Abolition by Cycloheximide of Caffeine-enhanced Lethality of Alkylating Agents in Hamster Cells

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ABSTRACT

Greatly enhanced lethality for Syrian hamster cells (baby hamster kidney cells and polyomavirus-transformed derivative of baby hamster kidney cells) that had been treated with alkylating agents or ultraviolet light is produced by caffeine. Thus, nitrogen mustard [methylbis(β-chloroethyl)amine] (HN2) at 0.5 μM decreased cell viability by only a few percent in the absence of caffeine. The addition of 2 mM caffeine during the first 24 hr after nitrogen mustard decreased viability more than 95%. Further kinetic studies of the caffeine effects using other alkylating agents indicate the existence of two modes by which damaged cells become caffeine sensitive. Treatment with ultraviolet light or nitrogen mustard makes cells immediately sensitive to caffeine; cells treated with methyl methanesulfonate or N-methyl-N'-nitro-N-nitrosoguanidine, on the other hand, express their maximal caffeine sensitivity only about 15 to 20 hr later. A low concentration of cycloheximide completely abolished this caffeine effect. When 0.36 μM cycloheximide was present, loss of viability was negligible even in the presence of caffeine. Based on further experiments involving the timing of caffeine and cycloheximide additions, caffeine does not affect viability by itself but is involved with some substance such as a protein the rapid synthesis of which is prevented by cycloheximide and clastogenic effects in cells exposed to various physical and chemical carcinogens. Among these agents are UV irradiation, X-rays, and a wide variety of alkylating agents (7, 32, 33), which are believed to produce their biological effects through their interactions with DNA. The molecular mechanism by which caffeine posttreatment decreases cell survival, increases chromosome aberrations, and modifies mutation and transformation frequencies is not understood. There are some indications of inhibition of postreplication repair (8, 10, 11, 17). Whether caffeine itself acts directly or serves to initiate processes of a more complex sort is not known.

In this laboratory, we have been studying the effect of caffeine on hamster cell lines treated with a variety of toxic chemicals including anticancer drugs. The studies have been carried out with BHK cells and also with pyBHK cells. Treatment with low concentrations (2 μM) of caffeine up to 24 hr do not have growth-retarding or toxic effects on these cell lines. Similar concentrations, however, produce a large increase in cell kill after treatment with HN2, UV, MMS, MNNG, etc. We report here studies that were aimed to provide better understanding to the mechanism of enhanced lethality caused by posttreatment with caffeine.

MATERIALS AND METHODS

Cell Culture. BHK cell line was obtained from the American Tissue Culture Collection, Rockville, Md. PyBHK cell line was obtained from Dr. Michael Stoker, Imperial Cancer Research Fund Laboratories, London, England. The cells were grown in tissue culture flasks or dishes in Dulbecco's modified Eagle's medium at 37°C in a humidified, 10% CO2 atmosphere. The medium was supplemented with 5% calf serum and 5% horse serum for BHK cells and 10% calf serum for pyBHK cells. The doubling time under these conditions for both cell lines is 12 hr. New cultures were started every 4 to 6 weeks from frozen aliquots. Each new batch of cells was determined to be mycoplasma free using the method of Schneider et al. (24).

Chemicals. HN2 was obtained from Merck, Sharp and Dohme (West Point, Pa.). MMS and MNNG were purchased from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). Caffeine and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, Mo.).

Drug and UV Treatment. Two methods of drug and UV treatment were used. With the first method, cells were plated at 1 × 10⁵/60-mm dish 24 hr before drug or UV treatment. Medium was changed, and required amounts of drugs were added to cultures. Cells were then incubated at 37°C for 1 hr. At the end of this period, cells were washed twice, trypsinized, and replated at 200/60-mm dish. Before UV irradiation, culture medium was replaced by warm sterile phosphate-buffered saline (137 mM NaCl: 2.7 mM KCl: 0.9 mM CaCl2: 8 mM Na2HPO4: 14.5 mM KH2PO4). A gemicillic lamp (1.0 watt/sq m) was used as the source of irradiation, and cells were exposed to the light 80 cm away from the source. The second method was similar to the first except that cells were plated at 200/60-mm dish 12 hr before drug treatment. Cultures were washed only once after drug treatment, and fresh medium was added back without replating. Both methods gave identical results. The end of drug or UV treatment is taken as t = 0 for all experiments. Cultures exposed to UV were subsequently covered with aluminum foil to avoid exposure to visible light.

