Kinetic Response of Human Lymphoid Cells to Adriamycin-DNA Complex in Vitro

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SUMMARY

The lysosomotropic agent adriamycin-DNA complex was analyzed for its effect on cell cycle progression of human lymphoid cells in culture by means of pulse cytophotometry. Complexing to DNA slightly reduced the perturbation effects previously reported for adriamycin alone. The major kinetic response was a G2 block, the magnitude and duration of which was dependent on drug concentration and duration of treatment. When high drug concentrations were maintained for a prolonged period of time, an additional, completely reversible block in G1 phase or at the G1-S boundary was observed, accounting for two-step G1 accumulation curves. Cell age markedly influenced the magnitude of G1 accumulation in that treatment of cells in S and early G2 phase was most effective.

INTRODUCTION

The introduction of anthracycline antibiotics into cancer chemotherapy has clearly improved response rates and survival in a variety of human cancers (4, 7). ADR2 has been particularly successful in the treatment of metastatic tumor of the breast, in sarcoma, in lymphoma, and in acute leukemia. Due to severe dose-dependent cardiomyopathy, the application of ADR has been limited to induction therapy rather than to long-term maintenance (8). Thus, not infrequently, ADR must be discontinued despite continuing tumor responsiveness. In 1972, Trouet et al. (10) developed their concept of "lysosomotropic" agent chemotherapy. Linkage of an antitumor agent to a carrier compound such as DNA impairs cellular uptake of this complex unless cells exhibit a marked endocytic activity with which some tumor cells are endowed. After incorporation into the cells, the complex must be digested by lysosomal enzymes to release the free drug. A differential endocytic activity of normal host and tumor cells would provide target cell selectivity. In a recent comparative analysis of ADR and ADR-DNA complex in L1210 leukemic DBA/2 mice, Trouet et al. (11) documented an increase in the therapeutic index for the lysosomotropic complex which was, unlike that for daunorubicin-DNA, independent of the route of administration. Atassi et al. (1) investigated the free drug and ADR-DNA complex in 2 different mice strains with L1210 leukemia and obtained therapeutic enhancement of the complex only in 1 strain. Subsequently, Rozenweig et al. (9) showed that the superiority of the lysosomotropic compound over ADR alone depends on strain of mice, L1210 line, and route of tumor inoculation. Preliminary pharmacokinetic studies by these authors in mice and humans indicate a delayed plasma clearance of ADR-DNA complex in a more linear curve compared to the biphasic disappearance curve of ADR alone. Cardiotoxicity could not be evaluated in this trial, since the total dose of ADR administered with the complex was too low.

Recently, we have analyzed the kinetic response of cultured human lymphoma cells to ADR and found a highly irreversible cell-cycle-stage-independent block in G2 phase (2). Prolonged exposure to high drug concentrations induced additional perturbation effects, namely, a delay in S phase transit and a reversible block at the G1-S transition or in G2. The present study was carried out to test the effect of complex formation of ADR to DNA on cell cycle progression as a function of drug concentration, exposure time, and cell age in this lymphoma cell line. For reasons of comparability, the concentrations of ADR in the complex were kept identical to those in the previous study. ADR-DNA complex induced kinetic responses qualitatively similar to free ADR. However, degree and duration of G1 accumulation were reduced, and cell age became an important determinant in that cells treated in S and early G2 phase were most effectively blocked in G2.

MATERIALS AND METHODS

A human lymphoma cell line (T~ cells) was grown as monolayer in Ham's F-10 medium supplemented with 20% fetal calf serum, glutamine, vitamins, and antibiotics. The environment was adjusted to 37°, and a 5% CO2 atmosphere was maintained. Under these conditions, recently tested kinetic parameters were: cell cycle time Tc = 31 hr with Tg, = 11 hr, Tn = 10 hr, Tg1 = 10 hr, and TM = 0.43 hr (2, 6). The cultures were allowed to reach exponential growth (48 hr), before drug perturbation experiments were started.

The influence of drug concentration and exposure time was analyzed in asynchronously growing cultures, both during drug incubation and after drug removal (2 washes with Hanks' balanced salt solution and replacement of fresh...
medium). TdR-synchronized populations [3 mM TdR for 24 hr (5)] were treated for 1 hr at various time intervals after release of the synchronizing agent; kinetic analyses were performed at 12-hr intervals for the 1st 60 hr and later on at 24-hr intervals for a total time period of 150 hr following release of TdR block.

Kinetic analysis utilized sequential DNA histogram measurements by PCP, with mithramycin as fluorescent dye; 30,000 to 50,000 cells were measured for each DNA histogram (3). For determination of MI, 3,000 cells were scored.

