Tumorigenicity in Vivo and Induction of Malignant Transformation and Mutagenesis in Cell Cultures by Adriamycin and Daunomycin

Hans Marquardt, Frederick S. Philips, and Stephen S. Sternberg

Summary

The two anthracycline antitumor antibiotics, adriamycin and daunomycin, have been tested for tumorigenic activity, and the results confirm previous findings that they can induce mammary tumors in female rats receiving single i.v. doses. Both substances are highly potent in producing malignant transformation and mutation in mammalian cell systems in vitro. Their transforming activity is comparable to that of the potent carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine. Actinomycin D, although similar to the anthracyclines in having high binding affinity for DNA, is only minimally effective in the same in vitro systems and its direct carcinogenic activity in vivo is moot. These results suggest that satisfactory correlations may be obtainable between tests for tumorigenicity in vivo and assays for transformation and mutagenesis in vitro, and that adriamycin and daunomycin may have carcinogenic potential in man.

Introduction

The long-term toxicity of antitumor agents, i.e., their oncogenicity, mutagenicity, and teratogenicity, is a subject of growing concern because of the increased survival of cancer patients which modern chemotherapy has made possible and because of the use of antitumor agents as immunosuppressants in organ transplantation, as well as in the therapy of certain nonmalignant diseases (28). It has been one of the intriguing results of cancer research that active cancer chemotherapeutic agents have been found to be carcinogenic. Haddow was the first to show that carcinogens can be effective against animal tumors (12) and, conversely, that antitumor agents can be carcinogenic (13). Many antitumor agents are now known to be carcinogenic in laboratory animals (31). From observations of patients who have been treated with such substances for nonmalignant diseases or for transplantation surgery, and from the occurrence of 2nd neoplasms after chemotherapy of primary neoplasms, it can be suspected that antitumor agents may also be carcinogenic in man (28).

Recently, the anthracycline antibiotics, daunomycin and adriamycin, which are known to be useful in the therapy of leukemia and some solid tumors, have been shown to induce mammary and renal tumors in rats after single i.v. doses (2, 21, 29). The mammary tumorigenic activity compares well with that of irradiation (26, 27). We now describe (a) a further study of in vivo tumorigenesis by these agents; and (b) their high capacity to induce malignant transformation and mutation in mammalian cells in vitro. We also show that the antitumor antibiotic, actinomycin D, is by contrast relatively ineffective in the same in vitro assays. As we shall discuss later, the last mentioned result suggests that the transforming and mutagenic actions of the anthracyclines are probably highly specific properties of these agents and not merely the consequence of high binding affinity for DNA.

Materials and Methods

Chemicals. Daunomycin and adriamycin were kindly provided by Dr. F. Arcamone (Farmitalia, Milano, Italy); actinomycin D was provided by Merck Sharpe and Dohme, Rahway, N. J. The antibiotics were dissolved in either (in vivo studies) 0.9% NaCl solution or (in vitro studies) phosphate-buffered saline (Grand Island Biological Co., Grand Island, N.Y.) containing, in mg/liter: KCl, 200; KH₂PO₄, 200; NaCl, 8000; and Na₂HPO₄·2H₂O, 1150). N-Methyl-N'-nitro-N-nitrosoguanidine was purchased from Sigma Chemical Co., St. Louis, Mo. Tissue culture media (Eagle's basal medium supplemented with 10% fetal calf serum and penicillin-streptomycin) were obtained from Grand Island Biological Laboratories.

Tumor Induction in Rats. As in the previous work (29), we used 6- to 7-week-old Sprague-Dawley virgin female rats (CD line; Charles River Breeding Laboratories, Brookline, Mass.). Groups of 20 to 25 rats received a single injection into the femoral vein of 10 or 5 mg of daunomycin per kg, 5 mg of adriamycin per kg, or 0.9% NaCl solution. Thereafter, they were maintained and observed as previously described (29). Animals were killed when debilitated, when they had externally apparent tumors, or at 1 year. Rats found dead were not included in the results. The animals were killed under ether anesthesia by exsanguination from the abdominal aorta. All tissues were examined grossly except for the central nervous system. All tumors or masses and all vis-
cera that appeared abnormal were examined microscopically.

