Enhancement by Caffeine of Neocarzinostatin Cytotoxicity in Murine Leukemia L1210 Cells

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ABSTRACT

Posttreatment incubation with nontoxic doses of caffeine resulted in enhancement of cell lethality and inhibition of cell growth in L1210 mouse leukemia cells which had been exposed to a protein antibiotic, neocarzinostatin. In addition, caffeine treatment appeared to inhibit the eventual maturation of newly synthesized DNA in L1210 cells following exposure to this antibiotic.

These results, indicating the existence of caffeine-sensitive repair in L1210 leukemia cells treated with neocarzinostatin, provide further evidence for DNA damage as a mechanism of the cytotoxic action of the antibiotic.

INTRODUCTION

There may be hardly any other drug that affects the genetic material in as many different ways as does caffeine (1,3,7-trimethylxanthine). Caffeine not only produces cell death, mutations, and chromosome aberration by itself at relatively high concentrations (13, 15) but also strongly modifies the lethal, mutagenic, and clastogenic effects of other agents at its nontoxic doses (10). Those agents with lethal effects that can be potentiated by caffeine in some mammalian cell systems include UV irradiation (27), X-rays (44), and a variety of alkylating agents (28, 31, 45). These physical and chemical agents have in common the characteristic that the principal cell target is believed to be DNA. Although the molecular mechanism for caffeine potentiation is not fully understood, published work seems to indicate that the process of postreplication repair of DNA damage is involved (17, 23).

NCS, an acidic protein isolated from Streptomyces carzinostaticus var. F 41 (12), has attracted notice because of a high chemotherapeutic index in experimental leukemia in mice (4) and its therapeutic efficacy in the treatment of acute leukemia (11). This antibiotic, cytotoxic in both bacterial and mammalian systems, has been shown to inhibit DNA synthesis at an early stage of S phase and also to arrest cells in the G2 phase of the cell cycle (8). NCS-induced breakage of existing DNA in cultured cells in vivo (2, 26, 40), together with degradation of extracted DNA in vitro (2, 40), suggests that either the whole or a particular part of the protein molecule interacts directly with cellular DNA and that this is the primary process in its lethal action. A correlation seems to exist between the ability of the drug to induce breakage of cellular DNA in HeLa cells and its inhibition of DNA replication and cell growth (3). However, recent findings that immobilized NCS bound to either agarose or Sepharose retains its lethal activity (16, 24) and also that higher levels (10 μg/ml or more) of NCS inhibit the formation of microtubular paracrystals induced by vinblastine sulfate (7) appear to support the possibility that NCS exerts its cytotoxic action by interacting with receptors on the cell membrane.

To characterize further the principal target for NCS action, we attempted to demonstrate the enhancement by caffeine of NCS cytotoxicity.

MATERIALS AND METHODS

Chemicals. Lyophilized NCS (Lot T-64) was the generous gift of Kayaku Antibiotics Research Co. Ltd., Tokyo, Japan. The antibiotic was stored in the dark at —20° and was dissolved in 0.9% NaCl solution just before use. Caffeine, obtained from Sigma Chemical Co., St. Louis, Mo., was dissolved in 0.9% NaCl solution and sterilized by filtration through a Millipore filter.

Cell Culture. The cell line L1210/V/C (38) was kindly provided by Dr. M. Shimoyama, National Cancer Center, Tokyo, Japan, who established this line from mouse lymphocytic leukemia L1210/V. The stock cultures in sealed flasks were maintained in suspension in Roswell Park Memorial Institute Medium 1640 (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% calf serum (Osaka University Research Institute for Microbial Diseases, Osaka, Japan) and kanamycin (60 μg/ml).