ADR-DNA complex was prepared as follows. ADR (Farmitalia Co., Milan, Italy) was dissolved in sterile distilled water to a concentration of 10 mg/ml. Herring sperm DNA (highly polymerized, type VII; Sigma Chemical Co., St. Louis, Mo.), at a concentration of 2.34 mg/ml in 0.9% NaCl solution, was kindly provided by Dr. A. Trouet, Laboratoire de Chimie Physiologique, Université de Louvain, Louvain, Belgium. The DNA solution was autoclaved for 15 min at 120°C and cooled slowly. One-tenth ml of a 10-mg/ml aqueous solution of ADR was dissolved in 5 ml of DNA solution. Further dilution was performed with 0.9% NaCl solution and medium, respectively, for the appropriate concentrations of ADR, i.e., 0.05, 0.25, and 0.5 μg/ml. Thus, a constant 20:1 molar ratio of DNA mononucleotide to ADR was maintained for all ADR concentrations (11). In a few instances, the effect of DNA in concentrations present together with the 3 different ADR concentrations was tested alone for perturbation of cell cycle progression.

Each experiment was carried out in duplicate samples. The evaluation of DNA histograms was performed as previously described (3). The standard deviation of the DNA histogram distribution did not exceed 1.5% for the G1, S, and (G2 + M) compartments.

RESULTS

Asynchronous Cells. Asynchronous cultures of T, cells were exposed to ADR-DNA complex with 0.05 μg ADR per ml for increasing time periods of 1, 3, 12, 24, and 48 hr (Chart 1). Cells were harvested and processed for PCP both during and after drug incubation. The presence of the drug is indicated by the solid lines; values obtained after drug release are connected by dashed lines. Cells were accumulated in the (G2 + M) compartment at the expense of the G1, S fraction. Since the MI of treated populations never exceeded control values (0.5 to 1%), the (G2 + M) fraction mainly represents G2 cells. Magnitude and duration of G2 accumulation increased with prolongation of treatment; a maximum of 85% was reached after incubation for 48 hr, 72 hr after drug addition. The rates of G1, S evacuation and G2 accumulation were not impaired by extension of drug exposure. Treatment for ≤12 hr resulted in eventual recovery of the G2 accumulation with subsequent decrease of the G1, S compartment size to pretreatment values. Incubation for 24 hr permitted only partial recovery with a residual G2 accumulation of 45%. There was virtually no reversal of the kinetic response induced by the 48-hr treatment. A transient increase of the S-phase compartment to 20 to 30% at 24 hr was followed by a steady decrease with minor fluctuations to minimum values of 2 to 5% after 48 hr drug exposure. The fluctuations in S-phase compartment size after treatment for 1 and 3 hr were similar to those observed in control (untreated) populations.

Incubation of T, cells with 0.25 μg ADR per ml in the lysosomotropic complex caused more pronounced compartment shifts (Chart 2). Thus, after treatment for 1 and 3 hr, G2 peak values of 35 and 55% were obtained at 24 and 36 hr, respectively, compared to 20 and 28% at 24 hr after exposure to 0.05 μg/ml. Complete recovery from G2 accumulation was only observed after 1 hr of treatment. Incubation for 3 hr resulted in a sustained G2 accumulation of 35%. Prolongation of drug exposure beyond 12 hr caused a biphasic-shaped curve of G2 accumulation with similar initial rates and intercepts plateauing at 40 to 45% at 24 hr. The duration of these plateau parts of G2 compartment curves was related to the time of drug exposure, i.e., 10 hr for 12 hr treatment, 24 hr for 24 hr treatment, and about 48 hr for 48 hr treatment. Thus, peak values of G2 accumulation after treatment for ≥12 hr were reached at 48 hr for 12 hr incubation (60%), at 72 hr for 24 hr incubation (70%), and at 170 hr for 48 hr incubation (85%). The inclinations of the 2nd slope of G2 increase were the same after 12, 24, and 48 hr incubation, indicating equal rates of G2 accumulation after a transient plateau. The size of the G1, S compartment declined steadily at an equal rate after 1 and 3 hr incubation, reach-
ing minimal values of 40 and 30% at 24 and 36 hr, respectively, with subsequent recovery to pretreatment values after 1 hr and to 60% after 3 hr treatment. Following an initial decline of 10% for ≥12 hr incubation, a plateau of 50% G1 compartment cells was reached at 12 hr from time of drug addition. This plateau lasted for 24, 36, and 60 hr for 12-, 24-, and 48-hr incubations, respectively. Subsequent evacuation rates were equal, with partial recovery after 12 hr treatment to about 40%. The final levels of residual G1 compartment values were inversely correlated to incubation time with 70% for 1 hr, 55% for 3 hr, 35% for 12 hr, 20% for 24 hr, and 10% for 48 hr treatment. The course of S-phase compartment size showed a transient increase, similar to treatment with 0.05 μg/ml. Evacuation occurred earlier after treatment for ≥12 hr (at 12 hr) compared to treatment for 1 and 3 hr (at 24 hr).

