Lethal and Kinetic Effects of Peptichemio on Cultured Human Lymphoma Cells

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SUMMARY

The lethal and cytokinetic effects of peptichemio (multipeptide complex of m-[di(2-chloroethyl)amino]-L-phenylalanine), a polypeptide derivative of L-phenylalanine mustard, were investigated on a human lymphoma cell line (T1 cells) by means of colony formation and serial DNA histograms. One hr of exposure of exponentially growing T1 cells to increasing concentrations of peptichemio resulted in a biphasic exponential survival curve. Prolongation of drug treatment effected progressive reduction of Do to a minimum of 0.25 μg/ml after 12 hr of incubation. One hr of exposure of synchronized cultures to 2.5 μg/ml killed cells in G1 and G2 more effectively than those in S phase. Cell cycle traverse was delayed in S and, more markedly, in G2. These kinetic perturbation effects increased with increasing drug concentrations and incubation times. Treatment of exponentially growing T1 cells with 10 μg/ml for >12 hr caused a largely irreversible S-phase arrest, reducing the degree of G2 accumulation observed after exposure to lower concentrations and/or shorter incubation times. The position of the cells in the cell cycle at the time of drug incubation (5.0 μg/ml, 1 hr) determined the extent and onset of subsequent G2 delay; cells in G1 and early S phase were blocked in the premiotic phase of their immediate lifespan, whereas late S and G2 cells underwent cell division without significant delay and were arrested in the G2 phase of the subsequent generation.

INTRODUCTION

PC is a multipeptide complex of m-[di(2-chloroethyl)amino]-L-phenylalanine (Chart 1) (7, 8) and thus is closely related to L-phenylalanine mustard, an alkylating agent that has been shown to interfere with cell cycle progression in S and G2 phase (2). The mechanism of action of PC has been attributed to alkylating and antimetabolic properties (8). In extensive clinical trials in Italy, PC induced tumor regression in patients with lung, breast, ovarian, and hematological malignancies (1, 5, 14, 16, 19). At present, the compound is undergoing Phase 1 and 2 clinical trials in the United States.

In this report, we have examined the effects of PC at the cellular level in a human lymphoma cell line (T1 cells). Lethal cell damage was assessed by reduction in colony-forming ability and perturbation of cell cycle progression by DNA distribution analysis. Drug concentration, exposure time, and cell cycle stage at the time of drug administration were important determinants for both cell survival and interference with cycle traverse in S and G2. The cell kinetic effects of PC resembled those observed with L-phenylalanine mustard and Yoshi 864 on this cell line (2, 3).

MATERIALS AND METHODS

Our studies were carried out with the use of T1 cells (18), the culture conditions, harvesting procedures, and present kinetic characteristics of which have been reported previously (9, 11).

Cell survival was assessed by the colony formation technique described in detail elsewhere (17). Drug effects on cell cycle progression were analyzed by serial measurements of cellular DNA distribution. Therefore, ethanol-fixed cells were stained with thymulin (6) and measured in a Phye ICP-11 pulse cytophotometer as previously described (4). We performed a DNA histogram evaluation using a modification of Fried's model (13). In addition, the [3H]TdR labeling index was routinely determined on replicate cultures to test whether cells with S-phase DNA content were actively replicating DNA. Therefore, cells were pulse labeled for 30 min with 1 μCi [3H]TdR per ml (specific activity, 6.7 Ci/mummole) prior to harvesting. Cytocentrifuge preparations were processed for autoradiography with Kodak nuclear track emulsion type NTB2 (diluted 1:3 in distilled water), exposed for 7 days, and stained with May-Grünwald-Giemsa. The labeling index was recorded on 200 cells (labeled cells >5 grains overlying the nucleus). Since G0 and mitotic cells cannot be distinguished on the basis of their DNA content, mitotic index determinations were performed on 3000 cells for each sample.

For analysis of both lethal and kinetic response of T1 cells to PC, 3 variables were tested. The influence of drug concentration and incubation time was investigated in asynchronous populations in their exponential phase of growth. The effect of cell cycle stage at the time of drug administration was examined on cell populations treated with a single...
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24-hr TdR block (3 mM) (12), which has been shown to yield a high degree of synchrony for 1 generation time (4). In particular, T
cells were treated for exactly 1 hr at the following times after release of thymidine block: 2 hr (early S), 6 hr
(mid-S), 10 hr (late S), 13 hr (early G\textsubscript{2}), 15 hr (late G\textsubscript{2}), 24 hr
(early G\textsubscript{1}) and 30 hr (late G\textsubscript{1}).

All experiments were carried out in duplicate, with triplicate and duplicate samples for colony formation and DNA
distribution analysis, respectively.

PC was obtained from Professor A. De Barbieri, Istituto
Sieroterapico Milanese S. Belfanti, Milan, Italy. All drug
solutions were prepared freshly immediately before each
experiment with Ham’s F-10 growth medium (enriched with
20% fetal calf serum and glutamine). Drug exposure was
terminated by decanting the drug-containing medium, rinsing
the Petri dishes twice with Hanks’ balanced salt solution,
and adding fresh medium.