Growth Curves. Exponentially growing cells were treated with NCS for 60 min at 37°, harvested by centrifugation, washed in fresh medium, and resuspended in growth medium without kanamycin. The cells, which were diluted so that 1.8 ml contained approximately 3.2 x 10^6 cells, were dispensed into a series of sealed test tubes (15 x 150 mm) with or without appropriate concentrations of caffeine in 0.2 ml. The cultures were incubated for 5 days at 37° in a revolving-drum incubator without dilution with fresh medium. Viable cell numbers in triplicate tubes were measured every 24 hr by counting the cells excluding 0.25% trypan blue in a hemocytometer.

Colony Formation. Following the exposure to NCS for 60 min at 37°, L1210 cells were washed, resuspended in Roswell Park Memorial Institute Medium 1640, and given appropriate dilution with the same medium. Two ml of the cell suspension were added to 0.5 ml of calf serum and 0.2 ml of various concentrations of caffeine, kept at 38°, and then added to 0.4 ml of 2.4% Bacto-agar (Difco Laboratories, Detroit, Mich.) that had been kept at about 50° beforehand. This complete soft-agar medium was poured into a Falcon No. 3001 tissue culture dish (35 x 10 mm) and left at room temperature for a few min to solidify. The dishes were incubated in a humidified incubator...
with a constant flow of 5% CO₂ in air at 37° for 14 days, by which time colonies of about 1 to 2 mm appeared and were counted. The surviving fraction was expressed as the ratio of plating efficiency of treated cells over that of control cells. Dose-response curves were analyzed by the use of the mean lethal dose, D₀, defined as the concentration required to reduce the survival to a factor of 0.37 in the linear portion of the curve; extrapolation number, n, defined as the intersection with the log survival axis; and quasithreshold dose, D₅₀, defined as the intercept of the extrapolation of the linear portion with the 100% survival ordinate.

**Alkaline Sucrose Gradients.** Methods developed by Sawada and Okada (35) were adopted with slight modification. Following the exposure to NCS for 60 min, L1210 cells were incubated at 37° in [³H]thymidine (10 μCi/ml; 20 Ci/mmol), with or without various concentrations of caffeine for 4 hr. The 5-ml gradients of 5 to 20% sucrose containing 0.3 M NaOH, 0.7 M NaCl, 0.001 M EDTA, and 0.01 M Tris (pH 13) were prepared over a 0.2-ml cushion of 2.3 M sucrose. At the top were 0.2 ml of 0.5 M NaOH, 0.14 M NaCl, 0.12 M sodium citrate, and 1% sodium deoxycholate into which approximately 2 × 10⁶ cells were gently layered. After remaining at 4° for 17 hr, the samples were spun for 120 min at 38,000 rpm in a Hitachi RPS 40 rotor. About thirty 10-drop fractions were collected directly from the top of the tube onto disc filters (Whatman No. 3MM; 2.4 cm in diameter) using a Mitsumi-Gradienter. The discs were washed with cold 10% trichloroacetic acid, ethanol, and acetone. After drying, the discs were counted in a scintillation spectrometer.

**RESULTS**

**Effect of Caffeine on Cell Growth after Treatment with NCS.** Growth curves for L1210 cells in a typical experiment in the presence of various concentrations of caffeine are presented in Chart 1. The control without caffeine showed a doubling time of approximately 24 hr under the condition used. After drying, the discs were counted in a scintillation spectrometer from the top of the tube onto disc filters (Whatman No. 3MM; washed with cold 10% trichloroacetic acid, ethanol, and acetone). The concentration of NCS is shown on each chart.

The control without caffeine showed a shoulder, with D₀, D₅₀, and n values of 0.018 μg/ml, 0.021 μg/ml, and 2.3, respectively. The shoulder was substantially reduced, and the slope became slightly steeper when the treated cells were plated into caffeine-containing medium; (D₀, 0.003 μg/ml; D₅₀, 0.013 μg/ml; n, 1.3).

