Lethal Activity of Camptothecin Sodium on Human Lymphoma Cells

B. Drewinko, E. J Freireich, and J. A. Gottlieb

INTRODUCTION

CS<sup>2</sup> is a plant alkaloid, derived from the Chinese tree *Camptotheca acuminata* (26), that has shown antitumor activity against experimental animal neoplasias (3, 11, 16, 25). It is a potent and rapid inhibitor of nucleic acid synthesis; protein synthesis is inhibited only after prolonged incubation with the drug (15, 16, 17, 18, 22). The inhibition of nucleic acid synthesis may be related to a direct effect on the DNA template (15, 18) and it has been reported that CS may cause DNA strand scissions (14). Although 10% of the intracellular CS may be bound to some subcellular macromolecule (21), it is not clear whether it complexes with DNA (11, 15, 18).

The experience with CS in human neoplasia chemotherapy has been disappointing (12, 13, 19, 20). Pharmacological studies have shown that relatively high levels of CS are achieved in human plasma after doses up to 10 mg/kg. These levels decline rapidly and are followed by a much slower plasma disappearance rate; the drug is highly bound to plasma proteins (2, 12). Although there is some knowledge of the effects of CS at the cellular level in mammalian cells (1, 11, 16, 18), there is no such information available on cells of human origin. Differences in the lethal mode of action on human tissue could account for the poor results obtained in the clinical situation. This report describes the survival behavior of a long-term culture of human lymphoma cells exposed to CS.

MATERIALS AND METHODS

An established culture of human lymphoma cells was utilized as the target cell line (23). The line produces a tumor-associated antigen common to lymphomas (24) and is grown in Ham's F-10 medium supplemented with 20% fetal calf serum, glutamine, vitamins, and antibiotics. Under these conditions the doubling time is about 53 hr, the length of the cell cycle is 27 hr; G<sub>1</sub> is 3.5 hr; S is 13.5 hr and G<sub>2</sub> is about 10 hr (9).

CS (NSC 100880) was obtained as a dry powder, manufactured by Ben Venue Laboratories, Inc., Bedford, Ohio, from the Cancer Chemotherapy Branch, National Cancer Institute, NIH. The drug was reconstituted with 10 ml of 0.9% NaCl solution and appropriate concentrations were prepared in growth medium. The pH ranged from 7.1 to 7.4.

Asynchronous cells were exposed to increasing concentrations (1 to 75 μg/ml) of CS for 1, 24, and 48 hr at 37° in a 5% CO<sub>2</sub>, in air, atmosphere. The drug was decanted, and the cells were washed twice with fresh medium and harvested as previously described (10). Single cell suspensions were counted with the aid of a Coulter Model F electronic particle counter (Coulter Electronics, Hialeah, Fla.); aliquots containing known cell densities were dispensed into 60-mm Petri dishes. Appropriate cell concentrations were chosen so that 50 to 100 colonies would appear after a 3-week incubation interval, regardless of the dose point (i.e., about 3000 cells were plated for a dose point with an expected cell kill of 90%). At that time, the supernatant medium was discarded and the colonies were rinsed with 0.9% NaCl solution, fixed in 0.5% crystal violet in 95% ethanol, and counted under a stereomicroscope. Cell survival was defined as the capacity of single cells to give rise to a colony of 50 or more cells. Controls (cells not treated with drug) were run in parallel for each experiment. Percentage survival was calculated in reference to controls with the aid of a Sigma 5 XDS computer. Each experiment was repeated at least twice with 3 dose or time experimental points and 6 control points each. The plating efficiency averaged 30%.

Synchronized cells were obtained by previously described

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The abbreviations used are: CS, camptothecin sodium; TdR<sup>-H</sup>, tritiated thymidine; LI, labeling index; MI, mitotic index.

