Dehydroretronecine-induced Rhabdomyosarcomas in Rats

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SUMMARY

Two groups of rats were given s.c. injections of either monocrotaline or its major detectable metabolite, dehydroretronecine, biweekly for 1 year. Tissues obtained from partial hepatectomies performed at 4 months on a portion of these animals showed that both compounds caused a decided inhibition of mitotic division in regenerating liver. Rhabdomyosarcomas developed at the site of dehydroretronecine injection in 51.6% of the rats and in 3.3% of the monocrotaline-treated rats. Metastatic lesions were recorded in 8.3% of these animals. In addition to the above, 10% of the monocrotaline-treated rats developed other tumors that included myelogenous leukemias, hepatocellular carcinomas, and pulmonary adenomas. These data indicate that either monocrotaline or its metabolite dehydroretronecine are capable of causing neoplastic transformations in the tissues of experimental animals.

INTRODUCTION

The pyrrolizidine alkaloids represent a large group of naturally occurring toxins that are capable of causing liver necrosis and cirrhosis in man and lower animals (5, 13). In addition, it has been shown that prolonged exposure to these alkaloids may be conducive to the development of tumors in experimental animals. Research to date on the oncogenic effects of these compounds has been limited to long-term exposure of experimental animals to the parent alkaid (9, 15, 16, 18). There are also data that indicate that these alkaloids per se are rather innocuous but must be metabolized in order to produce tissue changes (2). In this laboratory, the isolation, identification, and synthesis of dehydroretronecine (11), a major metabolite of the pyrrolizidine alkaloid monocrotaline, has made it possible to investigate the effects of a single metabolite on experimental animals (3, 12). In this report, data are presented that indicate that dehydroretronecine is a potent oncogenic compound capable of producing sarcomas in over 50% of the treated rats.

MATERIALS AND METHODS

Dehydroretronecine was prepared according to previously reported methods (1, 8) (Chart 1) and was identical to earlier preparations (7, 12) in melting point, mass spectra, and thin-layer chromatography. Because of its instability in aqueous solutions of acid pH, dehydroretronecine was dissolved in phosphate buffer (0.1 M, 4 mg/ml), pH 7, immediately prior to injection.

Male Sprague-Dawley rats weighing approximately 55 g were housed in a well-ventilated, windowless room that was illuminated from 6:00 a.m. to 6:00 p.m. daily. A commercial rat diet (Ralston Purina Co., St. Louis, Mo.) and water were supplied ad libitum. Two hundred rats were divided into 1 control group consisting of 50 animals and 2 experimental groups that were comprised of 75 animals/group. The animals of one experimental group were given s.c. biweekly injections for 4 months of 20 mg of dehydroretronecine per kg body weight and, for the succeeding 8 months, of 10 mg dehydroretronecine per kg body weight. The animals of the other experimental group were given s.c. doses of 5 mg monocrotaline per kg body weight biweekly for 12 months. The levels of monocrotaline and dehydroretronecine used in this experiment were based on previous observations that indicated that repeated exposures to these doses would not produce obvious injurious effects. The control animals were given biweekly injections of 0.1 M phosphate buffer, pH 7. After having been on this treatment regimen for 4 months, a partial hepatectomy (10) was performed on 15 of the dehydroretronecine-treated animals, 15 of the monocrotaline-treated animals, and 5 control animals in order to determine the effect of the drugs on hepatic mitosis and to evaluate tissue changes resulting from exposure to these compounds. After 36 hr of regeneration, portions of the liver were placed in Carnoy’s fixative and subjected to an acetocarmine squash procedure (17) for a count of the mitotic indices. All injections of dehydroretronecine and monocrotaline were discontinued after 12 months. During the succeeding 10 months, as the animals became moribund, they were sacrificed and tissues were obtained for histological and electron microscopic evaluation. Small segments of the tissues were placed in 10% neutral formalin for 24 hr and subsequently dehydrated. They were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin. In addition, sections of the tumor tissue were stained with phosphotungstic acid-hematoxylin stain (4). For electron microscopy, various areas of tumor tissue that appeared...
RESULTS

Throughout the 12 months of dehydroretronecine or monocrotaline administration, there were no indications of illness. In addition, the survival rate was equally as good in the experimental and control animals. After 4 months, it was observed that the dehydroretronecine-treated animals were approximately 50 g smaller than the animals of the other groups. Because of the decrease in weight gain, the dosage of dehydroretronecine was reduced by one-half during the remaining 8 months of the experiment. Following reduced dehydroretronecine administration, weights of the experimental and control animals were essentially the same by the 12th month.