**Sedimentation Profiles of Newly Synthesized DNA in L1210 Cells after Exposure to NCS.** To determine whether caffeine prevents nascent DNA from being joined together, the sedimentation profiles of DNA molecules, which were synthesized in the presence or absence of caffeine, were analyzed by an alkaline sucrose gradient technique. The representative growth rate during the 5-day culture. Chart 2 shows that exposure to NCS (0.01 μg/ml) for 1 hr hardly inhibited cell growth in the absence of caffeine, whereas this dose of NCS suppressed to an appreciable extent the growth rate in the presence of 0.5 mM caffeine at least for the initial 2 days. Although the cells which had been exposed to NCS (0.05 μg/ml) for 1 hr did not resume a normal growth rate, a partial increase was noted between Days 2 and 3 in the medium without caffeine. At this dose of NCS exposure, the subsequent treatment of the cells with 0.5 mM caffeine resulted in a progressive decrease in viable cell counts during the whole culture period. The statistical analysis of the results from the repeated experiments indicated that the differences in viable cell counts between caffeine-treated and nontreated groups were significant only at Days 2 and 3 (p < 0.05) for NCS (0.01 μg/ml) and at Days 3, 4, and 5 (p < 0.01) for NCS (0.05 μg/ml). The appearance of so-called giant cells was noted during the progressive decrease of caffeine-treated cultures.
results are presented in Chart 4. The sedimentation profile of DNA synthesized by the nontreated cells in the absence of caffeine (profile not shown) was indistinguishable from that of DNA synthesized in the presence of 1 mM caffeine, suggesting that caffeine at this dose does not affect the process of maturation of nascent DNA in nontreated control cells. Also there was no, if any, significant difference in the sedimentation profile of newly synthesized DNA between the nontreated cells and those treated with NCS (0.2 µg/ml), whereas the net incorporation of [3H]thymidine during 4 hr incubation was reduced in NCS-treated cells (60.8%) relative to those in untreated or caffeine-only-treated cells. On the other hand, it can be observed that the presence of 1 mM caffeine in the labeling medium resulted in the shift toward lower molecular weight in the sedimentation profile of the DNA synthesized in NCS-treated cells. A similar but less pronounced shift of the sedimentation profile was noted when the concentration of caffeine was lowered to 0.5 mM. It was apparent from the radioactivity incorporated into 2 x 10^5 cells that posttreatment incubation with caffeine partially reversed the decreased incorporation of [3H]thymidine in NCS-treated cells rather than suppress it further (see legend to Chart 4). One possible explanation for this result is the reduced pool size of endogenous thymidine and thus increases the availability of exogenously supplied [3H]-thymidine. We have estimated the effective pool sizes in L1210 cell cultures by varying the concentration of thymidine supplied to the culture, as described by Scudiero et al. (37). Pool sizes (P) are calculated from the equation

\[ P = \frac{T_2 - T_1}{Q - 1} \]

where Q is the ratio of radioactivity incorporated into cells at Specific Activities 1 and 2 (cpm1/cpm2) and T1 and T2 are the concentration of thymidine exogenously supplied at Radioactivities 1 and 2, respectively. The calculated pool size was larger in the culture treated with NCS plus caffeine than in the culture treated with only NCS (Table 2). Within the limit of the validity of the method used, we conclude, therefore, that the partial reversion in caffeine-treated cells of NCS-induced inhibition of [3H]thymidine incorporation does not result from the lowered level of endogenous precursor for DNA synthesis. Hence, the abortive maturation of nascent DNA in NCS plus caffeine-treated cells cannot merely be due to a decreased rate of DNA synthesis and may be ascribed to caffeine inhibition of postreplication repair of NCS-induced lesions.