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techniques (5, 7). Briefly, synchronized S-phase cells were obtained by a single treatment with 3 mM thymidine. Synchrony was monitored by pulse-labeling replicate cultures with TdR-3H, 1 µCi/ml (specific activity, 3.0 Ci/mmole), for 30 min at 2-hr-intervals after synchronization was achieved. After harvesting, slide preparations were made with a cytocentrifuge (Shandon Scientific Co., Sewickley, Pa.) (8), and the cells were stained with 2% aceto-orcein. Radioautograms were prepared by the Ilford K5 liquid emulsion technique and were exposed for 1 week. The preparations were developed in Kodak D19, fixed in acid fix, and mounted with permount. The LI (labeled cells = more than 5 grains overlying the nucleus) and the MI were scored. At the end of the synchrony procedure, 90% of the cells were in S phase and moved synchronously into G2 (80%), estimated from the LI and the MI. After a mitotic peak of 10 to 12% (the MI of asynchronous T1, cells ranges from 0.6 to 1.8), the cells were still partially synchronized in G1 (70 to 80%). Ten to 12 hr after the mitotic peak, the LI began to rise again, although achieving a much lower peak (about 55%) than initially synchronized cells in S. These procedures did not significantly alter the plating efficiency of T1 cells, although they produced a shortening of S and G2 phases and an elongation of G1 phase. Synchronized cells were exposed to 10 and 50 µg of CS per ml for 1 hr at regular intervals throughout the cell cycle. Colony-forming ability was determined as described above. In addition, dose-response survival curves were defined at selected points of the cell cycle, with the use of concentrations ranging from 1 to 75 µg/ml. Cells incubated for 2, 6, and 8 hr in fresh medium, after removal of the synchronizing agent, were selected as representative of early, mid-, and late S-phase cells. Cells incubated for 13 and 15 hr were considered G2-phase populations and, finally, G1 populations were produced by incubation for 19 and 48 hr under normal conditions (3 to 8 hr after the mitotic peak).

RESULTS

Asynchronous cells exposed to increasing concentrations of CS (1 to 75 µg/ml) for 1 hr (Chart 1) revealed an initial rapid decline in survival (mean lethal dose, D50 = 20 µg/ml, obtained by extrapolation) followed by a much slower decrease in survival (D50 = 226 µg/ml, also obtained by extrapolation). When the cells were exposed to CS for 24 and 48 hr, a similar biphasic survival curve was observed. The initial slope of the cells incubated for 24 hr had a D50 of 2.5 µg/ml, while the remainder of the curve had a D50 of 23 µg/ml. After 24 hr of incubation with CS, 5 and 75 µg/ml, the survival had decreased to 23 and 0.8%, respectively. The survival curve of cells incubated with increasing concentrations of CS for 48 hr had an initial D50 of 2 µg/ml, while the rest of the curve was characterized by a D50 of 23 µg/ml. A concentration of 75 µg/ml reduced the survivors by almost 3 log decades.

Synchronized cells exposed to a single CS dose of either 10 or 50 µg/ml revealed fluctuations in survival depending on the stage of the cell cycle. Early and mid-S were very sensitive to the drug, while the survival increased in late S through G2, reaching a maximum in G1 (Chart 2). The cell cycle stage sensitivity effect was further substantiated by determining dose-survival curves at various points of the cell cycle (Chart 3). It is evident that both early and mid-S-phase cells are considerably more sensitive than late S, G2, and G1 cells, with more than 1 order of magnitude of difference between the most sensitive and the least sensitive population. Interestingly, in all stages of the cycle, a biphasic survival curve was obtained, with an initial steep slope followed by a much more shallow one, which sometimes was barely perceptible.

DISCUSSION

We have studied the killing effect of CS on a human lymphoma cell line, as an in vitro model of the activity of the drug once it reaches the neoplastic elements in vivo, in order to define the lethal properties of CS at the cellular level. Cytotoxic effects were analyzed by assessing drug-induced inhibition of the colony-forming capacity of the target cells.

Asynchronous cells exposed to increasing concentrations of CS for 1 hr (Chart 1) revealed a biphasic survival curve. The initial slope was characterized by a D50 of 20 µg/ml (obtained by extrapolation); 10 µg/ml reduced the survivors to 65%. The secondary slope had a D50 of 226 µg/ml; 75 µg/ml decreased the survival to 50%. Exposing the cells to CS for 24 and 48 hr substantially increased the killing effect.
Effect of CS on Lymphoma Cells

100%
50%
10.

