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An Explanation for the S-Phase Specificity of the Cytotoxicity of Protein Synthesis Inhibitors*

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Abstract—*A pulse of the protein synthesis inhibitor cycloheximide is shown to induce chromosome aberrations in cultured cells of human origin. These aberrations do not appear until in excess of* 12 hr *after the pulse and their appearance coincides with the time when cells which had been in S-phase at the time of the cycloheximide pulse reach mitosis. We suggest that the reason why the protein synthesis inhibitor cycloheximide has previously been reported not to induce chromosome aberrations is due to the long delay of cells with damaged chromosomes in reaching mitosis.*

INTRODUCTION

NUCLEOSIDE analogues and antimetabolite drugs which inhibit DNA synthesis such as arabinofuranosylcytosine (ara-C), hydroxyurea, fluorodeoxyuridine and methotrexate all exhibit a characteristic S-phase specificity of cytotoxicity towards mammalian cells [1–3]. Another property that these compounds have in common is that they all induce chromosome aberrations [4–7]. In the case of ara-C, it has been specifically proposed that inhibiting DNA synthesis with ara-C induces chromosome aberrations in cells which were in the S-phase when the ara-C was added and that these chromosome aberrations are the direct cause of the S-phase-specific cell death [4, 8, 9]. In this model, the primary toxicity of ara-C is related to effects on DNA synthesis, the basic cellular activity by which the S-phase of the cell cycle is defined.

However, other authors consider that the cytotoxic effect on cells of the nucleoside analogues and of the antimetabolite drugs is primarily mediated by their effects on RNA metabolism and subsequently on protein synthesis and that any effect on DNA synthesis is of secondary importance [10, 11]. Hence, if a substance could be shown to exhibit cytotoxicity that was S-phase specific, and if this cytotoxicity was independent of any effect on DNA synthesis or on karyotypic stability, this would be a strong argument against the view that the cytotoxicity of compounds such as ara-C is, because of its S-phase specificity and because of the clastogenicity of the ara-C, necessarily related to some specific effect on DNA synthesis.

Protein synthesis inhibitors are cytotoxic to mammalian cells and this cytotoxicity has been shown to exhibit definite S-phase specificity [1]. Despite this S-phase specificity of cytotoxicity, several studies have reported that cycloheximide, a potent inhibitor of mammalian protein synthesis [1], does not induce chromosome aberrations [12–14]. Hence we considered that it was important to reexamine whether an inhibitor of protein synthesis such as cycloheximide could cause S-phase-specific cell death which was independent of any effect on DNA replication and on the stability and integrity of the chromosome complement.

MATERIALS AND METHODS

Cycloheximide and colcemid were purchased from the Sigma Chemical Co., St. Louis MO, U.S.A. [^{3}H]Thymidine (TdR) was from the Radiochemical Centre, Amersham, U.K.

Experiments employed Crow cells, a cell line derived from a human brain tumour in the laboratory of Dr. T. R. Bradely, Cancer Institute, Melbourne, Australia. Crow cells were grown as a monodisperse suspension culture in Alpha medium supplemented with 10% foetal calf serum (both from Flow Laboratories, Stanmore, N.S.W.,

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Australia). The cells grew with a doubling time of approximately 24 hr.

To define cells in S-phase at the time of the cycloheximide pulse, [^{3}H]TdR at 1 μCi/ml was added to the culture 30 min before the addition of the cycloheximide. After 1 hr with cycloheximide, cells were resuspended in fresh medium. Control cells were resuspended in fresh medium after the 30 min with [^{3}H]TdR. At the time of resuspension in the fresh medium and at 6-hr intervals thereafter, portions of the control and test cultures were made 1 μg/ml in colcemid. After 6 hr with colcemid, cells were fixed for c-metaphases and duplicate sets of slides were used for autoradiography and for scoring for chromosome aberrations. Autoradiography was performed using Kodak NTB-2 emulsion and D-19B developer and duplicate sets of slides were exposed for 3 days and 3 weeks.

All scoring of chromosome aberrations was performed by one person. Only well spread metaphases with approximately the average number of chromosomes (43–44) were scored. One hundred metaphases were scored from the first 6 control samples. Fifty metaphases were scored from the last two control samples and from each of the test samples. One hundred metaphases were scored for the frequency of labelled metaphases from all samples except for the 42- and 48-hr samples of cycloheximide pulsed cells where 50 were scored for each. For the determination of the percentage of cells arrested in metaphase in each sample, 200 cells were counted from each slide from non-overlapping fields which contained only intact interphase and metaphase cells.