There were no gross abnormalities in the animals that were sacrificed at 4 months. Megalohepatocytosis could be seen in the livers of monocrotaline-treated rats by light microscopy. However, only isolated, enlarged hepatocytes were observed in the livers of rats exposed to dehydroretronecine. The microscopic features of tissues from other organs in the experimental animals were normal. The mitotic indices per 1000 hepatocytes of the livers of animals following partial hepatectomy were 61.7 ± 8.7 S.D. for the control, and 11.99 ± 6.6 for the dehydroretronecine-treated group.

Two of the dehydroretronecine-treated rats developed well-circumscribed, firm nodules beneath the skin of the back between the shoulders by the 11th month of the treatment. During the succeeding 10 months following the cessation of dehydroretronecine injections, 29 additional rats developed similar tumors at the injection site (Table I). The average time required for the tumors to develop following the discontinuation of treatment was 181 ± 70 days (not including the 2 tumors that developed during the last month of treatment). However, only 2 monocrotaline-treated animals developed obvious tumors 6 and 8 months following the termination of injections. One tumor was located in the lower abdomen just anterior to the right thigh, while the other developed on the rib cage, posterior to the right elbow. Gross features of neoplasms in both groups of animals were similar, although there was considerable variation to size (30 to 300 g). The tumors were white or yellow on the cut surface and, except for focal necrotic areas, were firm.

The tumors in both groups of animals were composed of 2 distinct cell types, i.e., large, frequently multinucleated and small spindle or oval-shaped cells. The large cell had a round, oval, or strap-shaped profile. The former cells were over 75 μm in diameter, and the latter frequently exceeded 200 μm in diameter (Fig. 1). These cells were commonly multinucleated, with the nuclei randomly distributed throughout the cytoplasm, assuming a tandem-like arrangement or forming a chain-like array along the plasma membrane. On staining sections containing these large cells with phosphotungstic acid-hematoxylin, cross-striations characteristic of striated muscle were observed in the strap-like cells (Fig. 2). The predominant cell type in most of the tumors was relatively small with a spindle-shaped outline. Its eosinophilic cytoplasm was occasionally striated, and the basophilic nuclei were spindle-shaped, round, or oval with 1 or 2 nucleoli. These cells were invariably closely packed with few blood vessels and a limited amount of interstitium. Transitions between the smaller spindle-shaped cell and the larger multinucleated cell were apparent in many sections. All of the metastatic lesions were of the small cell variety.

Electron microscopically, the outline of large cells assumed many diverse shapes, with the border varying from smooth to highly irregular. Short segments of the rough endoplasmic reticulum were randomly dispersed throughout the cytoplasm, with the ribosomes along their outer border being prominent and particularly abundant (Fig. 3). Polyribosomes were abundant. There was a paucity of mitochondria, with entire cell profiles being devoid of this organelle. Those present were small oval structures containing few cristae. The Golgi complexes were small and sparse, and only isolated lysosomes were present.

The nuclei of these cells were decidedly irregular in outline (Fig. 3). The nuclei were most frequently multiple, and much of the chromatin was clumped into packets throughout the nucleoplasm. Multiple nuclei, some undergoing mitotic changes, were commonly present.

The major difference in the appearance of the larger and smaller cells that comprised these tumors was the single nucleus that prevailed in the smaller cells. These nuclei

<table>
<thead>
<tr>
<th>Type</th>
<th>Monocrotaline</th>
<th>Dehydroretronecine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhabdomyosarcomas</td>
<td>3.3</td>
<td>51.6</td>
</tr>
<tr>
<td>Rhabdomyosarcomas with</td>
<td></td>
<td>8.3</td>
</tr>
<tr>
<td>metastases</td>
<td></td>
<td>5/60</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>3.3</td>
<td>2/60</td>
</tr>
<tr>
<td>Acute myelogenous leukemia</td>
<td>3.3</td>
<td>2/60</td>
</tr>
<tr>
<td>Pulmonary adenoma</td>
<td>3.3</td>
<td>2/60</td>
</tr>
</tbody>
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Table I. Tumor incidence in rats 10 months following cessation of long-term treatment with pyrrolizidine alkaloids
comprised approximately one-half of the cell profile, which was not the case with the larger cells. Small segments of endoplasmic reticulum, abundant polyribosomes, and myofilaments and sparse populations of mitochondria were also characteristic of these cells. Cross-striations were also periodically seen in these cells (Fig. 4).

In addition to the 2 tumors that were recorded grossly in the monocrotaline-treated rats, 6 additional neoplasms were present in the internal organs of 4 animals that became ill during the 9th and 10th months following the cessation of treatment. Hepatocellular carcinomas were observed in 2 of these animals. In addition, one of the above-mentioned animals also had myelogenous leukemia and the other had a bronchial adenoma. One of the other animals that became moribund during this time also had myelogenous leukemia. An additional bronchial adenoma was also observed microscopically in 1 animal.

