ABSTRACT

The chemoresponsiveness of a Moloney sarcoma virus-induced osteosarcoma in the rat to i.v. Adriamycin therapy was demonstrated by (a) prolongation of life span, (b) reduction in tumor size, and (c) morphological alterations of the neoplasm. Adriamycin was given to groups of osteosarcoma-bearing rats i.v. or i.p. at 2 mg/kg/week for 10 weeks, at 1 mg/kg/week for 10 weeks, and at 1 mg/kg on Monday, Wednesday, and Friday followed by a week of rest (repeated to a total of 10 injections). Rats receiving 1 mg/kg/week had the most consistent response to Adriamycin treatment, with a significant prolongation of life span from 40 through 95 days and a significant decrease in tumor diameter from 7 days through 50 days after the start of treatment. Other Adriamycin regimens also produced a significant increase in life span and a reduction in tumor size, compared to results with rats in the placebo group. Radiographic evaluation of osteosarcomas demonstrated an increased radiodensity with Adriamycin therapy, compared to a rapid proliferation and destruction of preexisting bone in the placebo group. Microscopic and ultrastructural evaluation of osteosarcomas following various intervals of Adriamycin revealed necrosis of tumor cells, fibroblastic proliferation, and mineralization of osteoid matrix. Metastatic lesions in lung and sublumbar lymph node also were sensitive to Adriamycin therapy, as reflected by necrosis of tumor cells. Evidence of congestive heart failure and cardiomyopathy was observed in all groups of rats on Adriamycin chemotherapy. The sensitivity of the osteosarcoma-bearing New Zealand black rat to the antitumor and cardiomyopathic effects of Adriamycin provides an animal system for evaluation of the oncolytic activity of new analogs as well as their cardiotoxic potential.

INTRODUCTION

Of the primary cancers of the bone in humans, osteosarcoma occurs most frequently and has a poor prognosis; the 5-year survival rate with surgery and radiotherapy remains at or below 20% (9). Initial reports with immunotherapy and surgery in the treatment of osteosarcoma show no improvement over surgery alone (16). However, recent results with chemotherapy coupled with surgical ablation of the primary neoplasm have been encouraging (2, 3, 11, 21, 22). Methods, with citrovorum factor rescue, and ADR have presently shown the most encouraging responses (11).

Although a viral etiology for human osteosarcoma has not been conclusively demonstrated, bone tumors characterized as osteosarcomas have been produced by viruses (4, 6, 23) and other agents (12, 27) in several animal species. Recent reports with immunotherapy of experimental osteosarcoma have demonstrated the responsiveness of these tumors to cell-mediated and humoral immune mechanisms (8, 26). The chemoresponsive effect of experimental osteosarcomas to ADR and other chemicals appears to be variable and may not predict well for human studies (1, 25).

We have recently described an experimental osteosarcoma produced by inoculation of young NZB rats with MSV (17, 18). Rats inoculated at 4 days of age developed osteoproliferative osteosarcomas similar to human osteosarcoma, with a high rate of pulmonary and lymphatic metastases, elevations in urinary hydroxyproline excretion, and increased serum alkaline phosphatase and calcium levels (17). This investigation was designed to evaluate the chemoresponsiveness of this osteosarcoma model to ADR for (a) prolongation of life span, (b) reduction in tumor size and incidence of metastatic disease, and (c) morphological alterations of the osteosarcoma with chemotherapy.

MATERIALS AND METHODS

All rats in this study were inoculated at 4 days of age with a partially purified preparation of MSV i.t., as described previously (10, 17, 18). Rats were palpated at weaning (21 days of age), and those without palpable tumors were eliminated from the study.

ADR chemotherapy of rats with osteosarcomas was begun at 34 days of age (30 days postinoculation). Rats were given injections i.v. in the lateral tail vein or i.p., with a lyophilized preparation of ADR hydrochloride (the bulk drug was kindly provided by Adria Laboratories Inc., Wilmington, Del.). ADR was weighed in 10- and 5-mg aliquots on an electrobalance, placed in small glass vials, and stored in the dark at 1°. At the time of injection, ADR was reconstituted with 0.9% sterile NaCl solution at 1 mg/ml and given immediately.