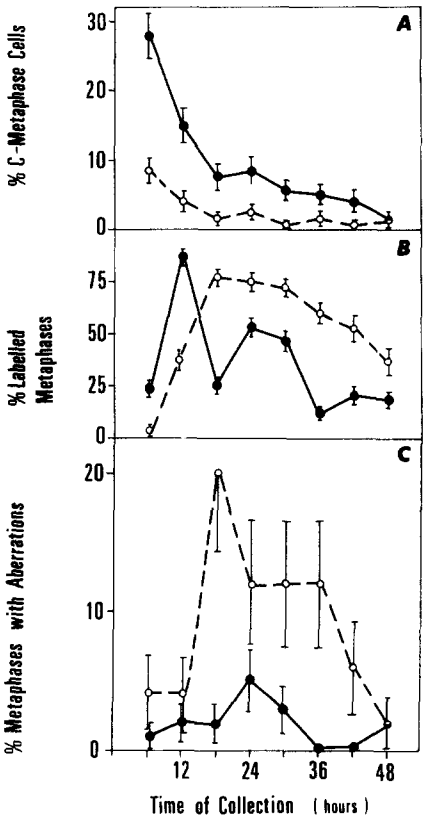

Fig. 1. Cells from control and cycloheximide pulsed cultures (100 μg/ml, 1 hr) were collected over 6-hr intervals and fixed for c-metaphases. Cells had been prelabelled with a 30 min pulse of [^{3}H] TdR in order to differentiate cells which had been in S-phase at time zero. (A) The proportion of cells arrested in c-metaphase in each sample; (B) the proportion of c-metaphases which were labelled and hence were c-metaphases of cells which had been in S-phase at time zero; and (C) the proportion of c-metaphases which contained aberrant chromosomes. Control cells: ●—●; Cycloheximide-pulsed cells: ○----○.

RESULTS

Logarithmically growing Crow cells were labelled with [^{3}H]TdR for 30 min. Half of the cells (control cells) were then transferred to normal growth medium (time zero). The other half of the cells (test cells) was made 100 μg/ml in cycloheximide after the labelling period and 1 hr later were transferred to normal medium (time zero for test culture). At 6-hr intervals from time zero to +42 hr, a portion of each culture was made 1 μg/ml in colcemid. After 6 hr in the presence of colcemid, cells were collected and fixed. Each cell preparation was scored for the proportion of cells arrested in metaphase by the colcemid during each 6-hr interval (Fig. 1A), for the fractions of those metaphases which were labelled (i.e., metaphases of cells which had been in S-phase at the time of the cycloheximide pulse) (Fig. 1B), and for the presence of chromosome aberrations (Fig. 1C). It was considered necessary to examine cells for up to 48 hr after the cycloheximide pulse because protein synthesis inhibitors have been shown to cause considerable delay in all phases of the cell cycle [15]. During the 48 hr of the experiment, control cells reached saturation density while the cell density in the cycloheximide-treated culture remained approximately constant.

Following the cycloheximide pulse there was a reduction to about 1/3 or 1/4 of control values in the proportion of cells reaching mitosis (Fig. 1A). This occurred within the first 6 hr. Hence G_2 cells as well as S-phase cells were delayed in reaching mitosis. While some control cells which had been in S-phase at time zero reached mitosis during the first 6 hr, the initial peak of labelled metaphases in

control cells (87% labelled) was present in the 6- to 12-hr sample (Fig. 1B). In the cycloheximide-pulsed culture, there was an immediate delay of S-phase cells reaching mitosis, followed by a peak of labelled mitoses in the 12- to 18-hr sample (Fig. 1B). The fraction of metaphases which were labelled in the drug treated culture remained high (60% or more) until 36 hr after the cycloheximide pulse (Fig. 1B). Hence cells in G_1 at the time of the cycloheximide pulse must also have been drastically delayed in cell cycle progression, since if they were not they would have reached metaphase well before 36 hr (normal cell doubling time less than 24 hr) and would therefore have reduced the fraction of metaphases which were labelled. Hence the 1 hr pulse of cycloheximide at 1000 μg/ml caused delays in G_1, S and G_2 phases.

There was a low level of metaphases from control cells which included chromosome abnormalities (14 metaphases containing chromosome aberrations observed in a total of 700 metaphases scored from 8 control samples) (Fig. 1C and Table 1).