Caffeine Sensitivity Studies. To study the time dependence of caffeine sensitivity of drug-treated or UV-irradiated cells, caffeine was added to pairs of plates at various times after drug treatment or UV irradiation for an equal interval of time. At the end of each interval, caffeine-treated cultures were rinsed once, and fresh medium without caffeine was added back. After 7 days of incubation, cultures were fixed and stained with crystal violet. Colonies of 100 or more cells were counted. Percentage of survival is calculated as the relative plating efficiency of drug-treated cultures to that of the untreated controls. Plating efficiency of untreated controls was 60 to 70%. Each experimen...
ment was repeated 4 to 5 times, and results of typical experiments are reported.

RESULTS

Time Dependence of the Potentiation by Caffeine of HN2 Lethality. When BHK or pyBHK cells were exposed to HN2, a dose-dependent increase in cell lethality was seen. At 1 μM HN2, cell survival was about 70% (Chart 1). When caffeine was present from 0 to 10 hr after HN2-treatment, cell survival was greatly reduced. If caffeine was added 10 hr after drug treatment, cell survival was between that for no caffeine treatment and that for caffeine treatment for the first 10 hr. Pretreatment with caffeine did not produce any lethality. These results suggest that the effect of caffeine on HN2-treated cells was time dependent. To obtain a more detailed picture of the time dependence of the caffeine effect, HN2-treated cells were pulsed with caffeine for 8-hr intervals up to 30 hr after HN2 was removed. Caffeine was effective in reducing survival only transiently in the first 12 hr after treating cells with 0.5 μM HN2 (Chart 2). After this time, no major enhancement of lethality was observed. Cell kinetic studies with synchronized cells show that HN2-treated cells were delayed for 6 hr in the first G2 period following treatment with 0.5 μM HN2 (14). Maximal caffeine sensitivity was observed during this G2 delay. Thus, the caffeine effect is observed during the first cell cycle after HN2 treatment. We infer that repair of DNA damage took place during the first cell cycle. Thus, few lethal lesions remained in the DNA after damaged cells had passed through the first cell cycle, and the cells lost their caffeine sensitivity.

Time Dependence of the Potentiation by Caffeine of Lethality of MMS and MNNG. Experiments with a different alkylating agent, MMS, produced strikingly different results than obtained with HN2. Posttreatment with caffeine from 0 to 10 hr after MMS created only a modest increase in cell lethality (Chart 3). However, if caffeine was given from 10 to 20 hr after MMS, there was a 4- to 5-fold-enhanced cell lethality. These results show essentially the reverse time dependence of those obtained with HN2-treated cells (compare Charts 1 and 3). To understand better the time dependence of caffeine sensitivity in HN2- and MMS-treated cells, 4-hr exposures to caffeine were applied at intervals after either HN2 or MMS treatment. These shorter exposures to 4 mM caffeine were not toxic to untreated cells (Chart 4). HN2-treated cells were most sensitive to caffeine during the first 4 hr (Chart 4). After this period, the caffeine effect gradually decreased, and by 12 hr most of the lethal enhancement due to caffeine had disappeared.

On the other hand, there was very little kill enhancement by caffeine during the first 4 hr after treatment with 0.7 mM MMS. However, the cell kill was increased significantly by caffeine in the following time intervals reaching a maximum at about 16 to 20 hr after MMS treatment. Only after about 24 hr did these cells start to become insensitive to caffeine again. With 2 mM caffeine, similar results were obtained although the effect was reduced.

Irradiation with UV, at doses which produced minimal cell kill, also made cells sensitive to caffeine again, with a time dependence like that of HN2-treated cells. They became most sensitive to caffeine immediately after irradiation (Chart 5A). MNNG-treated cells, on the other hand, were much like MMS-treated cells (Chart 5B). These results indicate that the agents in question cause cells to respond to caffeine with different kinetics.

Different Modes of Caffeine Sensitivity. Evidence for the
existence of 2 independent modes, by which cells treated with these agents become sensitive to caffeine, was obtained by subjecting cells to a combined treatment with both HN2 and MMS. These cells were then exposed to 2 mM caffeine for 6-hr intervals. As seen in Chart 6, the amount of cell kill observed at various intervals following a combined treatment approximated closely with that obtained by the sum of the individual drug treatments.

An obvious explanation which might account for the different caffeine sensitivity patterns could be that MMS- or MNNG-treated cells were temporarily growth arrested, and the effect of caffeine might be elicited at a later time after recovery from growth arrest. Examination of growth of cultures showed that at these low doses, MMS-treated cells continued to grow exponentially, like the untreated control cultures (data not shown).

