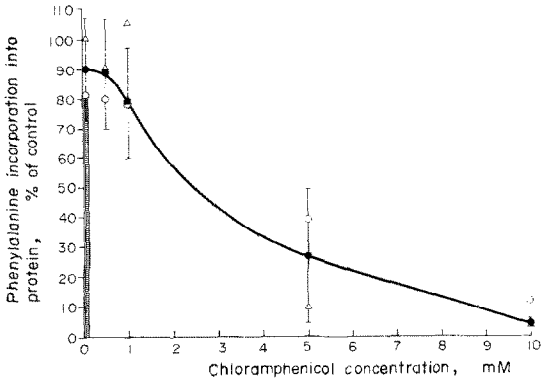

Fig. 1. The effect of increasing concentrations of chloramphenicol on the incorporation of phenylalanine into protein. Key—●: Mean of eight normal subjects ± 1 standard deviation. ○, △: Patients who had recovered from chloramphenicol-induced aplastic anaemia. Hatched areas represent range of plasma levels of chloramphenicol during therapy.

Table 3. The effect of chlorpromazine on the viability of granulocytes *in vitro*

Experiment No.	Viable cells (%) Control	Chlorpromazine (1×10^{-4} M)
1	95	67
2	90	62
3	94	81
4	92	86
5	89	87
6	84	74
7	96	88

(Details in text.)

and Kersten [21] who reported that 1×10^{-3} M chlorpromazine inhibited the aminoacylation of tRNA in subcellular preparations from rat brain and liver.

Previous workers have shown that chloramphenicol inhibits amino acid incorporation into protein by intact mammalian cells. A concentration of 1×10^{-3} M chloramphenicol inhibited protein synthesis in rabbit reticulocytes by 20 per cent [22] and by 13 per cent [23], and in rat adrenal gland by 40 per cent [24]. The inhibition of protein synthesis in human granulocytes by chloramphenicol could be an indirect effect since high concentrations are known to inhibit leukocyte respiration [25]. However, when 3×10^{-3} M chloramphenicol caused 76 per cent inhibition of protein synthesis in rabbit reticulocytes, the ATP content of the cells was decreased by only 15 per cent [22], suggesting that in this situation, at least, inhibition of respiration did not account for the whole of the effect on protein synthesis.

Although only bacterial ribosomes and mitochondrial ribosomes of eukaryotic cells are sensitive to inhibition by low concentrations of chloramphenicol, the activity of cytoplasmic ribosomes isolated from mammalian cells can be inhibited by high concentrations of the drug. At 1×10^{-3} M, chloramphenicol inhibited the protein synthetic activity of rabbit reticulocyte ribosomes by 40 per cent [26] and 10 per cent [27] suggesting that the observed inhibition of protein synthesis in human granulocytes by chloramphenicol could be due to a direct effect on cytoplasmic ribosomes. Attempts to isolate ribosomes from granulocytes have been frustrated by the high level of ribonuclease activity in these cells [28] and the lack of a sufficiently potent ribonuclease inhibitor.

It has been suggested [29] that the reversible depression of erythropoeisis, observed in most patients given large enough doses of chloramphenicol, is due to inhibition of protein synthesis by mitochondrial ribosomes, since chloramphenicol (1×10^{-4} M) has been shown to inhibit the incorporation of amino acid into protein by mammalian mitochondria [1–3]. Mitochondrial protein synthesis is not inhibited by cycloheximide even at high concentrations [30], therefore [U-^{14}C]phenylalanine incorporation into protein by mitochondria could contribute to the protein-bound radioactivity of the cycloheximide blank used in the present studies. If this contribution were significant the blank value would be reduced by chloramphenicol. It is of interest that this did not occur in our system.

The protein synthetic activity of granulocytes from patients who had recovered from an episode of drug-induced neutropenia was not abnormally sensitive to inhibition by the relevant drug. This might reflect the loss of abnormally sensitive cells during the period of marrow depression, followed by repopulation with clones genetically more resistant to drugs. An alternative explanation would be that myeloid precursor cells are sensitive to the action of myelotoxic drugs only at certain stages of the cell cycle, and that the cells which repopulate the marrow and refurbish the circulating granulocyte pool are derived from stem cells which were quiescent when the toxic injury occurred. The production *in vivo* of abnormal and toxic drug metabolites by sensitive individuals remains a further possibility not tested by studies *in vitro*.

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