Effects of mitochondrial inhibition by chloramphenicol on the mitotic cycle of human cell cultures

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Summary. Chloramphenicol, at concentrations of 500 $\mu\text{g/ml}$, had a marked inhibitory effect on mitoses in human fibroblasts. Metaphase and G2 appeared to be particularly sensitive to the action of the drug. With concentrations of 1000 $\mu\text{g/ml}$, or with 500 $\mu\text{g/ml}$ after prolonged exposure, the cells did not reach G2 and no mitoses were seen. The drug required several hours to produce its effect. Studies of oxygen uptake showed no immediate reduction in the respiratory activity of cells at concentrations of 500 $\mu\text{g/ml}$ or 1000 $\mu\text{g/ml}$ but cells which had been in contact with 500 $\mu\text{g/ml}$ of drug for 24 hours showed a marked reduction in respiratory activity. Since the primary effect of chloramphenicol is arrest of mitochondrial protein synthesis, it is concluded that continued synthesis of mitochondrial proteins in the maintenance of the respiratory chain are prerequisites for normal cell division in fibroblasts.

Chloramphenicol is a causative agent of bone marrow dysfunction, which in severe cases leads to aplastic anaemia (Pisciotta, 1971). At the cellular level, the primary effect of the drug is to arrest the synthesis of mitochondrial protein by reacting with the mitochondrial ribosome (Grivell, Reijnders, and de Vries, 1971). At high concentrations it has been claimed that chloramphenicol may also inhibit oxidative phosphorylation and electron transport. Blockage of mitochondrial protein synthesis inhibits the formation of active cytochrome oxidase (Kroon and Jansen, 1968) and it is thought that as cells divide there will be a progressive loss of functional mitochondria, which in turn will affect ATP dependent processes, including cell maturation (*cf.* Kroon and Arendzen, 1972). The question arises as to the rate of loss of functional mitochondria when replacement of the respiratory chain is precluded and attempts have been made to assess this in animals (Kroon, Gijzel, and Nijhoff, 1972).

In the experiments reported here, the effects of mitochondrial inhibition by chloramphenicol in cultures of human fibroblasts have been studied throughout the cell cycle with respect to nuclear DNA synthesis and mitosis. The results have been compared with those obtained with chlorimipramine, a direct inhibitor of mitochondrial function

(Wilkie and Delhanty, 1970; Mittwoch and Wilkie, 1971).

Materials and methods

Cell culture. Diploid human fibroblasts were derived from skin biopsies from four normal adults (two males and two females) using standard techniques (Harnden, 1960) with modifications made by J. D. A. Delhanty in 1974 (personal communication). In this variation the minced biopsy fragments are immobilized on the bottom of a Leighton tube under pressure of a coverslip. The cells were grown in Eagle's minimum essential medium supplemented with hepes buffer, glutamine, and antibiotics (penicillin and streptomycin). Biopsy specimens were set up in this medium plus 20% fetal calf serum; in subsequent passages 10% fetal calf serum was used. Cells used in experiments had undergone 21 passages (experiment 1), six passages (experiment 2), 11 passages (experiment 3), 12 passages (experiment 4), and 11–12 passages (oxygen uptake).

Experimental procedures. For experiments 1–4 confluent cultures were trypsinized (0.25% trypsin) and plated at concentrations of 10^5 cells/ml of medium into 30 mm Nunclon Petri dishes containing one coverslip each. Cells for experiments 1–3 were plated into medium containing 10% fetal serum, while for experiment 4, 20% human serum was used. Chloramphenicol and chlorimipramine were dissolved in the appropriate medium before treatment of cells, and this was added by itself to control cells.

Cells were harvested by placing coverslips in 95%

methanol, staining by the Feulgen reaction, and mounting in DPX. Mitoses were scored using an oil immersion objective. Partial synchronization following trypsinization resulted in a peak of mitoses approximately 38 hours after plating into 10% fetal calf serum and 32–34 hours after plating into 20% human serum.

Experiments were carried out as follows.

Experiment 1. Chloramphenicol was added 21 hours after plating at concentrations of 10, 100, and 1000 $\mu\text{g/ml}$, respectively (see Tables I and II).

Experiment 2. 500 $\mu\text{g/ml}$ of chloramphenicol was added at the time of plating, 4 hours after plating, and 19 hours after plating, respectively (see Tables III and IV).