A total of 54 rats were given injections of ADR or 0.9% NaCl solution (placebo) with 3 treatment schedules. Fifteen rats received ADR, 2 mg/kg/week; 14 rats received ADR, 1

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2 Recipient of Fellowship CA00549 from the National Cancer Institute. To whom requests for reprints should be addressed, at Department of Veterinary Pathobiology, College of Veterinary Medicine, The Ohio State University, 1925 Coffey Road, Columbus, Ohio 43210.
3 The abbreviations used are: ADR, Adriamycin; NZB, New Zealand black; MSV, Moloney sarcoma virus; i.t., intratibial(iy); MWF, Monday, Wednesday, and Friday.
mg/kg/week; and 13 rats received ADR, 1 mg/kg MWF, followed by 1 week of rest and then another MWF schedule followed by a week of rest to a total dose of 10 mg/kg. Twelve control rats received weekly injections of 0.9% NaCl solution i.v. for 10 weeks. All animals received a total of 10 injections. Careful technique in the injection of ADR permitted most i.v. injections, but perivasculitis was severe in some rats, resulting in necrosis of the tail. Injection of ADR i.p. was then selected as the alternative route of administration. Almost all rats in all treatment groups received the first 6 ADR injections i.v. Afterward, 40 (Injection 7) to 100% (Injection 10) of the rats in each group were given ADR i.p.

All rats in each group were observed daily for evidence of toxicity (e.g., terminal cachexia or death). Rats were weighed, and osteosarcomas were measured with Helios calipers in 3 different dimensions at weekly intervals (17) at the time of injection, from the initiation to the completion of injections (up to 63 days), and at 20-day intervals thereafter. Statistical analyses of the data were performed with Student's t test (24) and with 2 × 2 contingency tables (7).

Animals receiving ADR were necropsied at the time of death or at 150 days after the first injection (termination of the experiment). Portions of osteosarcoma, sublumbar lymph node, heart, lung, liver, kidney, spleen, duodenum, and tibial epiphysial plate in controls were collected in 10% neutral buffered formalin for histopathological examination. Lungs were cut in cross-sections at 3 planes each and examined for microscopic evidence of metastases. The hearts from 10 rats and the osteosarcomas from 12 rats were examined ultrastructurally. Portions of the apex, right and left ventricular free walls, and interventricular septum were sliced free and prepared for electron microscopic evaluation (19). Bone was decalcified for 7 days in 10% buffered EDTA. All tissues for light microscopy were embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin. Selected sections of myocardium also were stained with periodic acid-Schiff with diastase digestion. Myocardium and osteosarcoma collected for fine structural evaluation were minced immediately under fixative into 0.5-cu mm blocks, fixed in cold 3% glutaraldehyde with 0.1 M sodium cacodylate buffered at pH 7.4, postfixed in 1% osmium tetroxide in s-collidine, dehydrated in graded ethanols, transferred to propylene oxide, and embedded in "hard" Epon (osteosarcoma) or "soft" Epon (myocardium) (Shell Chemical Co., New York, N. Y.). Thin sections were cut with a diamond knife on an LKB ultramicrotome and floated on a water bath. Sections were stained with uranyl acetate and lead citrate and were examined with a Philips 300 electron microscope.

RESULTS

The accumulative mortality of tumor-bearing rats on ADR therapy or of rats receiving 0.9% NaCl solution placebo is presented in Chart 1. The administration of ADR in all regimens initially had an attenuating effect on the mortality rate compared to that of control rats. The mortality incidence in all ADR treatment groups was significantly less than that of controls (p < 0.05) from 40 to 50 days after initiation of treatment. Rats receiving ADR, 2 mg/kg/week, had significantly fewer deaths (p < 0.01) at 35 days after the start of treatment. The mortality rate for rats receiving ADR, 1 mg/kg/week, was significantly less (p < 0.025) from 40 through 95 days. At all points after 95 days from the start of treatment, there was no significant difference in the rate of mortality between ADR-treated groups and the placebo group.

The effect of chronic ADR administration on weight gain in osteosarcoma-bearing rats is depicted in Chart 2. No significant effect with any regimen of ADR therapy was observed on the rate of weight gain in tumor-bearing rats compared to rats receiving 0.9% NaCl solution. At 150 days after initiation of therapy, both groups of rats receiving a total ADR dose of 10 mg/kg had weight percentage increases greater than that of the placebo group, whereas the group given a total dosage of 20 mg/kg (2 mg/kg/week) had less than that of the placebo group.

Chart 3 illustrates the results of chronic ADR therapy regimens on mean tumor diameter, compared to results with rats receiving 0.9% NaCl solution. A progressive increase of mean tumor diameter was observed in the placebo group from initiation of therapy through 50 days, with a subsequent decline in mean tumor diameter. All ADR regimens produced an overall attenuation in mean tumor diameter, with the most consistent decrease observed in rats that received ADR, 1 mg/kg/week (p < 0.05 from 7 through 50 days after the start of ADR treatment). Rats treated at 2 mg/kg/week had significant (p < 0.05) regression in tumor size 14 through 50 days, except at Day 28, compared to controls. For rats receiving ADR, 1 mg/kg, on a MWF schedule, the duration of significant (p < 0.05) reduction in
tumor size was from 28 through 50 days. Most rats (9 of 12) with osteosarcomas receiving injections of 0.9% NaCl solution had rapidly growing neoplasms. The sharp decline in mean tumor diameter 50 days after the start of treatment in the placebo group reflects the spontaneous tumor regression rate in 25% (3 of 12) of the rats surviving the period of observation (Chart 1).