Compartment shifts after treatment with 0.5 μg ADR per ml (as ADR-DNA complex) were further enhanced (Chart 3). Again, as with 0.25 μg ADR per ml, both minimum G1 compartment values and maximum G2 values were incubation time dependent. Treatment for ≥12 hr induced a 2-step increase of the G2 compartment with equal initial and subsequent rates. The duration of the intercept plateau (between the initial and subsequent ascending limbs of the G2 accumulation curve) was positively correlated to exposure time with 24 hr for 12 hr incubation, 48 hr for 24 hr incubation, and 72 hr for 48 hr incubation. The G1 compartment declined promptly to 35 and 20% following treatment for 1 and 3 hr. Similar to treatment with 0.25 μg/ml, the initial decrease of the G1 compartment after ≥12 hr incubation with 0.5 μg/ml resulted in a plateau of 50% 12 hr from drug addition and a transient 20% increment to 70% at 72 hr in case of 48 hr drug exposure. Thus, subsequent decline was delayed for 24 hr for 12 hr treatment, for 60 hr for 24 hr treatment, and for 84 hr for 48 hr treatment. There was no significant difference in the inclination of all slopes of Gy, efflux. Minimum Gy, values of 20% were equal for 12, 24, and 48 hr treatment. The decrease of the S-phase compartment subsequent to a slight transient increase occurred at 12 to 24 hr after initiation of treatment. There were minor fluctuations of the S-phase compartment size exhibiting another peak at 48 hr (12 hr incubation), at 72 hr (24 hr incubation), and at 120 hr (48 hr treatment).

Maximum G2 accumulation and time to G2 maximum as functions of drug concentration for the different incubation times are summarized in Table 1. Concentration and exposure time are positively correlated to the magnitude of G2 accumulation. Extension of drug exposure to 48 hr abolishes the influence of concentration. The time course to G2 maximum is delayed by an increase in both concentration and incubation time.

When T1 cells were exposed to DNA for 1, 3, 12, 24, and 48 hr in concentrations used in 0.05, 0.25, and 0.5 μg ADR-DNA complex per ml (see "Materials and Methods"), compartment distribution profiles were equal to those of control populations.

Synchronized Cells. TdR-synchronized cultures were treated at selected stages of the cell cycle with 0.5 μg ADR-DNA complex per ml for 1 hr; i.e., 2 hr (early S phase), 6 hr (mid S phase), 10 hr (late S phase), 13 hr (early G2 phase), 15 hr (late G2 phase), and 24 hr (G1 phase) after TdR release. Compartment distribution was monitored at 12 hr and, from 60 hr after TdR release, at 24-hr intervals for a total period of 150 hr after release of TdR block.

Chart 4 shows DNA histograms of synchronized T1 cells harvested at the time of G2 maximum of control cultures

Table 1

<table>
<thead>
<tr>
<th>Exposure Time (hr)</th>
<th>Maximum G2 Accumulation (%)</th>
<th>Time to Maximum G2 Accumulation (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 μg/ml</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>0.25 μg/ml</td>
<td>28</td>
<td>24</td>
</tr>
<tr>
<td>0.5 μg/ml</td>
<td>32</td>
<td>36</td>
</tr>
</tbody>
</table>

* In case of prolonged G1 accumulation, the earliest G2 value with all subsequent G2 fractions not to exceed 10% was considered as maximum G2 accumulation.

#Time to maximum G2 accumulation was calculated from time of drug addition.
mements, accumulation of cells in the G₂ fraction indicates a delay in G₂ phase transit. Both magnitude and duration of G₂ block were a function of drug concentration and incubation time. There was no recovery from G₂ accumulation when T₁ cells were treated with ≥0.25 μg/ml for ≥3 hr (Chart 2). The pattern of G₂ accumulation was modified by

(81%) after treatment in early, mid, and late S phase. The G₂ compartment size of cells treated in early and mid S phase is reduced to 40 and 16%, respectively, due to retardation in late S phase. When treatment was initiated in late S phase, the delay in S-phase transit was only marginal. At the time of G₁ for control populations (i.e., 24 hr after TdR release), 97 to 98% of the cells were accumulated in G₂ phase, independent of whether the drug had been administered in early, mid, or late S phase (Chart 5).

Chart 6 outlines the magnitude of G₂ accumulation as a function of cell age. The total height of the bars indicates the maximal degree of G₂ accumulation achieved during the entire period of the experiment. There is a marked phase sensitivity in that S and early G₂ phase cells can be most effectively blocked in the immediate G₂ phase (95 to 100%). When treatment was initiated in late G₂ and G₁ phase, a maximum of 25 and 45% of the total cells was accumulated in G₂ phase at the time that control cells traversed into G₂ phase. The slashed segments of the bars indicate the extent of recovery following maximal G₂ accumulation, which amounted to 15 to 20%, when the drug was administered to cells in G₁, early S, and early G₂ phase.

