Actinomycin D: Drug Concentrations and Actions in Mouse Tissues and Tumors

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SUMMARY

Concentrations of actinomycin D-3H in tissues and organs of mice were measured at 30 min and other times up to 48 hr after injection (600 μg/kg). Although this dose is sublethal for mice, it is known to damage cells of the intestinal epithelium, spleen, thymus, and the sensitive Ridgway osteogenic sarcoma (ROS). It has little or no cytotoxic effects on mouse liver or the solid, transplantable 7,12-dimethylbenz(a)anthracene (DMBA)-induced tumor. By 30 min, the average concentrations of labeled antibiotic in liver, salivary gland, and spleen (1.5-1.7 μg/gm) were already considerably higher than blood concentrations (0.1 μg/ml). Concentrations in other tissues were less: small intestine (0.8 μg/gm), ROS tumor (0.4 μg/gm), and DMBA tumor and thymus (0.2 μg/gm). Thereafter, concentrations decreased in liver, salivary gland, small intestine, and DMBA tumor while ROS and thymus continued to accumulate the antibiotic. By 48 hr, high concentrations were localized in spleen, thymus, and ROS, all of which are considered to be highly sensitive to the agent. Shorter retention times may be associated with moderate damage in salivary gland and small intestine and with little or no damage in liver and DMBA tumors. Uridine-3H incorporation into RNA was estimated at 3 hr after actinomycin, when most tissues had maximal drug concentrations, and at 24 hr, when most were depleted of drug. The inhibitory effects of actinomycin on incorporation rates were correlated with tissue levels of the drug but not with the susceptibility of the tissue to cytotoxicity.

INTRODUCTION

It is now possible to correlate inhibition of RNA synthesis with uptake of actinomycin D and to associate cytotoxicity with prolonged drug retention in a variety of mouse tissues. A preliminary report has been published (17). Others have reported short term distribution studies with labeled actinomycin (10, 22); however, the correlations noted herein have not been described previously.

MATERIALS AND METHODS

ROS and DMBA tumor were transplanted into female AKR mice (Jackson) for experiments as previously described (18). Mice bearing either ROS or DMBA tumor or both (i.e., as double implants) were used during the 3rd or 4th week after implantation when tumors were well established. Tritium-labeled actinomycin D in 50% ethanol, obtained from Schwarz BioResearch, had a specific activity of 3.8 c/m mole and a specified radiochemical purity of about 95%. For uptake studies, samples were diluted 5-fold with pyrogen-free H2O and administered slowly in the tail vein in a volume of 0.1 ml/10 gm body weight.

Labeled actinomycin was extracted from tissues of treated mice with n-butanol and identified chromatographically on paper. All glassware was siliconized and procedures were carried out in semi-darkness. Tissues from 2 mice were iced, cleaned, and combined. Lymph nodes, connective tissue, and grossly necrotic tumor tissue were dissected out and discarded. Gall bladders were carefully separated from liver and usually discarded (see Discussion). Tissues were homogenized in ice with 4 volumes (or a minimum of 3.0 ml) of water to which was added 40 μg unlabeled actinomycin D in 0.2 ml H2O and a drop of caprylic alcohol. To each homogenate, 0.5 ml of 1% sodium lauryl sulphate was added and solutions were warmed to room temperature for 10 minutes. Five ml of butanol (equilibrated with 1% sodium lauryl sulphate) was added and actinomycin was extracted for 20 min before centrifugation. Each tissue was extracted 3 times and an aliquot of the combined butanol phases was counted using liquid scintillation techniques.

In preliminary studies a fourth extraction accounted for little of the total extractable radioactivity in tissues of mice treated with actinomycin D-3H for 3 or 24 hr: 0.4-3.2% from liver, 1.5-7.5% from small intestine, 1.6-3.3% from ROS, and 1.2-2.7% from DMBA tumor. Blood concentrations were measured in individual mice. Samples (0.25 ml) were diluted with 3.0 ml water; carrier (40 μg) and sodium lauryl sulphate were added as before and actinomycin was extracted once with butanol.