**DISCUSSION**

The present results demonstrate the existence of a caffeine-inhibitable restoration process against NCS-induced lesions in L1210 cell. Generally, the growth curves represent the population integrity of asynchronous cell population, while the colony-forming capacity represents the reproductive integrity of
individual cells. In this study, both of these parameters indicated that posttreatment of L1210 cells with nontoxic doses of caffeine enhanced NCS cytotoxicity. As to the colony-forming capacity of NCS-treated cells, we previously reported in a preliminary communication that posttreatment incubation of a shorter period (48 hr) with caffeine was also synergistic at 2 concentrations of NCS (34). Treatment of L1210 cells with NCS results in the dose-response curve which has a smaller shoulder followed by an exponential decrease in survival. The effect of caffeine on this NCS survival curve was a substantial reduction of the initial shoulder together with a slight decrease in the mean lethal dose ($D_0$). We adopted the nontoxic dose of caffeine in assessing the dose-survival relationship of NCS-treated cells, since we wanted to avoid any additional effect of caffeine cytotoxicity in the synergism. The quasithreshold dose ($D_0$) of NCS might have been completely abolished at higher concentrations of caffeine which themselves caused slight killing and inhibited cell growth. Although recovery from sublethal damage after X-ray treatment is known to be associated with the size of the initial shoulder of the dose-response survival curve (9), it is still not clear whether the same relationship is applicable to treatments with chemicals. The existence of a shoulder may reflect more about drug concentrations and the time required for entry of the drug into the cells and interaction with a target molecule than it does about the accumulation and repair of sublethal damage (1). Split-dose experiments with Chinese hamster cells showed no increase in survival in spite of the presence of an apparent shoulder and suggested that accumulation or recovery of sublethal damage did not exist for NCS in this type of cell (33). Therefore, the initial shoulder width on the NCS survival curve probably has nothing to do with sublethal damage and is related only to caffeine-sensitive recovery (27). It has been reported recently, however, that postirradiation incubation with caffeine results in complete removal of $D_0$ without significant alteration of $D_0$ in X-ray survival curves of a variety of mammalian cells (44). Thus far, the relationship between restitution of sublethal damage and caffeine-sensitive recovery still remains to be known. Split-dose experiment in the presence and absence of caffeine may elucidate this question.

Evidence for the existence of caffeine-sensitive recovery in mammalian cell was provided for the first time by Rauth (37), who showed that in mouse fibroblast L-cells the addition of caffeine to cultures of UV-irradiated cells resulted in an increase in killing. As to the molecular mechanism for this process, it has been shown that the enhancement in killing by caffeine may be due to the inhibition of postreplication repair which results in an increase in the amount of chromosomal aberrations in UV-irradiated cells (25). Moreover, the level of inhibition by nontoxic dose of caffeine of postreplication repair in UV-irradiated cells inversely correlates with $C_0$, the concentration of caffeine necessary for reducing $D_0$ to a factor of 0.37 (10). Postreplication repair has also been reported to be functioning against DNA lesions induced by X-ray (14) and alkylating agents (31). Our experiments showed that caffeine inhibited the eventual maturation of DNA strands synthesized during 4 hr postincubation of NCS-treated cells. The existence of postreplication repair has been demonstrated in 2 ways: (a) "filling-in of daughter-strand gaps," defined as an eventual increase in size of pulse-labeled DNA from damaged cells up to that from control cells after a chase in cold medium (17, 23); and (b) the recovery of the ability to synthesize high-molecular-weight DNA at late times after treatment (19, 23). The size of DNA obtained by continuous labeling of NCS-treated cells for 4 hr in this study probably reflects the combination of both events. Since the exact nature of postreplication repair mechanism has been in dispute, it might be argued that this mechanism depends on another type of repair process or that the effect of caffeine on postreplication repair secondarily arises from the primary inhibition by caffeine of excision repair or of the rejoining of single-strand DNA breaks. It was recently found that the presence of 4 mM caffeine for the first 3 days postincubation markedly reduced the survival of UV-irradiated adenosine 2 in the normal fibroblasts and in excision-proficient xeroderma pigmentosum variant cells but had no effect in excision-defective classical xeroderma pigmentosum fibroblasts (6). More recently, the removal of 7-bromomethylbenzanthracene from Chinese hamster cell DNA was also shown to be inhibited by the presence of a nontoxic dose of caffeine (30). In contrast to these findings, earlier publications demonstrated that in cultured mammalian cells caffeine does not affect dimer excision (29, 43), unscheduled DNA synthesis and repair replication induced by UV irradiation (5, 22) and alkylating agents (32), and the rejoining of $gamma$-ray-induced single-strand DNA breaks (18, 36). The unscheduled DNA synthesis in human unstimulated lymphocytes after treatment with NCS is resistant to 2 mM caffeine (42). The effect of caffeine on the rejoining of NCS-induced single-strand breaks of cellular DNA remains to be known. At present, the possibility is not excluded that as yet unknown mechanisms other than postreplication repair can be involved in caffeine-sensitive recovery.

