DNA Synthesis by L929 Cells following Doxorubicin Exposure

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ABSTRACT

Doxorubicin does not kill L929 cells at concentrations that profoundly reduce clonogenic survival. Instead, the cell and nuclear volume progressively increase for at least 1 week following drug exposure leading to the production of characteristic giant cells. The increase in nuclear volume is due to continued DNA synthesis and increase in chromosome number without entry into mitosis. The implications of this finding for in vitro chemosensitivity assays and for the mechanism of doxorubicin cytotoxicity are discussed.

INTRODUCTION

In vitro cytotoxicity assays have proven useful for both preclinical screening of potential chemotherapeutic agents and subsequent detailed studies of the mechanisms by which established drugs act. Assays based on clonogenic survival have been referred to as the “gold standard” in this regard (1) because they provide direct visual confirmation of reduced proliferative capacity following drug exposure. However, because clonogenic survival assays are extremely labor intensive and yield data with a large statistical variance, other methods of measuring cell survival have become popular. For example, inhibition of clonogenic survival and inhibition of DNA synthesis have been correlated with inhibition of clonogenic survival and has been used in a number of recent studies.

Doxorubicin is a major chemotherapeutic agent which has been studied by using cell survival assays based on both clonogenic survival and DNA synthesis. Many cell lines have been studied by both methods with generally consistent results, i.e., inhibition of clonogenic survival and inhibition of DNA synthesis were closely correlated (2–5). However, we noted that although L929 cells exposed to doxorubicin did not form colonies they also were not killed outright. Rather, after incubation for at least 1 week, they could still be found in the cultures as giant cells with a single large nucleus. We now report that after doxorubicin treatment nearly all L929 cells give rise to these giant cells by a mechanism that appears to involve continued DNA synthesis without nuclear division.

RESULTS

Fig. 1 confirms the previous incidental finding that L929 cells become progressively larger following doxorubicin (5 μg/ml) exposure. Approximate nuclear diameter measured with an ocular micrometer increased from 13 to 36 μm over the 8-day course of observation. Dead cells were not observed during the first week following drug exposure, but did become noticeable after 2 to 3 weeks of culture.

Fig. 2 shows an inverse relationship between appearance of these giant cells and the disappearance of clonogenic cells. As the number of giant cells increases, the number of colonies decreases in a dose-dependent manner. In such experiments, at least 60% of the clonogenic cells could be recovered as giant cells after 1 week of incubation. A few (<5%) multinucleate cells were initially present in the cultures and these were considered as giant cells to simplify the accounting. Thus, doxorubicin exposure converted clonogenic cells to giant cells with high efficiency and with little or no immediate cell killing.

Progressively increasing nuclear diameter suggested that DNA synthesis might be ongoing in drug-treated cells. Incorporation of [methyl-3H]thymidine was found to be suppressed immediately after drug exposure (Fig. 3), but gradually resumed and reached 80% of the rate in untreated control cultures by 3 days after exposure. DNA content analysis by flow cytometry (Table 1; Fig. 4) showed that immediately after exposure, when [methyl-3H]thymidine incorporation was strongly suppressed, ≥85% of the cells had a 2c DNA content. With continued incubation, cells with progressively greater DNA contents were observed until 8 days after exposure only 15–20% of the cells had the 2c DNA content and 46% of the cells contained a 4c DNA content. At the 8-day time point, DNA contents up to 8c were detected (25–34%).

Fig. 4 demonstrates the shift in DNA content following...
DNA SYNTHESIS AND DOXORUBICIN EXPOSURE

Fig. 1. L929 cell morphology following doxorubicin exposure. Subconfluent cultures were exposed to 5 μg/ml doxorubicin for 2 h at 37°C, rinsed, and incubated in growth medium for: A, 0 days; B, 4 days; or C, 8 days. Crystal violet stain, x315.

Fig. 2. Survival of L929 cells following doxorubicin exposure. Both clonogenic survival (○) and recovery of giant cells (●) are shown as a fraction of the number of cells plated prior to drug exposure.

Fig. 3. DNA synthesis by L929 cells following doxorubicin exposure. Subconfluent cultures were exposed to 5 μg/ml doxorubicin for 2 h at 37°C, rinsed, and incubated in growth medium. At the indicated times after exposure, cultures were labeled for 4 h with [methyl-3H]thymidine. Points, mean of trichloroacetic acid-precipitable cpm from triplicate cultures; bars, SE.