Experiment 3. 500 $\mu\text{g/ml}$ of chloramphenicol was added 26 hours after plating (see Tables V and VI).

Experiment 4. Chlorimipramine was added 17 hours after plating at concentrations of 5, 10, and 15 $\mu\text{g/ml}$, respectively (see Tables VII and VIII).

Densitometry. DNA values of Feulgen-stained interphase nuclei were measured with a Barr and Stroud integrating microdensitometer. The readings were converted into logarithms to the base 2 and delineated into the three major DNA classes—2C, 4C, and intermediate—as described previously (Mittwoch and Wilkie, 1971). Nuclei with the 2C amount of DNA were classed as G₁, those with twice this amount as G₂, while nuclei with intermediate values were regarded to be in the S phase of DNA synthesis. A small proportion of cells had DNA values greater than 4C and these must have been polyploid.

Oxygen uptake. Oxygen uptake was measured polarographically in a Clarke type oxygen electrode. At

least 6×10^6 cells in log phase (subcultured 2 days previously in 10% human serum in Eagle's MEM) were trypsinized in dilute (0.025%) trypsin in Hank's balanced salt solution. The cells were derived from two different skin biopsies and had undergone 11–12 passages. In the experiment involving a 24-hour pretreatment with chloramphenicol the drug was included in both trypsin and Hank's solutions. Cells were introduced into the electrode well which contained 1 ml Hank's solution. Chloramphenicol was added in aqueous solution. To arrest respiratory activity, 0.01 ml of 0.1% potassium cyanide solution was added.

Results

DNA synthesis and mitosis. The effect of the drug is clearly dependent both on concentration and on the length of time during which the cells are exposed to it. It will be seen from Table I that 500 $\mu\text{g/ml}$ of chloramphenicol after 15 hours' exposure caused a marked depression in the number of mitoses; in addition, the proportion of metaphases relative to other mitotic stages was significantly increased. The data suggest that an increase in metaphase index occurred also with lower doses of the drug. The data further suggest that the relative increase in metaphases is due to a shortage of pro-phases as well as one of ana- and telophases. The shortage of pro-phases in cultures treated with 500 $\mu\text{g/ml}$ of drug is reflected in a high proportion of interphase cells in the G₂ phase of the mitotic cycle (Table II). It seems, therefore, that at this concentration, chloramphenicol arrests cells in G₂ after the completion of DNA synthesis; while those

TABLE I
NUMBERS OF MITOSES IN CELLS TREATED WITH DIFFERENT CONCENTRATIONS OF CHLORAMPHENICOL
(drug added 21 hours after plating)

Concentration of Drug ($\mu\text{g/ml}$)	Duration of Culture* (hr)	Time in Contact with Drug (hr)	No. of Cells Counted	No. of Cells in				Mitotic Index† (%)	Metaphase Index‡ (%)
				Pro-phase	Meta-phase	Ana-phase	Telo-phase		
0	36	—	2000	11	25	5	16	2.85	
0	38	—	2000	12	13	3	17	2.25	
			Total 4000	23	38	8	33		0.37
10	36	15	2000	19	31	4	21	3.75	
10	38	17	2000	8	13	3	7	1.55	
10	40	19	2000	8	14	3	5	1.50	
			Total 6000	35	58	10	33		0.43
100	36	15	2000	5	40	2	18	3.25	
100	38	17	2000	9	21	6	13	2.45	
100	40	19	2000	7	15	1	9	1.60	
			Total 6000	21	76	9	40		0.52
500	36	15	2000	4	1	0	0	0.25	
500	38	17	6000	2	24	3	4	0.55	
500	40	19	2000	2	2	0	1	0.25	
			Total 10,000	8	27	3	5		0.63
1000	36	15	2000	0	0	0	0		
1000	38	17	2000	0	0	0	0		
1000	40	19	2000	0	0	0	0		
			Total 6000	0	0	0	0	0.00	

* Time between plating and harvesting cells.

† Proportion of mitoses among total number of cells.

‡ Proportion of metaphases among total number of mitoses.