RESULTS

Lethal Effects

Asynchronous Cells. Chart 2 shows the concentration-
dependent survival of asynchronous exponentially growing
T
cells after 1, 3, and 12 hr of incubation with PC. The 1-hr
treatment curve shows a logarithmic reduction in colony
formation with a D\textsubscript{0} of 1.0 \(\mu\text{g/ml}\). After an inflection point
at 2.5 \(\mu\text{g/ml}\), further increments in concentration are associated
with lesser degrees of cell kill (\(D\textsubscript{0} = 3.2 \mu\text{g/ml}\)). Prolongation
of drug exposure for 3 hr effects a steeper although still biphasic dose-dependent survival curve with an inflec-
tion point at the same concentration as for the 1-hr treat-
ment curve. After 12 hr of treatment, the biphasic exponen-
tial curve disappears, and the survival is characterized by a
simple exponential function (\(D\textsubscript{0} = 0.25 \mu\text{g/ml}\)).

Chart 3 presents the survival of asynchronous exponentially
growing T
cells as a function of increasing incubation times for 2 different concentrations (1.0 and 2.5 \(\mu\text{g/ml}\)).
Following an initially steep logarithmic reduction in sur-
vival, there is no further significant increase in cell kill with
prolongation of treatment beyond 12 hr. Control cultures
were treated for 1 hr with supernatant medium from cells
treated for 1, 3, 12, 24, and 48 hr. The survival of these
cell control cells was similar to that obtained after 1 hr of treat-
ment with freshly prepared drug only for the 1- and 3-hr
supernatant fluids. In contrast, the killing efficiency of sup-
ernatant medium after \(\geq 12\) hr of incubation was com-
pletely abrogated.

Synchronized Cells. Synchronized cells treated with PC,
2.5 \(\mu\text{g/ml}\), for 1 hr showed fluctuations in survival as a
function of position in the cell cycle with a \(> 10\)-fold differ-
ence between the most (G\textsubscript{1} and G\textsubscript{2}) and least (S phase)
sensitive cells (Chart 4). This cell cycle stage dependency was somewhat reduced upon increase of drug concentration to 5.0 \( \mu \text{g/ml} \) with larger standard errors, compared with the lower drug concentration.

**Effects on Cell Cycle Progression**

**Asynchronous Cells.** \( T_1 \) cells in exponential phase of growth were incubated with increasing concentrations of PC (0.5, 1.0, 2.5, and 10.0 \( \mu \text{g/ml} \)) for various time periods (1, 3, 12, 24, and 48 hr). The basic kinetic response pattern evidenced from serial DNA histograms consisted of compartment shifts out of \( G_0 \) into \( S \) and \( G_2 + M \). The mitotic index never exceeded control values of 0.5 to 1%, so that any increase of the \( G_2 + M \) fraction was due to an increase of cells in \( G_2 \). Treatment of asynchronous \( T_1 \) cells with PC, 0.5 and 1.0 \( \mu \text{g/ml} \), induced moderate and highly reversible accumulations of cells in \( S \) and \( G_2 \), the magnitude of which was incubation time dependent.

Exposure to PC, 2.5 \( \mu \text{g/ml} \) (Chart 5), caused a rapid decrease of the \( G_1 \) compartment to 25% within the 1st 24 hr after initiation of treatment, which was accompanied by an increase of the \( S \)-phase compartment. These changes were independent of duration of drug exposure. For all incubation times, the peak of \( S \)-phase accumulation was reached at 24 hr. The subsequent rapid decrease of the \( S \)-phase fraction was associated with an increase of cells in \( G_2 \). Treatment for \( \geq 24 \) hr delayed the onset of \( G_2 \) accumulation; however, the slopes of \( G_2 \) increase appeared equal for all incubation times. The final compartment distribution was a function of exposure time; prolongation of treatment from 1 to 12 hr progressively increased the residual \( G_2 \) population while reducing the residual \( G_1 \) population. There was no significant difference between treatments for 12, 24, and 48 hr.

Chart 6 plots compartment distribution over time for \( T_1 \) cells exposed to PC, 5.0 \( \mu \text{g/ml} \), for increasing time periods. The pattern of compartment fluxes is similar to that observed after treatment with 2.5 \( \mu \text{g/ml} \) (see Chart 5). The extent of final \( G_1 \) evacuation and \( G_2 \) accumulation increased further for short-term exposures of 1 and 3 hr, with some reversibility (20%) of compartment changes after 1 hr of treatment. The transient \( S \)-phase accumulation shows only a slight increase compared to the lower concentration of 2.5 \( \mu \text{g/ml} \).