**Effect of Cycloheximide on Caffeine-enhanced Lethality.**

The enhanced lethality caused by caffeine in these HN2-treated cells was completely abolished by the presence of 0.36 μM cycloheximide (Chart 7). This concentration of cycloheximide inhibited protein synthesis (³⁵S]leucine incorporation) by about 85% and DNA synthesis ([³H]thymidine incorporation) by about 50% during a 24-hr period in cells treated with 0.5 μM HN2 and similarly for untreated cells. It decreased the growth rate of these cells by 50% over 2 days. This cycloheximide concentration did not affect survival of HN2-treated cells in the absence of caffeine (Chart 7). Cycloheximide at 0.18 μM was able to afford a substantial protection from the caffeine effect (data not shown). Also, puromycin (1 μg/ml) gave 2-fold protection. Cycloheximide also protected cells from caffeine following UV irradiation or treatment with MMS or MNNG (Table 1). Elimination by cycloheximide of enhanced lethality suggests that the synthesis of macromolecules such as proteins is important in this activity of caffeine. One possibility is that caffeine does not
act by itself but in cooperation with some macromolecule(s) that must be produced by damaged cells.

**Time Dependence of Cycloheximide Protection.** When caffeine was kept on the HN2- and MMS-treated cells continuously for 28 hr and 0.18 \( \mu \text{M} \) cycloheximide was added for 8-hr intervals, cycloheximide was able to substantially reduce the caffeine effect only during the time when cells were most sensitive to caffeine (Chart 8). Thus, HN2-treated cells were protected by cycloheximide only during the first 12 hr, whereas MMS-treated cells were protected during the 12- to 24-hr period.

These results indicate that cycloheximide cannot provide protection from the effect of caffeine by merely delaying growth. For if so, upon the removal of cycloheximide from HN2-treated cells after the first 8 hr, cells would still be killed by the continuous treatment with caffeine in the subsequent 20 hr. Furthermore, cycloheximide was unable to protect MMS-treated cells from caffeine during the first 12 hr, confirming that the basis of cycloheximide protection is not delaying growth to allow additional time for excision repair prior to S phase.

In addition, since 0.36 \( \mu \text{M} \) cycloheximide slowed the growth rate of HN2-treated cells by 50\%, all cells must have passed through one cycle during the 24 hr when caffeine was present. Therefore, slower growth does not appear to be responsible for the elimination of the effect of caffeine.

**Effect of Cycloheximide on the Recovery Process.** One alternative mechanism by which cycloheximide could prevent the lethal effect of caffeine is indirect. Assume that caffeine perturbs a recovery process so as to produce errors in the repaired DNA. Then, if cycloheximide simply blocked the entire recovery process by inhibiting DNA synthesis (4) or synthesis of new protein required for inducible repair (34), it would also block the defective repair created by caffeine. This seems not to be the case, since the recovery process seemed to be complete in 24 hr in the presence of this low concentration of cycloheximide. After HN2-damaged cells were incubated for 24 hr in the presence of cycloheximide, their initial sensitivity to caffeine had disappeared (Table 2), similarly to 24 hr incubation without cycloheximide. Table 2 also shows the cycloheximide alone did not produce lethality, and it blocked the enhanced lethality created by caffeine during the first 24 hr. The drugs did not markedly affect survival of cells not treated with HN2. These results indicate that cells passed beyond the caffeine-sensitive stage in 24 hr even in the presence of cycloheximide. Recovery itself does not seem to have been blocked by this 24-hr treatment. Therefore, the effect of cycloheximide must be on the functioning of caffeine itself.

Since MMS- or MNNG-treated cells became caffeine sensitive 12 hr after drug treatment, we surmised that the progression of these cells to a "caffeine-sensitive" state might be necessary for these cells to become sensitive to the lethal effect of caffeine. Caffeine must be interfering with the recovery process following drug treatment because (a) caffeine sensitivity appears only after drug treatment and (b) the enhanced lethality caused by caffeine is only transiently expressed. If the activation of such a recovery process were induced after drug treatment and took 12 hr, one would see a delayed appearance of caffeine sensitivity. On the other hand, if the repair or recovery processes were activated immediately following the introduction of lesions into DNA, caffeine sensitivity would be manifested much sooner. The following experiment was performed to investigate this question. Cells treated with MNNG (0.2 \( \mu \text{g/ml} \)) were sensitive to 24-hr exposure to 2 \( \mu \text{M} \) caffeine added after drug treatment (Table 3). Exposure to caffeine during the 24- to 48-hr interval did not cause any significant change in cell survival. This result confirms the observation made in the kinetic studies (Chart 5B) that by the end of 24 hr most of the recovery has already taken place. When MNNG-treated cells were incubated with cycloheximide for 24 hr after drug treatment, followed by exposure to caffeine during the 24
to 48 hr interval, a 7.5-fold decrease in survival was observed. These results indicate that with MNNG-treated cells, unlike the HN2-treated cells, the progression to the caffeine-sensitive state was either halted or delayed in the presence of cycloheximide.