**In Vitro Studies.** The M2 clone of mouse fibroblasts was used to determine malignant transformation. It was originally obtained from C3H mouse prostate and was established as a line by procedures described by Chen and Heidelberger (4). This clone has been found to be susceptible to chemical transformation (16, 18). In this work, cells were used between the 11th and 24th passages. The transformation assay was performed as previously reported (18). For assay of transformation and for estimation of plating efficiency, 10^9 and 10^10 cells, respectively, were plated into 60-mm dishes and treated with test compounds 24 hr later. One day later, 24 hr after the addition of the test compounds to the nutrient medium, the compounds were removed by change of medium; thereafter, the medium was changed twice weekly. After 7 to 14 days, dishes plated with 10^9 cells were fixed and stained, and colonies were counted for the determination of plating efficiency. After 56 days dishes plated with 10^10 cells were fixed, stained, and scored for transformed, piled foci. Piled-up foci of morphologically transformed cells, normal-appearing areas of the same dish, and areas from control dishes were ring-isolated. The isolated cells were passaged twice and inoculated s.c. into inbred male C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, Maine). Each mouse received 10^6 cells and was observed for 6 months.

V79 Chinese hamster cells were used to determine chemically induced mutation. These cells provide a model system for assay of mutagenesis that was developed by Chu and Malling (6). The model uses change from 8-azaguanine susceptibility to resistance as a marker for mutagenesis. The cells were kindly provided by Dr. E. H. Y. Chu, Department of Human Genetics, University of Michigan, Ann Arbor, Mich. Before use, these cells were cloned by ring isolation in a medium containing thymine, hypoxanthine, aminopterin, and glycine, which eliminates spontaneous 8-azaguanine-resistant mutants (14). The mutagenesis assay was performed as previously reported (14). Cytotoxicity was measured by plating 10^9 cells into 60-mm dishes containing 8-azaguanine-free medium. The test compounds were added 18 hr later; 3 hr thereafter, the contents of the dishes were replaced with fresh 8-azaguanine-free medium. After incubation for 6 to 8 days, the culture dishes were fixed and stained, and colonies were counted. Cytotoxicity was expressed as the percentage of colonies in the treated dishes as compared to the controls. The average plating efficiency of control dishes was 88%. Mutagenicity was measured by plating 2.5 x 10^9 cells into 60-mm dishes containing 8-azaguanine-free medium. The cell number was determined 18 hr later using 2 dishes (usually 5 x 10^9 cells), and the remaining dishes were treated for 3 hr with test compounds. The medium was then replaced with fresh 8-azaguanine-free medium without test compounds, and the cells were incubated for an additional 48 hr. Thereafter, the dishes were refed every 2 days with medium containing 8-azaguanine (20 μg/ml). At 10 to 14 days after the initial addition of 8-azaguanine, the dishes were fixed and stained, and the number of resistant colonies was determined. The mutation frequency was calculated per 10^9 survivors; the background spontaneous mutation rate in controls was 10 colonies/10^6 survivors.

**RESULTS**

**Tumor Induction in Rats.** Both daunomycin and adriamycin were active in inducing rat mammary tumors after single i.v. doses (Table 1). By the end of 1 year, there were 18 fibroadenomas and 7 adenocarcinomas in 34 adriamycin-treated rats, and 16 fibroadenomas and 3 adenocarcinomas in 17 adriamycin-treated rats. Although this finding confirms, in principle, the observations made by Bertazolli et al. (2), the distribution between fibroadenomas and adenocarcinomas in the 2 studies is different. We are unable at present to explain the discrepancy. We also failed to confirm our earlier observation that daunomycin is active in inducing rat kidney tumors (29). In the previous study, kidney tumors developed rather late after treatment with daunomycin (>300 days). It seems likely that early development of mammary tumors in this work, which we did not observe with daunomycin in our previous study, resulted in decreased numbers of animals available for observation at 300 or more days.

**In Vitro Studies.** Assays of malignant transformation by daunomycin, adriamycin, or actinomycin D are presented in Table 2 and Chart 1. The results show that both adriamycin and daunomycin are powerful transforming agents. This is in contrast to the minimal activity of actinomycin D. Exposure to concentrations of adriamycin or daunomycin as low as 0.001 to 0.01 μg/ml caused transformation of M2 cells with frequencies equivalent to that of the potent carcinogen N-methyl-N'-nitro-N-nitrosoguanidine (18). Price et al. (23) found adriamycin similarly potent as a transforming agent in rat embryo cells. Cells morphologically transformed by adriamycin and daunomycin were tested for their capacity to induce tumors in isologous nonirradiated mice. At 4 to 8 weeks after the s.c. inoculation of cells obtained from 2 daunomycin-transformed clones and from 2 adriamycin-transformed clones, sarcomas were found in all the treated mice (3 to 4 mice/clone). Sixteen mice, receiving cells either from normal-appearing areas of treated cultures or from control cultures did not give rise to any tumors. Such results are in agreement with previous findings showing that morphologically transformed foci of mouse prostate fibroblasts, whenever isolated and tested, uniformly give rise to tumors in isologous mice (5, 18).