To estimate recovery of added tritiated actinomycin, tissues were homogenized in water as before and 0.125 μg actinomycin D-3H and caprylic alcohol were added to cold preparations; after 10 minutes at room temperature, sodium lauryl sulphate was added as before and actinomycin was extracted once with butanol.
and carrier actinomycin (40 μg) were added. After a single extraction the butanol layer contained 74% of the added activity from liver, 56% from small intestine, 66% from ROS, 61% from DMBA tumor, and 79% from blood and saline. In a similar experiment, recoveries after 3 extractions with butanol were 96% from liver, 95% from intestine, 90% from ROS, and 92% from DMBA tumor. Recoveries of added actinomycin D-3H from liver and ROS were compared with methods described by Weissbach et al. (22) who described partial extraction with neutral ethylacetate and complete extraction with acidified ethylacetate; similarly, three extractions with butanol or with neutral ethylacetate were comparable to a single extraction with acidified ethylacetate. We also confirmed the decreased extractability of actinomycin added to liver or ROS homogenates after 30 min at 37°C; here also, we found that three extractions with neutral ethylacetate or with butanol were as complete as one extraction with acidified ethylacetate.

Values reported in the text are corrected for quench and counting efficiency, but not for recovery.

Butanol extracts (50 μl) from tissues were chromatographed on Whatman #1 paper in an ascending system (4). The organic phase of the following solvent mixture was used: 2% p-toluene sulfonic acid (200 ml), ethylacetate (50 ml), n-butyl ether (50 ml). The solvent front was allowed to run 23 to 25 cm before papers were removed and dried. After identification of added carrier actinomycin under visible and ultraviolet light, the extracts from tissues at 3 and 18 hr after treatment (see Table 1) were chromatographed; activity maxima in all chromatograms corresponded with the visible spots of added actinomycin and indicated the probable identity of the two. There was no evidence from these studies or from those of others (22) that actinomycin was metabolized or altered in the animal or during the extraction and chromatographic procedure.

In preliminary studies, we measured the disposition of actinomycin D-3H in mice bearing ROS and DMBA tumors at different times after treatment. Mice received actinomycin D (600 μg/kg; 2 mc/kg) i.v.; tissue from 2 mice was pooled for each determination. ROS, Ridgway osteogenic sarcoma; DMBA, sarcoma originally induced with 7,12-dimethylbenz(a)anthracene.

RESULTS

Chromatography and Distribution of Actinomycin D-3H in Tissues of Mice. Chart 1 shows the activity of a chromatogram of an actinomycin D-3H sample. Maximal activity at 17–19 cm corresponds with the visualized spot of carrier actinomycin D (Rf 0.7). The activity present at the origin is found in all samples of actinomycin-3H that we have tested; the amount is variable and appears to increase upon exposure of labeled actinomycin to light. The extracts from tissues at 3 and 18 hr after treatment (see Table 1) were chromatographed; activity from 1 experiment at 3 hr is shown in Chart 2. The extracts from other tissues (thymus, salivary gland, and spleen) at 3 and 24 hr were likewise chromatographed with similar results. Activity maxima in all chromatograms corresponded with the visible spots of added actinomycin and indicated the probable identity of the two. There was no evidence from these
Chart 2. Chromatography of tissue extracts. Pairs of mice (doubly implanted with ROS and DMBA tumors) were killed 3 hr after receiving 600 μg (2 me) actinomycin D-3H/kg. Tissues were extracted 3 times with butanol and chromatographed as described in Methods. Counts in a blank channel are indicated by the dashed lines (in liver panel). ROS, Ridgway osteogenic sarcoma; DMBA, sarcoma originally induced with 7,12-dimethylbenz(α)anthracene.

High initial concentrations were observed in liver, spleen and submaxillary salivary gland. Concentrations in liver subsequently fell off most rapidly; in salivary glands the decrease was slower and in spleen there was even an increase up to 3 hr. Moderate initial concentrations in small intestine were maintained for only 3 hr, after which the rate of decrease was as in liver. Relatively low initial concentrations were obtained in the ROS tumors; DMBA tumor and thymus had even lower initial concentrations compared to other tissues. Afterwards, both ROS and thymus eliminated the drug so that by 48 hr the concentrations reached were similar to those retained by spleen. In contrast, the other tissues and the DMBA tumor lost actinomycin, so that all had similarly low concentrations by 24 and 48 hr.