Further escalation of drug concentration to 10.0 \( \mu \text{g/ml} \) (Chart 7) did not affect the course of \( G_2 \) efflux. However, in contrast to treatment with lower concentrations, prolongation of treatment beyond 3 hr induced a sustained \( S \)-phase accumulation; e.g., for a 48-hr treatment, 40% of the total population was comprised in \( S \) phase at the end of the experiment (150 hr). Along with this effect on the \( S \)-phase compartment, there was a change in the \( G_1 \) accumulation pattern as a function of duration of treatment: maximal values of \( G_2 \) accumulation were observed after short-term treatment of 1 and 3 hr. Incubation of \( T_1 \) cells for 12 hr slightly retarded the rate of \( G_2 \) accumulation; 24 and 48 hr of treatment further delayed the onset of compartment transition into \( G_2 \) phase and resulted in \( G_2 \) increments of only 50 to 60%, compared with 85 to 95% for shorter drug exposures. There was no significant difference between DNA histogram-derived \( S \)-phase fractions and \( [\text{H}] \text{TdR} \) labeling index after treatment with concentrations up to and including 5.0 \( \mu \text{g/ml} \); a discrepancy between these 2 parameters emerged in the case of prolonged exposure (24 and 48 hr) to PC, 10 \( \mu \text{g/ml} \) (Chart 8).

**Synchronized Cells.** To analyze the effects of cell age on the kinetic response of \( T_1 \) cells to PC, synchronized cultures were exposed to 5 \( \mu \text{g/ml} \) for 1 hr at selected stages of their cell cycle (Chart 9). Cells treated in early and mid-\( S \)-phase with a more pronounced \( G_2 \) increment of 70% in the progeny after exposure of progenitor cells in early \( G_2 \). Incubation of cells in \( G_1 \) effects a kinetic response within the immediate life cycle, very similar to the cycle progression pattern of daughter cells through \( S \) and \( G_2 \) after treatment in late \( S \) phase. The same phase sensitivity pattern with less pronounced, and more transient \( G_2 \) accumulation was observed after 1 hr of treatment with PC, 2.5 \( \mu \text{g/ml} \).

**DISCUSSION**

Although PC is a congener of \( L \)-phenylalanine mustard, there are essential differences for both lethal and kinetic effects induced by these 2 compounds. Thus, the dose-response survival curve of PC lacks a threshold part observed after treatment with \( L \)-phenylalanine mustard (\( D_\text{50} = 0.5 \mu \text{g/ml} \) (2)). This indicates the inability of \( T_1 \) cells to absorb sublethal damage induced by PC. Instead, a bi-
phasic exponential-type curve was observed that suggested the presence of populations with differential sensitivity to PC. Extension of drug exposure to 12 hr abolished this differential sensitivity, resulting in a single exponential survival curve. There was no further reduction in survival, when the period of drug incubation was prolonged from 12 to 48 hr. This was due to inactivation of PC as indicated by the progressive loss of killing efficiency of drug-containing supernatant medium after ≥12 hr of incubation. For cells treated with L-phenylalanine mustard, a similar exponential plateau-type time-response survival curve was observed (2). In contrast to PC, there was no drug inactivation which...
Cytotoxicity and Cell Cycle Perturbation of PC

Hr after TdR-Release

Cytotoxicity and Cell Cycle Perturbation of PC equally and maximally susceptible to lethal cell damage, while L-phenylalanine mustard exerted a similar reduction in survival only after exposure in early G1 phase (2).

Interference with cell cycle traverse by PC resembled perturbation effects induced by other agents investigated in this cell line (2, 10). Thus, the major kinetic response was a block in G2 transit, the extent and duration of which was a function of drug concentration and exposure time. The compartment shift out of G1 into G2 phase was modulated by a concentration and incubation time-dependent retardation in S-phase traverse, accounting for a delay in onset of G2 accumulation. There was no perturbation of the efflux of cells out of G1 into S phase at any concentration and exposure time studied. This indicates that PC does not affect initiation of DNA replication but, rather, slows down DNA synthesis; after a >12-hr treatment with 10 μg/ml, a largely irreversible block of cell traverse in S phase develops, as indicated by the discrepancy between S-phase fraction and [%H]Tdr labeling index (Chart 8). As with L-phenylalanine mustard (2) and Yoshi 864 (10), both magnitude and time of manifestation of G2 block were a function of cell cycle stage at the time of drug administration. Whereas, for L-phenylalanine mustard and Yoshi 864, only the cycle stages of greatest lethal susceptibility coincided with those sensitive to induction of G2 block in the immediate life-span, for PC there was an additional stage-sensitive cytotoxic effect in G2, which was not accompanied by a transit delay in the same stage.

In conclusion, while sharing certain features of lethal and kinetic effects with the related compound L-phenylalanine mustard (i.e., similar D0, S, and G2 delay), T1 cells lack the ability to absorb sublethal damage from PC characteristic for alkylating agents investigated in this cell line. The phase sensitivity patterns for lethal and kinetic response do not coincide and are different from those observed for L-phenylalanine mustard.

With regard to clinical application of the above information, our data suggest that, dependent on the tumor cell kinetics, a 24- to 48-hr continuous infusion at an intermediate dose level should be explored. Such a schedule may be advantageous to sterilize tumor cells with less sensitivity to PC. Due to the largely irreversible block of cycle progression in G2 phase, PC should not be considered for use as a synchronizing agent.

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