Contaminations of population in different stages of the cycle. This is substantiated by the fact that S-phase cells (sensitive) and G2- and G1-phase cells (less sensitive) demonstrated biphasic curves. If these cells had been contaminated with each other in our experiments (i.e., late G2 or early G1, when almost no labeled cells were noted by radioautographic techniques) a continuous exponential curve would have been obtained, the slope of which being the summation of the sensitive and less-sensitive cell-killing slopes. Yet, in all cases we obtained biphasic curves. A similar observation on the same cell line was made with respect to the lethal effects of another chemotherapeutic drug, bleomycin (4).

Our results on synchronized cells are similar to those observed on nonhuman mammalian cells grown in vitro, in that CS appears as an S-phase-sensitive drug (1, 11, 18). The killing effect is rapid and prolonged incubation is not necessary to elicit cell lethality. This is in keeping with the known effects of CS at the molecular level, that is, rapid DNA inhibition independent of precursor molecule inhibition, probably by interference with the DNA template (11, 15, 16, 18). It has also been indicated that cell lethality may be directly related to the inhibiting effect of CS on DNA synthesis (18). These considerations could explain the poor results obtained with CS in human tumors when used as a single agent. CS, being a cell cycle-sensitive agent, could hardly be efficacious in solid tumors, which composed the vast majority of neoplasias in which the drug was tested (13, 19, 20), since most solid tumors have a very small fraction

Chart 2. The effect of 2 concentrations of CS on synchronized T1 cells.

(more than 100-fold after 48 hr of incubation for each concentration point), again in a biphasic fashion. Such curves could be interpreted as representing 2 cell populations, one sensitive and the other less sensitive, within the seemingly homogeneous cellular population. Conversely, the possibility that CS is a cell cycle-specific drug, killing only the cells in the population located in that stage, should also be considered. As was shown with synchronized cells, CS is more lethal on early and mid-S-phase cells, but also kills cells in other stages. Hence, although CS is not a phase-specific drug it can be considered an S-phase-sensitive agent. Pulse-labeling experiments have shown that asynchronous T1 cells contain about 26% cells in S phase. Continuous labeling indicates that after 24 hr, 75%, and after 48 hr, 82% of the cells pass through S phase (6). Thus the killing effect of CS after 1, 24, and 48 hr of incubation was always greater than the proportion of cells in S phase. The difference could be accounted for by its efficacy on cells in other stages of the cell cycle. The presence of a steep and a more shallow slope in the survival curves for all time-exposure modalities, as well as in dose-response survival curves obtained on synchronized cells, suggests the existence of 2 populations with differential sensitivities to CS in the T1 cell line. In experiments involving synchronized cells, there is always some degree of uncertainty about the grade of synchronization achieved by the techniques used. Furthermore, synchronization is an ephemeral attainment since soon, after normal progression is allowed, cells enter into asynchronous growth as a result of the random rates of cellular growth. However, in our system, and judging by the temporal markers used (LI and M1), there was a substantial accumulation of phased cells in the different stages of the cell cycle. Therefore, we believe that biphasic curves elicited in all stages of the cell cycle are the result of 2 separate populations of cells in the T1 cell line and are not due to

Chart 3. The effect of increasing concentrations of CS at selected periods of the cell cycle.
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of proliferating cells. Such cells could be the only ones sensitive to the drug. Even if these cells are completely eradicated by a course of treatment with CS, a great number of viable cells would remain which could repopulate the tumor mass. Clearly, under the usual clinical criteria of evaluation of solid tumor response, CS could not emerge as a valuable addition to the chemotherapy arsenal. Gallo et al. (11) reported that peripheral blood lymphocytes exposed to CS, before phytohemagglutinin stimulation, could not divide after stimulation, hence suggesting CS activity on nonproliferating cells. However, CS may persist within these cells for a considerable length of time, (21) exerting its lethal effect on DNA synthesis and blocking cell progression (18) as soon as stimulated lymphocytes exit the resting stage. Conversely, neoplastic G0 cells may have very little bound CS left at the time they reenter cycle, due to degradation processes, the concentration of which might be insufficient to render these neoplastic cells nonviable. Therefore, it is still doubtful that CS has any significant activity on nonproliferating neoplastic cells, but it most certainly appears potentially effective on proliferating populations, with a major activity on S phase.

It appears, then, that CS may still be proven useful if used in chemotherapeutic regimens utilizing drug combinations wherein CS represents an S-phase-sensitive agent, or in rapidly proliferating tumors containing a greater proportion of cells in S phase.

REFERENCES


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