Chart 4 shows the uptake of the drug by tissues 3 hr after various doses of actinomycin. Pairs of mice were given 200, 400, or 800 μg/kg and killed after 3 hr. The experiment was not replicated because values 3 hr after the 600 μg/kg dose (taken from data in Chart 3) affirmed the results: concentrations in each tissue increased with increasing doses. Similarly drug concentrations in both ROS and DMBA (Chart 5) were proportional to the amounts administered. Through the 6-fold range tested, the average concentrations were 2.17 times (range 1.69–2.74) higher in ROS than in DMBA.

Uridine Incorporation into RNA. Uridine-3H was given to mice bearing DMBA tumors to establish a relationship between tissue concentrations of actinomycin and the ability of the agent to inhibit RNA synthesis. Three hr after a dose of actinomycin (800 μg/kg) RNA synthesis was partially inhibited in liver, salivary gland, spleen, and intestine (Table 2). In thymus, increased incorporation 3 hr after treatment may be indicative of a decreased pool of RNA precursors; the same may also be true for DMBA tumors at both 3 and 24 hr after the drug was given. By 24 hr, spleen was the only
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Chart 4. Tissue concentrations 3 hr after administration of varying doses of actinomycin D-3H. Pairs of mice (bearing ROS tumors) were given actinomycin D-3H (0.67 mc/kg) diluted with carrier to final doses of 200, 400, or 800 μg/kg. Points shown at 600 μg/kg were taken from data at 3 hr presented in Chart 3. Data from Ridgway osteogenic sarcoma (ROS) are shown in Chart 5.

Table 2

<table>
<thead>
<tr>
<th>Tissues</th>
<th>3 hr</th>
<th>24 hr</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n = 6)</td>
<td>(n = 4)</td>
<td>(n = 5)</td>
</tr>
<tr>
<td>Liver</td>
<td>11.1 ± 2.0a</td>
<td>35.9 ± 7.9</td>
<td>41.9 ± 16.7</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>7.2 ± 1.9a</td>
<td>23.9 ± 3.3</td>
<td>24.6 ± 7.6</td>
</tr>
<tr>
<td>Spleen</td>
<td>76 ± 16</td>
<td>80 ± 10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>165 ± 45</td>
</tr>
<tr>
<td>Small intestine</td>
<td>23.2 ± 4.2a</td>
<td>41.0 ± 4.9</td>
<td>45.1 ± 12.9</td>
</tr>
<tr>
<td>Thymus</td>
<td>22.0 ± 2.8a</td>
<td>14.6 ± 1.9</td>
<td>15.6 ± 3.7</td>
</tr>
<tr>
<td>DMBA tumor</td>
<td>8.6 ± 2.1b</td>
<td>10.9 ± 0.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.5 ± 0.5</td>
</tr>
</tbody>
</table>

Uridine-3H incorporation into RNA in tissues of mice at different times after treatment with actinomycin D.

Mice bearing DMBA tumors received actinomycin (800 μg/kg, i.v.) or saline. After 160 min or 24 hr uridine-3H (20 μc/mouse, i.p.) was given and mice were killed 20 min thereafter. Tissues from treated and concurrent control mice were analyzed individually. Each treated group was compared with the control group for estimates of probability (P). DMBA, sarcoma originally induced with 7,12-dimethylbenz(a)anthracene.

<sup>a</sup> P < 0.01.
<sup>b</sup> P > 0.01 < 0.05; for all others, P ≥ 0.05.

tissue shown in Table 2 which retained actinomycin and in which uridine incorporation into RNA was still inhibited. ROS, in other experiments, was similarly inhibited at 24 hr.