**DISCUSSION**

The exact means by which monocrotaline is converted *in vivo* to dehydroretronecine is not known. However, it seems likely that it is first dehydrogenated to form monocrotaline pyrrole, and subsequently hydrolyzed to dehydroretronecine. A number of experiments on this compound have been conducted in our laboratory since it was determined to be the major detectable metabolite of monocrotaline. Dehydroretronecine has been shown to be much less toxic than the parent alkaloid. Relatively large doses of dehydroretronecine given to rats produced no liver necrosis or pulmonary vascular lesions, which commonly occur in monocrotaline-intoxicated animals (11). Both compounds do, however, cause an inhibition of regenerating rat liver cells and are conductive to the development of megalohepatocytosis. Dehydroretronecine has also been shown to have a particular affinity for the gastric mucosa, which is not a prominent feature of monocrotaline (12). In addition to the stomach being the major site of tritiated dehydroretronecine localization, there is a decided reduction in mitosis by the gastric epithelium which is responsible for thinning of the mucosa, ulceration, and hemorrhage (3).

Monocrotaline, like some of the pyrrolizidine alkaloids, is also capable of producing neoplastic transformations in the tissues of exposed animals. Newberne and Rogers (15) were able to produce liver tumors in 10 of 50 rats that had intragastric administration weekly with monocrotaline for 42 weeks.

In the experiment being reported, the incidence of tumors produced by s.c. injections of monocrotaline is somewhat lower than that reported by Newberne and Rogers. Differences in dosage, route, and frequency of administration may have been responsible for the variation in tumor incidence in these 2 studies. In addition, tumors observed by these scientists were limited to the liver, whereas the rats given s.c. injections of monocrotaline in the presently reported experiment developed neoplasms at other sites (Table 1). Those given dehydroretronecine showed a greater frequency of sarcomas at the site of injection within 10 months following cessation of treatment than did monocrotaline-treated animals. Moreover, tumors were continuing to appear in the surviving animals even toward the termination of the experiment. These data indicate that dehydroretronecine is an active carcinogen. However, it has been proposed that monocrotaline must be metabolized before it is capable of causing similar tissue changes. The necessity for metabolism prior to becoming an active carcinogen or the fact that the dose administered was smaller would be one explanation as to why the metabolite produced more tumors at the site of injection than did the parent alkaloid during the period of experimentation.

It is of interest that dehydroretronecine is rapidly absorbed from the site of injection and accumulates in the various tissues throughout the body (12), yet tumors appear only at the site of injection. A high concentration of the compound at the site of injection as well as the dilution that would take place in the blood prior to its association with extravascular cellular components may be instrumental in determining the tissues that are affected by tumors. The possibility exists that, following absorption, dehydroretronecine is further metabolized to a compound that maintains its antimitotic potentials but is devoid of its tumor-producing ability. Since the greatest concentration of radioactivity following the injection of tritiated dehydroretronecine is in the gastric mucosa, and no tumors were seen in this area, the possibility exists that the affected cells disappear through maturation before tumors develop. In the same context, skeletal muscle that had been in contact with dehydroretronecine would survive for a sufficient period for tumors to be manifested.

Svoboda and Reddy (18) observed tumors in the pyrrolizidine alkaloid-treated rats only after treatment had been discontinued. They proposed that the development of tumors in these animals could be related to the cessation of the antimitotic effects of the alkaloid as would be expressed by animals that were no longer being treated. This explanation does not seem to be the entire answer, in that the antimitotic effects of the pyrrolizidine alkaloids, including dehydroretronecine, may persist in the liver for an indefinite period following exposure to these compounds. If in fact dehydroretronecine is an alkylating agent, as has been shown to be the case for the parent alkaloids (7), it would seem that the inhibition of mitoses as well as the development of tumors could occur simultaneously, depending on the macromolecules that were affected.

Further experiments will determine whether dehydroretronecine has a particular affinity for the skeletal musculature or whether the rhabdomyosarcomas that developed in rats of the present study resulted from the repeated localized exposure.

**REFERENCES**

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Fig. 1. Numerous multinucleated giant cells varying considerably in size and shape predominated in some areas of the tumors produced on the back of rats by dehydroretronecine injections. Note the strap-like cell with tandem nuclei (arrow) that is quite characteristic of rhabdomyosarcomas. H & E, × 330.

Fig. 2. Cross-striations of the myofilaments (arrow) were occasionally observed in the neoplastic cells. Phosphotungstic acid-hematoxylin, × 510.

Fig. 3. The giant tumor cells contained large, irregularly shaped nuclei. Multiple nucleoli (arrows) are common in these cells. Note the sparsity of organelles in the cytoplasm. Uranyl acetate, × 11,290.

Fig. 4. Cross-striations in the myofibrils of a small tumor cell. Uranyl acetate, × 8,230.
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Cancer Res 1975;35:997-1002.

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