Table 1. Frequency and types of aberrations in control and cycloheximide-pulsed cell samples

	Control metaphases	Cycloheximide-pulsed metaphases
Total number metaphases scored	700	400
Total number aberrant metaphases scored	14	36*
Gaps and breaks (Chromatid-type)	12	36
Gaps and breaks (Chromosome type)	2	10
Acentric fragments	0	6
Exchanges	0	0

*$P<<0.005$ for increase in frequency of aberrations scored in all samples of cycloheximide-pulsed cells compared to frequency in all control cell samples.

Cycloheximide-pulsed cells showed a similar low frequency of aberrant metaphases for at least 12 hr after the drug treatment (Fig. 1C). Hence cycloheximide does not appear to induce chromosome aberrations in G_2 cells. This absence of induction of aberrations during the first 12 hr was followed by a peak of aberrant metaphases. (20% with aberrations) in the 18-hr sample (Fig. 1C). This peak of aberrations coincided with the peak of labelled metaphases (Fig. 1B), the time at which cells which had been in S-phase at the time of the cycloheximide pulse reached mitosis. The frequency of metaphases with aberrations remained significantly above control values up until 36 hr after the drug pulse, falling to control values in the 42- to 48-hr sample (Fig. 1C). Thus the frequency of aberrations in cycloheximide-treated cultures parallelled the appearance in metaphase of the cells which had been in S-phase at the time of the cycloheximide pulse.

Table 1 shows the types of aberrations observed in all metaphases scored from control and test cultures. A total of 700 metaphases were scored from the 8 control samples and 400 from the 8 drug-treated samples. In the cycloheximide-pulsed culture, the most frequent aberration was chromatid gaps and breaks (scored together because of the uncertainty in some instances whether gaps and breaks represented different lesions). Chromosome gaps and breaks were also detected, together with a lower frequency of acentric fragments and exchanges. The increase in frequency of metaphases containing chromosome aberrations in all test samples relative to all control samples was highly significant ($P<<0.005$).

DISCUSSION

These results clearly demonstrate that cycloheximide causes chromosome aberrations in the cell line employed in these experiments. We have no reason to believe that this cell line is any way special. The time of the peak of aberrant metaphases coincides with the time of the peak of labelled metaphases, indicating that the chromosome aberrations are primarily present in the cells which were in S-phase at the time of the cycloheximide pulse.

The reason why the clastogenicity of protein synthesis inhibitors was not detected previously [12–14] is readily apparent from the prolonged delay in the appearance of damaged metaphases (more than 12 hr) and that after 42 hr the frequency of damaged metaphases returned to control values. This delay in the appearance of damaged metaphases is due to the delaying effect of the cycloheximide on progression through both S and G_2 phases.

Protein synthesis inhibitors have been shown to induce S-phase specific cytotoxicity [1] and we have shown here that the protein synthesis inhibitor cycloheximide induces S-phase specific chromosome aberrations.

Although this does not prove that the S-phase specific cytotoxicity of protein synthesis inhibitors is mediated by chromosome aberrations, it does mean that cycloheximide can no longer be considered to be an exception to the observation that compounds which exhibit S-phase cytotoxicity also induce chromosome abnormalities. This S-phase-specific induction of chromosome aberrations at least provides a feasible explanation for the pronounced S-phase specificity of cytotoxicity of protein synthesis inhibitors [1]—an observation which would be difficult to understand if this cytotoxicity was not somehow mediated by some effect on DNA replication.

We have previously suggested that the S-phase cytotoxicity of ara-C and of ara-A is mediated by chromosome aberrations which result from a pathological derangement of the pattern of replication of the chromosomal DNA caused by the ara-C or ara-A blocking DNA replication [8, 9, 16]. Protein synthesis inhibitors also block DNA replication, probably via a resultant depletion of DNA packaging proteins [17]. Using Crow cells, we have demonstrated that indirectly inhibiting DNA replication with a pulse of cycloheximide also results in the same type of pathological derangement of the DNA replication pattern as we have demonstrated to occur following a pulse of ara-C and of ara-A (Woodcock and Cooper, manuscript submitted).

We suggest that there may be a basic identity of the molecular mechanisms producing the chromosome abnormalities induced in S-phase cells by a direct inhibition of DNA replication with ara-C or ara-A or by an indirect inhibition of DNA replication with an inhibitor of protein synthesis such as cycloheximide.

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