Chart 6, shows the relationship between early actinomycin concentrations in tissues and uridine incorporation. Values for effects on uridine incorporation at 3 hr (except ROS) are from Table 2; concentrations of actinomycin at 3 hr after 800 μg/kg are taken from data shown in Charts 4 and 5 (extrapolated in FEBRUARY 1968

Chart 5. ROS and DMBA concentrations 3 hr after administration of varying doses of actinomycin D-3H. Mice were given actinomycin D-3H (0.67 mc/kg) diluted with carrier to final doses of 200, 400, 800, or 1200 μg/kg. Each point is from pooled tumors from 2 mice bearing single tumors. ROS, Ridgway osteogenic sarcoma; DMBA, sarcoma originally induced with 7,12-dimethylbenz(a)anthracene.

Chart 6. Uridine incorporation and drug concentrations in mouse tissues after actinomycin D-3H. See text for description. ROS, Ridgway osteogenic sarcoma; DMBA, sarcoma originally induced with 7,12-dimethylbenz(a)anthracene.

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DISCUSSION

At 30 min, the earliest time taken for distribution studies, all of the selected organs and tissues had drug concentrations that were higher than blood. The highest initial levels were found in liver, salivary gland, and spleen. Of these, the liver is least susceptible to the cytotoxic effects of the agent (11, 16, 21) and it was depleted of drug most rapidly. Spleen is one of the most susceptible organs (11, 18), and it retained the highest concentrations for prolonged periods of time. Salivary gland, which released actinomycin more slowly than liver, is known to be susceptible to the antibiotic. Cytotoxicity, however, is usually delayed 24 hr or more and occurs more prominently in rats given maximally tolerated doses than in mice (5, 6). Within this group of tissues, there appears to be a good correlation between drug retention and cytotoxicity.

The tissues with lowest initial uptake of actinomycin may also be examined for evidence of such a correlation: thymus, ROS, and DMBA tumors. During the first 6 hr, ROS contained the highest drug levels, whereas thymus and DMBA tumors regularly contained less. Thereafter, concentrations in DMBA tumor fell off, and cumulation continued in both ROS and thymus. By 24 and 48 hr, ROS and thymus held relatively high concentrations (0.5-0.8 μg/gm), comparable to spleen, and significantly more than DMBA (0.1 μg/gm or less) and the other tissues. Again, ROS and thymus are like spleen, highly susceptible to cytotoxicity by the agent, whereas DMBA tumor is relatively resistant (15, 18).

Prolonged retention of the drug was associated with cytotoxicity in spleen, thymus, and ROS. We then asked whether there is a correlation between the proximal biochemical action of the agent and cytotoxicity. For these experiments, the effect on RNA synthesis was estimated from the incorporation of uridine into RNA during a 20-min pulse. Of the 3 tissues that are more susceptible and which demonstrate prolonged retention, only spleen was inhibited at 3 hr. Incorporation of uridine was also inhibited in liver and salivary gland, which are less susceptible to cytotoxicity, and in small intestine (discussed below). Thus, the proximal effect on RNA synthesis at 3 hr could not be used to predict which of these tissues would prove susceptible to intoxication. The biochemical effect is of course related to the drug concentration: tissue levels that exceed 0.8-1.0 μg/gm inhibit uridine incorporation. ROS, thymus, and DMBA did not have such high levels at 3 hr and so uridine incorporation was not inhibited.

In thymus and DMBA tumor there was evidence suggestive of decreased ribonucleotide pool sizes. It is possible that low concentrations of actinomycin may interfere with de novo synthesis of pyrimidine ribonucleotides. Wheeler and Bennett (23) reported evidence of similar changes in purine ribonucleotide pools in L. leichmannii and H. Ep-2 cells after low doses of actinomycin.

The group of tissues with higher drug retention at 48 hr is most severely damaged by actinomycin (11). In sublethally intoxicated mice, spleen cells are among the last to recover and ROS may regress completely (18). Prolonged retention probably accounts for the sensitivity of this tumor to the drug. A similar correlation between drug retention and sensitivity of transplantable mouse leukemias has been reported by Kessel (7). Thymus also shows extensive damage (11, 18); but the onset is delayed for several days, probably due to slow accumulation of the drug.