TABLE II
STAGES IN MITOTIC CYCLE IN INTERPHASE CELLS TREATED WITH DIFFERENT
CONCENTRATIONS OF CHLORAMPHENICOL

Concentration of Drug ($\mu\text{g/ml}$)	Duration of Culture* (hr)	Time in Contact with Drug (hr)	No. of Cells† in				G2/Total
			G1	S	G2	Polyploidy	
0	15	36	72	12	16	0	0.16
0	17	38					
10	15	36	105	14	29	2	0.19
10	17	38					
10	19	40					
100	15	36	113	8	29	0	0.19
100	17	38					
100	19	40					
500	15	36	72	12	65	1	0.43
500	17	38					
500	19	40					
1000	15	36	89	41	19	1	0.13
1000	17	38					
1000	19	40					

* Time between plating and harvesting cells.

† Fifty cells were measured from each culture; G1 corresponds to 2C, G2 to 4C, S to intermediate values.

cells which enter mitosis may be further arrested in metaphase.

At the still higher dose of 1000 $\mu\text{g/ml}$, DNA synthesis had not been completed (Table II) and there were no mitoses (Table I) at the end of the experiment.

When cells were plated in the presence of 500 $\mu\text{g/ml}$ of chloramphenicol, they became attached to the cover slips but there were no mitoses (Table III) nor was there any evidence of DNA synthesis (Table IV). The same result was obtained when the drug was added 4 hours after plating. When

the drug was added 21 hours after plating, the results were similar to those shown in Tables I and II, ie, there was a low mitotic index with arrest of interphase cells in G2. Although in Table III the increase in metaphase index is insignificant, this may be due to the small numbers of mitoses in this experiment.

The results of adding chloramphenicol to cells only a few hours before mitosis are shown in Tables V and VI. A depression of mitotic index is not evident until 7 hours after the drug was added when the number of prophase began to fall relative

TABLE III
NUMBER OF MITOSES IN CELLS TREATED WITH 500 $\mu\text{g/ml}$ CHLORAMPHENICOL
(drug added at different times after plating)

Duration of Culture* (hr)	Time in Contact with Drug (hr)	No. of Cells Counted	No. of Cells in				Mitotic Index† (%)	Metaphase Index‡ (%)
			Pro- phase	Meta- phase	Ana- phase	Telo- phase		
4	0	1000	0	0	1	0	0.1	0.40
19	0	1000	0	0	0	0	0.0	
36	0	1000	4	3	1	0	0.8	
38	0	1000	3	8	3	6	2.0	
40	0	1000	6	8	2	2	1.8	
		Total 5000	13	19	7	8		
4	4	Total 5000	0	0	0	0	0.0	
19	19							
36	36							
38	38							
40	40							
19	15	Total 4000	0	0	0	0	0.0	
36	32							
38	34							
40	36							
36	17	1000	0	2	1	0	0.3	0.47
38	19	5000	10	13	1	4	0.56	
40	21	1000	2	1	0	0	0.3	
		Total 7000	12	16	2	4		

* Time between plating and harvesting cells.

† Proportion of mitoses among total number of cells.

‡ Proportion of metaphases among total number of mitoses.

TABLE IV
STAGES IN MITOTIC CYCLE IN INTERPHASE CELLS TREATED WITH
500 µg/ml OF CHLORAMPHENICOL
(drug added at different times after plating)

Duration of Culture* (hr)	Time in Contact with Drug (hr)	No. of Cells† in				G2/Total
		G1	S	G2	Polyploidy	
4 19	0 0	90	3	7	0	0.07
36 38 40	0 0 0	115	4	29	2	0.19
4 19	4 19	86	1	8	5	0.08
36 38 40	36 38 40	122	3	23	2	0.15
19	15	44	0	6	0	0.12
36 38 40	32 34 36	129	2	17	2	0.11
36 38 40	17 19 21	76	8	59	7	0.39

* Time between plating and harvesting cells.

† Fifty cells were measured from each culture; G1 corresponds to 2C, G2 to 4C, S to intermediate values.

to that in the control culture. An arrest of cells in G2 was not detectable. These results suggest that chloramphenicol needs to be in contact with cells for several hours before it inhibits cell division.

When the effect of chlorimipramine (Tables VII and VIII) is compared with that of chloramphenicol (Tables I and II) several differences become apparent. Firstly, chlorimipramine inhibits cell division at very low concentrations; second, the arrest in metaphase seems to be of longer duration; third, although there is a marked reduction in the number of prophases, with chlorimipramine there is

no evidence of an arrest of cells in G2. It would seem that the cells are arrested in G1, bearing in mind that this stage in the control is constantly being added to by the products of mitosis (Table VIII).