**DISCUSSION**

Although the actual mode of action of caffeine on drug-treated animal cells is not understood, it is widely believed that caffeine interferes with the DNA repair process. This assumption is at least operationally correct, since the caffeine effect on cell survival can be seen only in cells pretreated with drugs such as alkylating agents and at doses of caffeine which alone do not have much lethal effects. Also, if a time interval is allowed to elapse before caffeine is added to drug-treated cells, no significant increase in lethality is seen. Presently available data strongly suggest that in mammalian systems, caffeine might inhibit postreplication repair (2, 10, 16, 22, 31).

To our knowledge, there has been no extensive study on the time dependence of caffeine effect on drug-treated cells in vitro. Previous investigations used protocols in which cells were treated with caffeine continuously for a long period of time. Tolmach et al. (30) reported the effect of short-term caffeine treatment on X-ray-irradiated HeLa S3 cells.

Studies presented here describe a detailed examination of the time dependence of caffeine effect on drug and UV-treated hamster cells. Our results indicate the existence of 2 types of mechanisms through which sensitivity to the lethal effects of caffeine might arise. Cells treated with HN2 or UV become sensitive to caffeine immediately after exposure to the DNA-damaging agents. Most of the caffeine sensitivity is seen during the first 8 hr. By the end of 12 hr after drug treatment, these cells become relatively insensitive to caffeine. The doubling time of BHK and pyBHK cells under these conditions is approximately 12 hr, indicating that most of the caffeine-sensitive recovery must take place within the first cell-doubling period following HN2 or UV treatment. With the use of synchronized mouse L cells, Rauth has observed that UV-irradiated or mitomycin C-treated cells were no longer sensitive to caffeine after they have been incubated for one generation’s time in the absence of caffeine (6, 20, 21). In human lymphocytes treated with mitomycin C, multiple chromosome breaks appeared only if both mitomycin C and caffeine were present in the first cell cycle (25).

Delayed expression of caffeine sensitivity was observed in the case of MMS- or MNNG-treated cells. Our data show that cells treated with these drugs were most sensitive during the 16- to 20-hr period after drug treatment. For the first 4 hr after exposure to MMS or MNNG, no enhanced lethality was seen when caffeine was added. No difference was observed in the pattern of the 2 types of caffeine sensitivity when normalized for increased cell number at later time points. Roberts et al. (22) described studies with MNU-treated V79 cells, in which maximal chromosomal aberrations were seen in the presence of caffeine about 30 to 40 hr after drug treatment. Caffeine treatment in these experiments, however, was continuous; therefore it is not clear whether this caffeine effect was exerted before the actual appearance of chromosomal aberrations. These authors also reported that the ability of caffeine to sensitize sulfur mustard-treated cells persisted for only the first 20 hr, whereas MNU-treated cells remained sensitive to caffeine for nearly 50 hr after drug treatment. These studies, although determining the time after which drug-treated cells were no longer sensitive to the lethal effects of caffeine, did not clearly mark the time of peak sensitivity because caffeine treatment was continuous. Also, the drug doses used in these experiments would kill 50% or more of the cells without post-treatment of caffeine. Drug concentrations used in our experiments produced very little kill in the absence of caffeine. The results of Roberts et al. (22) suggest that sulfur mustard-treated cells might behave like UV- or HN2-treated cells, while MNU-treated cells would be similar to those treated with MMS or MNNG. It is noteworthy that the lesions in the DNA caused by the agents in the first group are bulky, whereas the agents of the second group introduce more subtle modifications.