Table 1 Percentage of cells in each DNA compartment following doxorubicin treatment

<table>
<thead>
<tr>
<th>Time post-treatment</th>
<th>5 μg/ml</th>
<th>10 μg/ml</th>
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<tbody>
<tr>
<td>6 h</td>
<td>G1</td>
<td>G2 + M</td>
</tr>
<tr>
<td>1 day</td>
<td>85.6</td>
<td>8.1</td>
</tr>
<tr>
<td>2 days</td>
<td>52.5</td>
<td>38.1</td>
</tr>
<tr>
<td>4 days</td>
<td>21.2</td>
<td>68.4</td>
</tr>
<tr>
<td>8 days</td>
<td>18.6</td>
<td>53.2</td>
</tr>
</tbody>
</table>

Table 1 *Cells were treated with doxorubicin for 2 h at 37°C, washed, and incubated in drug-free medium. The cells were harvested, fixed, and stained as described in “Materials and Methods.”

peak is evident, suggesting a G2 + M tetraploid population. The majority of the population has shifted to tetraploidy by day 8.

Karyotype analysis attempted within 1 day after doxorubicin exposure failed to show any metaphase cells. Colchicine block and karyotype analysis at later times after drug exposure identified occasional cells with very large numbers of condensed chromosomes consistent with the 4c to 8c DNA contents observed by flow cytometry. Since very few cells were found in any stage of mitosis, doxorubicin does not appear to block events within the M phase per se. We can conclude that the initial G2 arrest and eventual giant cell formation in the L929 system depend on continued DNA synthesis without initiation of mitosis.
Colonies may pass unnoticed. Closely following the fate of these colonies. In this context, the fate of the cells that do not form cell populations and with attention focused on the surviving based assays use an interval of about 4 days between drug arrest within 24 h of drug exposure, but this study was not cin action. Crissman et al. (10) demonstrated G2 or M phase indicated.

DNA content of 10,000 cells was analyzed by flow cytometry at 'he time intervals medium. Cultures were trypsinized to form single cell suspensions, washed, fixed, and stained with propidium diiodide as described in "Materials and Methods." These findings may explain why, for certain cell systems, in vitro cytotoxicity assays based on DNA synthesis do not correlate well with clonogenic survival. Although DNA synthesis-based assays use an interval of about 4 days between drug treatment and determination of labeled thymidine incorporation (1–5), DNA synthesis would not appear to be suppressed in the L929 system even after this interval. At the present time, relatively few cell systems have been examined by both methods under all relevant conditions. Thus, the possibility that apparent resistance to a chemotherapeutic agent is, in fact, due to exceptionally prolonged DNA synthesis by nonclonogenic cells requires especially careful consideration. These multiple rounds of DNA synthesis produce a tetraploid population which may be unstable, leading to chromosome loss and rearrangement. Resistant clones could then be selected from such genetically modified cells.

A similar phenomenon has been observed in other systems. The DNA tumor virus, simian virus 40, stimulates one round of cellular DNA synthesis in most cells (11, 12). However, 20–30% of the infected population are induced into successive rounds of DNA synthesis without intervening mitoses, leading to a population of tetraploid-polyploid cells. Division of Ehrlich ascites tumor cells after treatment with nitrogen mustard (13) and of neuroblastoma cells with sulfur mustard (14) is almost completely inhibited, but DNA synthesis continues and nuclear size increases greatly. That a virus and several drugs affect the regulation and the progress through the cell cycle in a similar manner suggests that they may act through a common pathway.

The findings may also contribute to better understanding of the mechanism of doxorubicin cytotoxicity. It is widely believed that this drug kills cells by inhibiting DNA synthesis, so that continued increase of the remaining cellular constituents leads to eventual death by "unbalanced growth" (15, 16). Our alternative hypothesis based on the response of L929 cells is that cell death results from continued DNA synthesis that is not coordinated with nuclear division. The generality of this mechanism must be evaluated with a variety of chemotherapeutic agents and cell systems. However, the possibility that doxorubicin may specifically block a critical step in the sequence of events leading up to initiation of mitosis is intriguing.

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REFERENCES

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