Recovery. Cells treated with chloramphenicol showed a considerable ability to recover from its effects. When cells were plated in 1000 µg/ml and left in contact with the drug for 72 hours and then rescued a proportion of the cells was found to be in DNA synthesis or mitosis after a further period of 48 hours.

TABLE V
NUMBERS OF MITOSES IN CELLS TREATED WITH 500 µg/ml CHLORAMPHENICOL
(drug added late in mitotic cycle)

Duration of Culture* (hr)	Time in Contact with Drug (hr)	No. of Cells Counted	No. of Cells in				Mitotic Index† (%)	Metaphase Index‡ (%)
			Pro- phase	Meta- phase	Ana- phase	Telo- phase		
5 26	0 0	2000 2000 Total 4000	6 18 24	11 27 38	5 5 10	3 6 9	1.2 2.8	0.47
28 30 31 33 37	0 0 0 0 0	2000 2000 2000 2000 2000 Total 10,000	18 19 8 15 17 77	21 24 9 20 10 84	4 6 0 3 3 16	5 25 14 17 9 70	2.4 3.7 1.6 2.8 1.8	0.34
28 30 31 33 37	2 4 5 7 11	2000 2000 2000 2000 2000 Total 10,000	19 11 6 5 2 43	30 41 15 17 5 108	1 5 0 0 1 7	5 27 9 11 7 59	2.8 4.2 1.5 1.6 0.8	0.50

* Time between plating and harvesting cells.

† Proportion of mitoses among total number of cells.

‡ Proportion of metaphases among total number of mitoses.

TABLE VI
STAGES IN MITOTIC CYCLE IN INTERPHASE CELLS TREATED WITH 500 $\mu\text{g/ml}$
CHLORAMPHENICOL
(drug added late in mitotic cycle)

Duration of Culture* (hr)	Time in Contact with Drug (hr)	No. of Cells† in				G2/Total
		G1	S	G2	Polyploidy	
5 26	0 0	79	2	17	2	0.02
28 30 31 33 37	0 0 0 0 0	185	10	51	4	0.20
28 30 31 33 37	2 4 6 7 11	179	20	51	0	0.20

* Time between plating and harvesting cells.

† Fifty cells were measured from each culture; G1 corresponds to 2C, G2 to 4C, S to intermediate values.

Oxygen uptake. Trypsinized cells in Hank's solution introduced into the chamber of the oxygen electrode took up oxygen at the rate of 28 nmol $\text{O}_2/\text{min}/10^6$ cells. When chloramphenicol was introduced to a concentration of 500 $\mu\text{g/ml}$, there was no detectable effect on oxygen uptake. The addition of a further 500 $\mu\text{g/ml}$ of chloramphenicol, making a concentration of 1000 $\mu\text{g/ml}$, also produced no detectable effect. The system continued to take up oxygen at the initial rate until the supply was exhausted. Oxygen uptake was arrested to a large extent when potassium cyanide was added to the cells. This shows that the oxygen uptake in the electrode system is mainly due to mitochondrial

respiration. It was concluded that chloramphenicol had no direct inhibitory effect on mitochondrial function.

When cells which had been growing in medium containing 500 $\mu\text{g/ml}$ chloramphenicol were compared with untreated cells, it was found that the untreated cells took up oxygen at the rate of 32 nmol/min/ 10^6 cells whereas the rate for treated cells was 15 nmol/min/ 10^6 cells. It was concluded that the inhibition of mitochondrial synthesis by the drug depressed the respiratory rate or, alternatively, that the respiratory chain has a limited existence and may undergo partial degradation in these conditions. This latter situation has been established in yeast