The difference between actinomycin D and the anthracyclines in transforming activity is closely paralleled by a major difference in capacity to induce mutation. As shown in Chart 2, the mutagenic activity of actinomycin D was negligible. Only at the concentration of 0.01 μg/ml did the mutation frequency exceed the control rate of 10 per 10^9 surviving cells. By contrast, both daunomycin and adriamycin induced major changes in mutation rate in a dose-dependent manner in the concentration range between 0.01 and 0.1 μg/ml. From the data shown in Chart 2, it may also be seen that the absolute number of mutants was significantly greater than control in dishes treated with adriamycin, 0.1 μg/ml, and with daunomycin, 0.01 and 0.05 μg/ml.
**Oncogenicity of Adriamycin and Daunomycin**

**Table 1**

*Tumors in rats given single i.v. doses of daunomycin, adriamycin, or 0.9% NaCl solution*

The 2 daunomycin groups and the controls each consisted initially of 20 rats; the adriamycin group, of 25 rats. Another group of 20 rats received adriamycin in the dose of 10 mg/kg. All died without tumors within the 1st 15 weeks.

<table>
<thead>
<tr>
<th>No. of tumors after injection of</th>
<th>Daunomycin</th>
<th>Adriamycin, 5 mg/kg (15 of 17 animals developed tumors)</th>
<th>0.9% NaCl solution (5 of 20 animals developed tumors)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mg/kg (12 of 16 animals developed tumors)</td>
<td>6 (175–266)</td>
<td>2 (77, 261)</td>
<td>1 (333)</td>
</tr>
<tr>
<td>5 mg/kg (15 of 18 animals developed tumors)</td>
<td>6 (88–119)</td>
<td>3 (71, 198, 277)</td>
<td>2 (53, 77)</td>
</tr>
</tbody>
</table>

Mammary tumors
- Fibroadenoma: 6 (175–266)
- Carcinoma: 5 (88–119)

Adrenal carcinoma
- Epidermoid carcinoma: 1 (365)
- Liposarcoma: 1 (308)
- Unclassified s.c. malignant tumor: 2 (170, 283)

Lipoma: 1 (202)
Schwannoma: 1 (322)
Cervical polyp: 1 (350)

* Numbers in parentheses, days on which animals were killed.

**Table 2**

*Malignant transformation in vitro of mouse M2 fibroblasts induced by daunomycin, adriamycin, or actinomycin D*

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration (μg/ml)</th>
<th>% Plating Efficiency</th>
<th>Transformed foci/disks treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (phosphate-buffered saline)</td>
<td>0.2</td>
<td>23</td>
<td>0/15</td>
</tr>
<tr>
<td>N-methyl-N'-nitro-N-nitrosoguanidine</td>
<td>0.2</td>
<td>14</td>
<td>12/9</td>
</tr>
<tr>
<td>Actinomycin D</td>
<td>0.0001</td>
<td>21</td>
<td>2/13</td>
</tr>
<tr>
<td></td>
<td>0.0005</td>
<td>16</td>
<td>2/17</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>11</td>
<td>0/17</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Adriamycin</td>
<td>0.0001</td>
<td>22</td>
<td>1/5</td>
</tr>
<tr>
<td></td>
<td>0.0005</td>
<td>21</td>
<td>3/7</td>
</tr>
<tr>
<td></td>
<td>0.001</td>
<td>17</td>
<td>9/15</td>
</tr>
<tr>
<td></td>
<td>0.005</td>
<td>15</td>
<td>13/11</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>10</td>
<td>5/3</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Daunomycin</td>
<td>0.001</td>
<td>21</td>
<td>11/13</td>
</tr>
<tr>
<td></td>
<td>0.002</td>
<td>18</td>
<td>6/12</td>
</tr>
<tr>
<td></td>
<td>0.005</td>
<td>16</td>
<td>15/11</td>
</tr>
<tr>
<td></td>
<td>0.01</td>
<td>9</td>
<td>4/5</td>
</tr>
</tbody>
</table>

* Each datum is the composite of 2 or 3 separate experiments with, initially, 4 culture dishes for the determination of plating efficiency and 6 culture dishes for the determination of transformation per experiment. For brevity, the S.D. values of the plating efficiencies were omitted from the table; they ranged between 2.3 and 4.1% of the means.