Radiographic evaluation of tumor-bearing rats receiving ADR, 2 mg/kg/week, illustrated evidence of tumor regression from 7 through 50 days after the start of ADR therapy (Figs. 1 to 3). By comparison, radiographic evaluation of an osteosarcoma in the placebo group at the beginning (Fig. 4) and end (Fig. 5) of this evaluation period demonstrated progressive tumor growth and lysis of preexisting bone of the tibia. Not only did many of the regressing tumors under ADR therapy become progressively more radiodense, but also there was a reduction in total tumor size to the extent that only a very small, barely palpable enlargement remained. These animals lived through the period of observation with virtually complete tumor regression and no evidence of metastatic disease.

Light and electron microscopic evaluation of osteosarcomas from rats showed evidence of sensitivity of the primary neoplasm to ADR chemotherapy. The morphological features of an osteosarcoma from the placebo group is illustrated in Fig. 6. With ADR, tumor cells often were observed in varying stages of degeneration. Broad zones of necrosis were observed most frequently in rats receiving 2 mg/kg/week (Fig. 7). Many areas of the osteosarcomas were replaced by collagen-producing fibroblasts (Fig. 8). Extensive mineralization of osteoid matrix was observed in tumors of varying size in rats receiving both 1 and 2 mg/kg/week (Fig. 9). Broad zones of osteoid matrix with mature osteoblasts aligned along the surface were observed in most tumors from rats receiving ADR chemotherapy. Fine structural features of osteosarcoma cells from rats receiving ADR included cytoplasmic vacuolization, mitochondrial degeneration, myelin figure formation, and lysis of tumor cells (Fig. 10).

Metastases of osteosarcoma were identified in all groups of rats and included gross and/or histopathological evidence of neoplastic cells in sublumbar lymph nodes and/or lungs (Table 1). The percentage of the incidence of metastases was diminished with all schedules of ADR chemotherapy, compared to that of placebo (p < 0.05). Many of the metastatic lesions in the lungs of rats receiving 2 mg/kg/week were very small and appeared to be undergoing degeneration (Fig. 11). One enlarged sublumbar lymph node in a rat receiving 1 mg/kg/week had a focus of mineralization of osteoid matrix (Fig. 12). Similar lesions were not observed in the placebo group.

Cardiotoxicity of ADR was defined by 2 parameters: gross evidence of congestive heart failure (e.g., ascites, pleural effusion, and/or cardiomegaly) and histopathological evidence of cardiomyopathy, including the presence of focal degenerating myocytes by the periodic acid-Schiff stain with diastase digestion, interstitial and intramyocytic vacuolization, and myocardial edema (Table 1). Both congestive heart failure and cardiomyopathy were observed in all groups of rats on ADR chemotherapy, but there was no evidence of cardiotoxicity in the placebo group. For all ADR treatment groups, a high percentage of cardiomyopathy (67 to 91%) was observed in those hearts examined histopathologically.

DISCUSSION

The results of this study show that the chronic administration of low doses of ADR can significantly reduce the mortality rate of osteosarcoma-bearing NZB rats. Chronic ADR administration had no effect on body weight gains compared to that of the placebo. All ADR therapy regimens had a significant effect in reducing the size of primary osteosarcomas at certain time points and in reducing the incidence of pulmonary metastases, but 1 mg/kg/week gave the most consistent effect in reducing the size of the primary tumor. Present data suggest that ADR-induced cardiotoxicity may be an important factor in causing the death of the osteosarcoma-bearing rat at an accumulative dosage of 10 or 20 mg/kg.

Recent reports about the use of ADR chemotherapy in human osteosarcoma have been encouraging. Many of the
studies have used multidrug chemotherapy including ADR and methotrexate to effect regression of metastases (11, 21, 22). One investigation (21) recommends the use of aggressive presurgical chemotherapy to induce primary osteosarcoma regression, followed by en bloc resection of the neoplasm with total knee prosthetic replacement. These studies and the results of our investigation demonstrate the sensitivity of osteogenic sarcoma to ADR and the potential usefulness of ADR in combination with surgery and additional chemotherapy for providing improved long-term survival to patients with this disease.