The group of tissues with lower retention at 48 hr can be subdivided into those in which cytotoxicity develops (small intestine and possibly salivary gland) and those with little or no cell death (liver and DMBA tumor). The high concentration of drug found in liver during the first 3 hr is sufficient to inhibit RNA synthesis; however, this action is reversible. Similarly, nucleolar changes (20, 21) and losses of nuclear RNA (13) in liver are probably reversible also. It seems unlikely that a major portion of actinomycin in liver was bound to DNA because the organ secretes actinomycin into bile in large quantities. In one experiment, gall bladders were taken from mice sacrificed 3 hr after doses ranging from 200 to 800 μg/kg (see Chart 4). The bladders were cleaned, opened in liquid scintillation vials, and counted directly without complete extraction or corrections for quenching; the radioactivity in these bladders (5 mice) accounted for 3-10% of the total injected dose. A previous unpublished study in this laboratory (F. S. Philips and J. E. Sodergren) demonstrated that biliary secretion in rats represents an important route of excretion of unchanged actinomycin. Thus at least part of the actinomycin load in liver is free for excretion (2, 9).

DMBA tumor is like liver insofar as it is also resistant to cytotoxicity. The tumor, however, takes up much less drug than does liver. The failure by DMBA to take up and to retain actinomycin may account for its resistance. Thus, to reach concentrations in DMBA which are comparable to effective levels in ROS probably requires the administration of doses which would be lethal to the host. The resistance of the DMBA tumor is possibly related to the relative inability of the drug to penetrate the tumor cell or, if it does, to reach the DNA. In studies which are in progress, actinomycin binds to isolated DNA from DMBA tumor as avidly as to DNA from ROS. Presumably retention would be similar in cells of the 2 tumors if the DNA were equally accessible and if the bond formations were the same; this will be the subject of a future communication.

Small intestine and salivary gland also fail to retain high
concentrations of actinomycin. Cytotoxicity in these organs probably represents special cases. For example, there is evidence that sensitive proliferative cells in the intestinal crypts may show evidence of cell death as early as 2-4 hr after DNA synthesis is interrupted. The reason for susceptibility of these cells is not known, but early cell death in the mucosa occurs after treatment with mitomycin C (19), hydroxyurea (12), arabinosylcytosine (3) and other agents that inhibit DNA synthesis. Similarly an early inhibition of DNA synthesis occurs in the intestine after actinomycin (19). It seems reasonable to expect that longer retention of actinomycin would be occasioned by higher doses which are lethal to the animal in 3 or 4 days (i.e., 1 or 2 times the LD_{50}). Since the mitotic cycle requires only 12-14 hr in mice (8), retention of effective concentrations for this period would probably be irreparable. But in the current study, retention for 6 hr is compatible with recovery of the epithelium after sublethal doses.

There is little basis for understanding cytotoxicity in salivary gland. It is one of the few, nonproliferative organs which is susceptible to actinomycin (5, 6), although salivary cells do have the capacity to synthesize DNA and to divide upon stimulation (1). It is, of course, one of the most active tissues synthesizing protein and it may require continuous synthesis of large quantities of RNA for synthesis of secretory protein as well as for maintenance. High concentrations of actinomycin in salivary glands are retained for more than 6 hr after 600 μg/kg in mice, and high concentrations may be held even longer in the more susceptible rats given maximally tolerated doses. As in pancreas and liver (20), changes in nucleolar morphology have also been described for acinar cells in rat parotid glands. This common effect is presumably due to inhibition of RNA synthesis rather than to a direct effect on their exocrine functions.

The results described here indicate that RNA synthesis is inhibited in tissues that acquire concentrations of the agent exceeding 0.5-1.0 μg/gm. The inhibition is reversible and is not antecedent to cytotoxicity except upon prolonged retention of the drug. How long the drug must be retained to intoxicate cells is probably related to factors that are unique to the cells themselves. As an example, cells of regenerating liver can survive inhibition of DNA synthesis far longer than those of the intestinal crypts (12, 14, 16, 19). Such differences may be due in part to the mitotic cycle. Alternatively, it may be due to the rate of turnover of RNA or to associated phenomena. In the case of ROS, cell death appears to be related to the prolonged retention of actinomycin and to an intolerable loss of RNA (15). The loss of RNA is probably due to increased ribonuclease activity because RNA synthesis continues even after cell death has become widespread within the tumor.

REFERENCES

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