We have used murine leukemia L1210 cells, because of the facts that a wealth of experimental chemotherapy data already exists describing their susceptibility to the currently available anticancer drugs (39) and that principles on treatment schedules deduced from this experimental leukemia can be well extrapolated to clinical chemotherapy of human cancer including acute leukemia. It should be borne in mind, however, that rodent cells in cultures exhibit only marginal DNA excision repair capacity as compared with human cells (19, 43). Furthermore, caffeine inhibits postreplication repair extensively in

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**Table 2**

**Effective thymidine pool size in L1210 leukemia cell cultures**

The effective thymidine pool ($P$) was measured as described by Scudiero et al. (37). L1210 cells (4 x $10^5$ cells/ml) were treated for 30 min with caffeine, NCS, or NCS plus caffeine and then labeled for 30 min with $[^3H]$thymidine (10 μCi/ml) at 0.263 μM ($T_1$) or 1.502 μM ($T_2$). The cells were then harvested and washed, and total acid-insoluble radioactivity (cpm, or cpm$_2$) in 4 x $10^5$ cells was determined. The ratio of radioactivity incorporated (Q) at the 2 different concentrations of exogenous thymidine ($T_1$ and $T_2$) was obtained from cpm$_1$/cpm$_2$ and was utilized in the equation

$$P = \frac{T_2 - T_1Q}{Q - 1}$$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>cpm$_1$</th>
<th>cpm$_2$</th>
<th>Q</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>33,211</td>
<td>23,849</td>
<td>1.39</td>
<td>2.89</td>
</tr>
<tr>
<td>Caffeine (1 mM)</td>
<td>35,107</td>
<td>21,433</td>
<td>1.64</td>
<td>1.68</td>
</tr>
<tr>
<td>NCS (0.2 μg/ml)</td>
<td>22,766</td>
<td>16,718</td>
<td>1.36</td>
<td>3.16</td>
</tr>
<tr>
<td>NCS (0.2 μg/ml) + caffeine (1 mM)</td>
<td>26,809</td>
<td>22,193</td>
<td>1.21</td>
<td>5.70</td>
</tr>
</tbody>
</table>

* Mean from triplicate cultures.
rodent cells but very slightly in human cells with the exception of xeroderma pigmentosum cells (20). Therefore, the simple extrapolation of experimental results from rodent cells to human cells may be inappropriate as far as DNA repair process is concerned. The combined use of caffeine with NCS does not seem practicable at this point in time.

Recent studies using DNA repair-deficient bacteria have suggested that a rec A gene-mediated process plays a major role in the recovery from NCS-induced lesions in DNA (41). Moreover, the rejoining of the NCS-induced single-strand breaks in DNA was noted with the use of HeLaS\textsubscript{2} cells (26), and unscheduled DNA synthesis was detected in human peripheral lymphocytes treated with the antibiotic (42). Although it has been exceptionally reported that caffeine also enhances the growth-inhibitory effects of the antimetabolites which do not directly interact with existing DNA (21), there is considerable evidence that DNA is a critical target for the primary mechanism of the lethal action of NCS.

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REFERENCES


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