The reason for delayed expression of caffeine sensitivity in MMS- and MNNG-treated cells is not clear. This is similar to the observation that DNA synthesis in MMS-treated cells was inhibited in the second cell cycle after MMS treatment (18, 19). This perhaps is related to a delay in appearance of certain lesions in the DNA such as strand breaks that arise due to the slow removal of N7-methylguanine which is the major product of alkylation (15, 26). N7-methylguanine has been shown not to interfere with DNA replication (9) and therefore could be tolerated during the first round of DNA synthesis. However, spontaneous (3) and enzymatic (27) depurination will eventually lead to strand breaks in DNA which interferes with DNA synthesis. Thus, the delayed caffeine sensitivity and inhibition of DNA synthesis might reflect the kinetics of removal of N7-methylguanine, which has been reported to take approximately 36 hr (27).

Indications that the appearance of caffeine sensitivity in MMS- or MNNG-treated cells might be dependent on induced process(es) were obtained by subjecting the drug-treated cells to cycloheximide treatment before the addition of caffeine (Table 3). This aspect of the phenomenon requires further investigation.

We have reported previously that caffeine potentiates the lethality of alkylating agents by preventing damaged cells from delaying in G2 phase (14). This G2 delay would allow additional time for DNA repair before mitosis and could be the basis of a surveillance mechanism proposed by Tobey (29). Caffeine induces damaged cells to undergo mitosis before the completion of DNA repair. This premature mitosis gives rise to shattered chromosomes, nuclear fragmentation, and cell death (14). Unlike what Domon and Rauth (6) had reported, this effect of caffeine is G2 specific.5

With the use of cycloheximide at concentrations that inhibit protein synthesis without completely stopping cells from

### Table 3

<table>
<thead>
<tr>
<th>Posttreatment</th>
<th>0–24 hr</th>
<th>24–48 hr</th>
<th>+MNNG</th>
<th>−MNNG</th>
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<td>75</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
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<td>7</td>
<td>104</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>51</td>
<td>108</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>10</td>
<td>93</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caffeine + cycloheximide</td>
<td>75</td>
<td>104</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caffeine + cycloheximide</td>
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<td>94</td>
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**Kinetic Studies of Caffeine-enhanced Lethality**

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growth, we probed the possibility that caffeine acts in cooperation with some macromolecules that must be produced by damaged cells. Low concentration of cycloheximide protected drug-treated cells from the lethal effects of caffeine posttreatment (Chart 7; Table 1).

With synchronized cells that were damaged with alkylating agents, we have shown that the protection by cycloheximide was also G2 specific.6 Thus, damaged cells were protected fully from caffeine by cycloheximide added only during the G2 delay. This is consistent with the results reported here (Chart 8) that cycloheximide is not merely delaying growth sufficiently so that damaged cells were prevented from entering into the caffeine-sensitive period (G2) when caffeine was present or allowing additional time for excision repair of DNA damage prior to S phase. In addition, we also eliminated the possibility that cycloheximide simply blocks the entire recovery process which might be sensitive to caffeine (Table 2).

Two models can be proposed to explain these results. The first model is that the action of caffeine in enhancing lethality, and probably on the other DNA damage-related events such as chromosome breakage, mutation, and transformation, depends on protein(s) the synthesis of which is strongly inhibited by cycloheximide. Alternatively, the synthesis of this protein(s) might be only moderately inhibited, but it might not increase appreciably when cycloheximide is present because of its lability; a labile enzyme or other protein is a possibility of this sort (23). In any case, caffeine does not act alone (e.g., by binding to damaged DNA) to cause these effects independently of some other material.

The second model is that the caffeine-sensitive point is in late G2. Thus, cycloheximide added during the G2 delay prevented damaged cells from progressing through G2 to the caffeine-sensitive point. This may provide additional time for DNA repair; therefore, after cycloheximide is removed, cells are no longer sensitive to caffeine.

Al-Bader et al. (1) reported that at least 9 proteins that were present in normal synchronized G2 cells were missing in cells arrested in G2 by the nitrosourea cis-4-[3-(2-chloroethyl)-nitrosouarno]carbonyl]amino]cyclohexaneacrylic acid. These results are in good agreement with our observation that cycloheximide retains HN2-treated cells in G2 in the presence or absence of caffeine.4 In addition, caffeine did not cause G2-arrested cells to enter mitosis immediately but usually after a 3- to 4-hr lag.5 This suggests that caffeine does not exert its effect directly, by for example binding to DNA (5), but through induction of some protein(s). Experiments are now in progress to test this hypothesis by analyzing protein synthesis in damaged cells during the G2 delay in the presence or absence of caffeine.

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REFERENCES


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