TABLE VII
NUMBERS OF MITOSES IN CELLS TREATED WITH CHLORIMIPRAMINE

Concentration of Drug ($\mu\text{g/ml}$)	Duration of Culture* (hr)	Time in Contact with Drug (hr)	No. of Cells Counted	No. of Cells in				Mitotic Index† (%)	Metaphase Index‡ (%)
				Pro-phase	Meta-phase	Ana-phase	Telo-phase		
0	34	—	1000	5	2	0	0	0.7	0.45
0	36	—	1000	0	2	0	4	0.6	
0	38	—	1000	2	10	1	9	2.2	
0	40	—	1000	4	9	1	2	1.6	
			Total 4000	11	23	2	15		
5	34	17	1000	1	0	0	0	0.1	0.42
5	36	19	1000	3	2	0	3	0.8	
5	38	21	3000	2	12	2	8	0.8	
5	40	23	1000	1	2	0	2	0.5	
			Total 6000	7	16	2	13		
10	34	17	1000	0	0	0	0	0.0	0.76
10	36	19	1000	0	0	0	0	0.0	
10	38	21	5000	5	16	0	1	0.44	
10	40	23	1000	0	6	0	0	0.6	
			Total 8000	5	22	0	1		
15	34	17	1000	0	0	0	0	0.0	1.00
15	36	19	1000	0	0	0	0	0.0	
15	38	21	5000	0	22	0	0	0.44	
15	40	23	1000	0	2	0	0	0.2	
			Total 8000	0	24	0	0		

* Time between plating and harvesting cells.

† Proportion of mitoses among total number of cells.

‡ Proportion of metaphases among total number of mitoses.

TABLE VIII
STAGES IN MITOTIC CYCLE IN INTERPHASE CELLS TREATED WITH CHLORIMIPRAMINE

Concentration of Drug ($\mu\text{g/ml}$)	Duration of Culture* (hr)	Time in Contact with Drug (hr)	No. of Cells† in				G2/Total
			G1	S	G2	Polyploidy	
0	17	0	45	0	5	0	0.10
0	34	0	158	9	33	0	0.11
0	36	0					
0	38	0					
0	40	0					
5	34	17	169	10	19	2	0.10
5	36	19					
5	38	21					
5	40	23					
10	34	17	177	6	17	0	0.08
10	36	19					
10	38	21					
10	40	23					
15	34	17	183	3	14	0	0.07
15	36	19					
15	38	21					
15	40	23					

* Time between plating and harvesting cells.

† Fifty cells were measured from each culture; G1 corresponds to 2C, G2 to 4C, S to intermediate values.

cell mitochondria in the presence of chloramphenicol and erythromycin, when cytochrome oxidase and cytochrome *b* are lost after about 12 hours (Stone and Wilkie, 1974).

Discussion

These results provide conclusive evidence that chloramphenicol acts as a mitotic inhibitor in human fibroblasts. Although the most striking effects were obtained with concentrations of 500 $\mu\text{g/ml}$ and above, the results shown in Table I do not preclude the possibility of some mitotic inhibition with 100 $\mu\text{g/ml}$ of drug or even less. Mitotic metaphase and the G2 phase preceding mitosis seem to be the two stages which are most sensitive to the drug. Clearly, an arrest of cells in metaphase could tend to increase the mitotic index, while an arrest in G2 would decrease it.

Chlorimipramine also arrests cells in metaphase, but an accumulation of cells in G2 could not be detected with this drug. These findings are in agreement with previous results (Wilkie and Delhanty, 1970; Mittwoch and Wilkie, 1971). Chlorimipramine has a direct effect on oxygen uptake and it would seem that if enough energy is available for DNA synthesis, the cells are able to proceed through G2 until metaphase. By contrast, chloramphenicol affects cellular respiration indirectly.

The fact that no direct effect on oxygen uptake was seen with high concentrations of chloramphenicol indicates that the mitotic inhibition results from arrest of mitochondrial protein synthesis. Since cells treated with chloramphenicol for a long time show a reduced respiratory activity, it seems that

synthesis of the respiratory chain is a prerequisite for normal cell division in fibroblasts.

An arrest of cells in metaphase is known to occur from the action of a variety of agents, including colchicine (Hughes, 1952), barbiturates (Caratzali and Roman, 1969), and vinblastine (Baserga, 1969). Other agents, including cycloheximide and mengovirus have been reported to block G2. Recent results by Paton, Silver, and Allison (1974) suggest that a delay in G2 is also seen in fibroblasts from patients with Down's syndrome. This finding is of interest in view of the known association between Down's syndrome and leukaemia. Although the relationship between cause and effect is not understood either in the case of chromosome abnormalities and malignancies nor in toxic drug reactions such as bone marrow dysfunction resulting from the use of chloramphenicol, it seems likely that an undue prolongation of the time taken to complete the mitotic cycle in rapidly growing tissues would interfere with the normal rate of cell proliferation and also lead to imperfect cellular differentiation.

This work was supported by a grant from the Cancer Research Campaign. The integrating microdensitometer was provided by the University of London Central Research Fund.

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