DISCUSSION

The major kinetic response obtained after treatment of T₁ cells with ADR-DNA complex was accumulation in G₂ at the expense of the G₁₉₅ compartment. Since, for a given generation time, the durations of the different phases of the cell cycle determine the distribution of cells in these compart-
an impaired evacuation of the G_{10} compartment after treatment with \( \geq 0.25 \) \( \mu g/ml \) for \( > 12 \) hr. This is indicative of a block in G_{1} or at the G_{1} - S transition which cannot be discriminated by the PCP technique. In the presence of the block in G_{1} or at the G_{1} - S interphase, S phase cells continue to traverse into G_{2} phase, resulting in the initial G_{2} accumulation with a subsequent plateau. The equal inclinations of G_{1} evacuation and G_{2} accumulation curves, independent of drug concentration and incubation time, suggest: (a) an equally effective block in G_{2} phase for all concentrations and incubation times studied; (b) no significant delay in S phase transit, since there was no difference in time of onset of G_{2} accumulation for the various conditions investigated; (c) equal and probably complete recovery from the transient block in G_{1} (or at the G_{1} - S interphase); (d) no impairment of S phase transit for cells temporarily arrested in G_{1} (or at the G_{1} - S transition). The documented delay in S phase transit after perturbation of synchronized cells with 0.5 \( \mu g \) ADR-DNA complex per ml for 1 hr (Chart 4) may imply that TdR synchronization influenced the kinetic response to subsequent drug treatment and/or that this delay may be short-lived and thus may not have been detected in the experiments on asynchronous populations. There was a pronounced phase sensitivity with S-phase cells and cells in early G_{2} phase being most susceptible to the kinetic effect of ADR-DNA complex.

Compared to ADR alone, the presented data on ADR-DNA complex are very similar in that both the free drug and the complex induce cell cycle progression delay in G_{2} phase and a reversible block of the G_{1} - S transition and/or in G_{2} phase after treatment with \( \geq 0.25 \) \( \mu g/ml \) for \( > 3 \) hr (2). However, extent and duration of G_{2} block are more closely concentration and exposure time related, with a smaller degree of G_{2} accumulation after short-term treatment even with 0.5 \( \mu g \) ADR-DNA per ml. In the absence of a significant delay in S phase transit, G_{2} accumulation was not observed until 12 hr from the time of administration of ADR-DNA complex. This delayed onset of G_{2} increase was independent of drug concentration and exposure time. This is in contrast to results obtained with the free drug where treatment with 0.05 \( \mu g/ml \) induced a rapid G_{2} accumulation within the 1st 12 hr of incubation. The most striking difference is the marked phase sensitivity of the G_{2} block displayed after treatment with the lysosomotropic compound, whereas ADR alone induced a similar magnitude of G_{2} accumulation independent of the stage of the cell cycle where treatment had been commenced.

The difference in kinetic response after complexing with DNA may be related to impaired cellular uptake of the complex, suggested by the delayed manifestation of the G_{2} block. This may be a function of cell age, with facilitated incorporation of the drug into S-phase cells. Studies to measure the incorporation rate for both ADR and ADR-DNA complex as a function of cell age are currently under way. The observed differences may also be attributed to impaired cleavage of the DNA carrier from the active drug. We did not find a kinetic response after treatment with DNA alone in the concentrations present in the ADR-DNA complex. Therefore, it appears unlikely that DNA counteracts the effect of ADR. This possibility could be tested by different schedules of combination treatment with ADR and DNA sequentially applied as single agents.

Preliminary results on lethal damage assessed by colony formation indicate that ADR-DNA complex is less effective than ADR alone on an equimolar basis. There was no correlation between cytotoxicity and the extent of cell cycle progression delay in G_{2} phase. Moreover, a high degree of reversibility of G_{2} block (i.e., Chart 1, 12 hr incubation with 0.05 \( \mu g/ml \) was not associated with unimpaired proliferative capacity (90% cell kill). Thus, although cells were only temporarily accumulated in G_{2} phase and were able to resume further cell cycle progression, their growth potential was compromised. A detailed analysis of the effects of ADR-DNA complex on the survival of cultured lymphoma cells as a function of drug concentration, exposure time, and cell age will be the subject of a forthcoming publication.

The cell cycle dependency of ADR-DNA complex in inducing a G_{2} block in this human lymphoid cell line may be disadvantageous in the treatment of human cancer where the majority of tumor cells is in G_{10}, which phase, in this cultured cell line was least sensitive to the perturbation effects of ADR-DNA. Altered pharmacokinetics of the complex, however, may compensate for the drawbacks of phase sensitivity documented in vitro.

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