**DISCUSSION**

The present results demonstrate the high oncogenic potential of daunomycin and adriamycin, and the data obtained in *in vitro* assays suggest that they may be directly acting carcinogens. Their transforming and mutagenic activity compares well with that of the potent carcinogen, N-methyl-N'-nitro-N-nitrosoguanidine. Such data suggest that patients treated with the substances should be closely monitored in anticipation of the possible appearance of newly induced tumors, and that the drugs should be re-

μg/ml. Chart 2 also shows that the positive control, N-methyl-N'-nitro-N-nitrosoguanidine, was active in the present experiments, as previously reported (14). A close comparison of the relative mutagenicity of the anthracyclines with that of N-methyl-N'-nitro-N-nitrosoguanidine is not justified at this time, since we have not explored whether the 48-hr “expression-time” (1) for the induction of mutant colonies that we have used herein is the optimum for each of these compounds and since we have not yet determined whether parent and mutant cells are equally susceptible to the lethal effects of these agents.
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Chart 1. Malignant transformation in vitro of mouse M2 fibroblasts induced by daunomycin, adriamycin, or actinomycin D. The data were calculated by using the mean plating efficiency values and the number of transformed foci per dishes tested, as shown in Table 2. For comparison, the transforming activity of N-methyl-N'-nitro-N-nitrosoguanidine is also presented (C).

Chart 2. Cytoxicity (percentage of survivors) and mutagenicity (induction of resistance to 8-azaguanine) induced in V79 Chinese hamster cells by treatment with daunomycin, adriamycin, or actinomycin D. Each datum is the composite of 3 separate experiments with 2 culture dishes for the determination of plating efficiency (survivors) and 8 culture dishes for the determination of 8-azaguanine-resistant colonies per experiment. For brevity, the S.D. values were omitted from the chart; they ranged between 3.2 and 5.6% (percentage of survivors), and 8.2 and 15.7% (8-azaguanine-resistant colonies) of the means. For comparison, the data for N-methyl-N'-nitro-N-nitrosoguanidine are also presented (C). The background spontaneous mutation rate is 10 8-azaguanine-resistant colonies per 10⁶ survivors.

stricted to the therapy of diseases with poor prognosis.

The mechanism of action by which daunomycin and adriamycin elicit their oncogenic effects is unknown. It is generally believed that, in their ultimate state, carcinogens or their metabolically activated derivatives are chemically reactive, electrophilic substances that react covalently with nucleophilic macromolecules, especially DNA (19). In regard to the anthracycline antibiotics, there is as yet no evidence for such covalent interaction with DNA (8). However, Di Marco et al. (9) have succeeded in generating firm linkage between daunomycin and DNA by photoirradiating daunomycin-DNA complexes. This observation and the observed oncogenicity of the anthracyclines indicate the need for more thorough investigation of the possibility of their metabolic activation.

In considering the mode of carcinogenic action of the anthracyclines, it may be instructive to compare their effects with those of actinomycin D. The 3 substances have high affinity for DNA. Association constants for their "strong-binding" interactions are similar in magnitude, and there is substantial evidence that the interactions involve intercalation of chromophoric regions between base pairs of the DNA double helix (8, 11). Presumably as a consequence of the binding, the template functions of DNA are disturbed. In the case of actinomycin D, DNA-directed RNA synthesis is selectively more sensitive than is DNA synthesis (24), but no such clear difference in sensitivity has been established in studies with the anthracyclines (8).

Despite the similar affinity of the anthracyclines and actinomycin D for binding to the DNA double helix, there are significant differences in the basic biological effects of the agents. Adriamycin and daunomycin cause chromosomal disintegration (28), and break DNA strands in vivo (25), but actinomycin D has little or no such effects (25, 28). Previous work has already shown that actinomycin D is only weakly mutagenic (7); this is in keeping with our present results and is in contrast with the high mutagenic activity of the anthracyclines in Salmonella typhimurium (20) and in V79 cells (see Chart 2). Tumors have been induced by treatment with actinomycin D, but only at injection sites. Peritoneal mesotheliomas appear after repeated i.p. injections, but not when the agent is given by other routes (30). Sarcomas occur at s.c. injection sites (10, 15). We have not seen tumors in rats receiving maximally tolerated doses by repeated intragastric intubation (22). It is conceivable that the tumors caused by actinomycin D are in nonspecific response to prolonged local injury and regenerative hyperplasia. If the substance is a direct carcinogen, its tumorigenic activity in vivo is limited and its capacity for malignant transformation in vitro is minimal.

These data demonstrate a satisfactory correlation between in vivo tumorigenicity and in vitro assays for malignant transformation and mutagenesis. On the one hand, the limited capacity for tumor induction by actinomycin D is matched by minimal effects in vitro; on the other hand, adriamycin and daunomycin induce a significant number of tumors after injection of single doses and the agents have high transforming and mutagenic activity. The correlation supports the use of the in vitro tests as rapid screening assays for carcinogenic potential. The need for such methodologies becomes increasingly evident as we become more conscious of the warnings given by Boyland (3) and others that 60 to 90% of all human cancer may be caused by environmental chemicals. With regard to the specific problem posed by the carcinogenicity of the anthracyclines, in vitro assays could be used advantageously in developing derivatives with effective cytotoxic, i.e., antitumor, activity with reduced oncogenic potential.
ACKNOWLEDGMENTS

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REFERENCES


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