Previous reports from our laboratory have shown that the NZB rat is sensitive to the cardiotoxic effects of both subacute high-dose (19) and chronic low-dose (20) ADR i.v. Rats with osteosarcomas in this investigation, receiving a total ADR dose of either 10 or 20 mg, were also sensitive to this toxic side effect. The data suggest that rats with osteosarcoma may have a higher incidence of cardiomyopathy at the ADR schedule of 1 mg/kg MWF than was shown in previous studies with control rats (20). Other investigators (5) have shown increased cardiac uptake of ADR in mice bearing Lewis lung carcinoma and B16 melanoma, compared to that of controls. The observation of cardiotoxicity in the rat (13, 20) makes this animal model particularly useful as a system for monitoring objective tumor response as well as the development of cardiotoxicity with new ADR analogs. Recent studies (14, 15) have shown that the ADR-induced cardiotoxicity in mice may be related to lipid peroxidation in myocardium, suggesting that treatment of osteosarcoma-bearing rats with α-tocopherol may allow sustained objective remission of osteosarcoma without cardiotoxicity with similar regimens of ADR.

Objective tumor remission with chemotherapy in our study was accompanied by increased radiodensity and necrosis of tumor cells with mineralization; this is similar to the response that has been described when aggressive chemotherapy preceded subtotal en bloc resection of the primary osteosarcoma (21). For long-term remission in human patients, surgical resection of the primary lesion and metastases is important, since metastatic lesions may recur within 3 to 14 months with resistance to chemotherapy; this may partially explain the ultimate high mortality in our treatment groups in spite of a lesser total dosage of ADR (10 mg/kg).

This study has demonstrated the usefulness of MSV-induced osteosarcoma as an experimental model to investigate the sensitivity to ADR chemotherapy. No attempt was made to reduce the initial tumor load by subtotal or total resection. Although ADR therapy was instituted well after the primary osteosarcoma was palpable, objective chemoresponsiveness has been demonstrated with this animal model.

MSV-induced osteosarcoma in NZB rats has considerable potential as an animal model system for investigating different chemotherapeutic regimens for osteosarcoma. Total resection before and after instituting chemotherapy may be useful in determining which approach best prolongs life span. The initiation of chemotherapy with younger rats bearing smaller osteosarcomas may also enhance chemoresponsiveness, by limiting the potential for continued growth of the primary neoplasm or development of metastases. Finally, the sensitivity of the NZB rat to the cardiotoxic effects of ADR provides a model system for evaluating the oncolytic activity of new ADR analogs as well as their cardiotoxic potential.

REFERENCES
Figs. 1 to 3. Radiographs illustrating sequential regression of osteosarcoma in a rat receiving ADR, 2 mg/kg/week.

Fig. 1. Osteosarcoma with osteogenic activity 7 days after the start of ADR injections. × 1.

Fig. 2. Osteosarcoma is smaller and more radiodense 21 days after the start of ADR. × 1.

Fig. 3. Small osteosarcoma elevated above the diaphyseal area (arrow) 50 days after the start of ADR. × 1.

Figs. 4 and 5. Radiographs illustrating the appearance of an osteosarcoma in a rat from the placebo group.

Fig. 4. Osteosarcoma with considerable osteogenic activity 7 days after the start of injections with 0.9% NaCl solution. × 0.9.

Fig. 5. Progressive growth of osteoproliferative osteosarcoma with destruction of preexisting tibial bone 50 days after the start of injections with 0.9% NaCl solution. × 0.9.

Fig. 6. Osteosarcoma from placebo group, composed of large pleomorphic and small fusiform tumor cells with an abundant osteoid stroma. H & E, × 330.

Fig. 7. Extensive area of coagulation necrosis in an osteosarcoma 26 days after the start of ADR treatment. H & E, × 130.

Fig. 8. Scattered neoplastic cells (arrow) with marked proliferation of fibroblasts in an osteosarcoma from a rat 21 days after the start of ADR, 2 mg/kg/week. H & E, × 330.

Fig. 9. Mineralization of osteoid matrix in the osteosarcoma of a rat receiving ADR, 2 mg/kg/week. H & E, × 130.

Fig. 10. Electron micrograph of cells in an osteosarcoma from a rat receiving ADR, 1 mg/kg/week. Degeneration of neoplastic cells includes cytoplasmic vacuolization (v), mitochondrial degeneration, myelin figure formation (arrow), nuclear degeneration (N), and cell lysis. Uranyl acetate and lead citrate, × 5700.

Fig. 11. Vacuolization (v) and pyknosis (arrows) of tumor cells in a pulmonary metastasis from an osteosarcoma in a rat receiving ADR, 2 mg/kg/week. H & E, × 330.

Fig. 12. Mineralization of osteoid matrix (O) in a metastatic focus of osteosarcoma in the sublumbar lymph node from a tumor-bearing rat treated with ADR, 1 mg/kg/week. H & E, × 330.
Chemoresponsiveness of Moloney Sarcoma Virus-induced Osteosarcoma to Adriamycin in the Rat

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