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The Ohio State University,
Ph.D., 1977
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PATHOLOGIC CHARACTERIZATION AND CHEMORESPONSIVENESS
TO ADRIAMYCIN OF MOLONEY SARCOMA VIRUS-INDUCED
OSTEOSARCOMA IN THE RAT

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

Harry Marshall Olson, B.S., D.V.M.

* * * * *

The Ohio State University

1977

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ACKNOWLEDGMENTS

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"Chemical and Morphologic Alterations of Rabbit Bone Induced by Adriamycin." Calcif. Tiss Res. 18, 47 (1975).


FIELDS OF STUDY

Major Fields: Veterinary Pathology and Experimental Oncology


Studies in Endocrine Pathology. Professor Charles C. Capen
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Chart 2: Weight gain in all groups of tumor-bearing rats receiving three schedules of ADR chemotherapy or saline placebo.

Chart 3: Mean tumor diameter of groups of rats receiving three schedules of ADR chemotherapy or saline placebo.

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Figure 2: Osteosarcoma is smaller and more radiodense after 21 days of ADR.

Figure 3: Small osteosarcoma elevated above the diaphyseal area after 50 days of ADR.

Figure 4: Regression of osteosarcoma to small, radiodense mass after 80 days of ADR.
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INTRODUCTION

Osteosarcoma is an important neoplastic disease in young adults, characterized by a rapid progression with the development of metastases and a very low survival rate. Recent reports have suggested a viral etiology for human osteosarcoma, but no viruses have been isolated from human osteosarcoma tissue. Osteosarcomas have also occurred in children following $^{224}$Ra therapy for tuberculosis and other diseases, and have been associated with high plasma somatomedin levels. Current therapeutic regimens have been moderately successful in offsetting the high mortality associated with this disease. Surgical ablation accompanied by radiotherapy has produced a 5 year survival rate of only 20%. Preliminary results indicate that immunotherapy of osteosarcoma does not substantively increase the survival rate. At the present time, surgical ablation followed by chemotherapeutic regimens to destroy metastatic foci provides the best therapeutic result. These clinical results suggest that a well-characterized and reproducible animal model of osteosarcoma would be useful in: (1) further understanding the biological behavior of this neoplasm, and (2) developing new therapeutic regimens for osteosarcoma.
Bone tumors characterized as osteosarcomas have been produced by viruses,\textsuperscript{2,9-12} chemicals\textsuperscript{13,14} and radiation\textsuperscript{12,15} in several species. Most of these have been induced by the intravenous or intraperitoneal instillation of the inciting agent, resulting in multicentric osteosarcomas,\textsuperscript{9-12,15} unlike the typical situation in man. Some of these tumor systems are also characterized by the concurrent development of sarcomas in tissues other than bone\textsuperscript{9,10,13,14} and by long latent periods.\textsuperscript{9,12,15} We produced osteosarcomas by the intratibial instillation of Moloney sarcoma virus (MSV) in neonatal rats, as reported by Ikemoto \textit{et al.}\textsuperscript{16} In a preliminary report,\textsuperscript{17} we noted an age-related susceptibility to MSV-induced osteosarcomas in the rat and indicated that rats inoculated at 4 days after birth (compared to 1 day) develop more osteoproliferative bone neoplasms. The objective of this investigation was to characterize the MSV-induced osteosarcoma in rats inoculated at 1 day and 4 days of age by macroscopic, radiographic, light and electron microscopic parameters and by selected biochemical indices reportedly altered in human osteosarcoma.

\textbf{Materials and Methods}

\textbf{Animals and Virus Preparation}

One day old (<24 hours) and 4 day old New Zealand black (NZB) rats from a breeding colony (kindly supplied by Mr. Clarence Reeder, Drug Research and Development Branch, National Cancer Institute) were inoculated by intratibial instillation of a partially purified preparation of MSV (courtesy of Dr. David Howell, Viral Oncology, N.C.I.). The inoculum or standard viral preparation (SVP) consisted of the MSV (lot
MSV-B-77, from BALB/c mice), titered at $10^{5.7}$ to $10^{6.5}$ focus forming units/ml, diluted 1:7 with a diluent consisting of 2% inactivated fetal calf serum and 1% antibiotics without mycostatin in sterile physiological saline. Rats received a constant volume (either 0.025 or 0.05 ml) of SVP in one or both tibias. Control rats received an equal volume of diluent with citrate buffer approximating the concentration in the SVP by intratibial instillation.

**Experimental Design and Sample Preparation**

Pathogenesis experiment and survival study. Rats inoculated at 1 (4 litters, 32 rats) and 4 (5 litters, 38 rats) days of age were monitored for the development of osteosarcomas by palpation and radiography. Rats were palpated daily from 5 days post-inoculation (pi) until day 30 pi and were radiographed on days 8, 10, 12, 15 and 20 pi, and thereafter at 10 day intervals until day 100 pi. Radiographs were taken with a General Electric Model 11AA-3A X-ray unit (25 or 30 KV, 5 ma, 10 or 12 sec/exposure) and Kodak no-screen medical X-ray film (Ready Pack NS 2T). Tumor-bearing rats (18 rats from day 1, 14 rats from day 4) were then monitored on a daily basis for mortality rates. Cumulative mortality data were tabulated and rats were necropsied for evaluation of the occurrence of metastatic disease.

Tumor associated cachexia, tumor development and biochemical studies. Beginning at 30 days pi, tumor-bearing and control rats were weighed at 10 day intervals on a Mettler P 1000 N top loading balance. At days 25 and 30 pi, and subsequently at 10 day intervals, tumor-bearing and control rats were placed in metabolism cages to collect
24 hour urine samples under toluene. Aliquots of urine were assayed for total urinary hydroxyproline (HOP) and creatinine (CR). Final values were expressed as a HOP to CR ratio to correct for variation in urine concentration. At these same time intervals, tumors were measured in three dimensions (length, width and thickness) with Helios stainless steel calipers and the average tumor diameter computed.

Serum samples were collected at 10 day intervals from day 30 pi, under light ether anesthesia from the retro-orbital sinus or terminally from the abdominal aorta. Serum calcium was determined by atomic absorption spectrophotometry (Perkin-Elmer model 303) and alkaline phosphatase by the method of Hausamen et al. Statistical analyses of data were performed using Student's t-test and 2 X 2 contingency tables.

Light and electron microscopic study. Tissues were collected from rats that died and from rats that were killed at specific time points pi. At necropsy, sections of osteosarcoma, sublumbar lymph node, lung (including bronchial lymph nodes), thymus, spleen, liver, kidney, intestine and thyroid were fixed in 10% neutral buffered formalin, decalcified where necessary for 7 days in 10% buffered EDTA, embedded in paraffin, sectioned at 6 µ and stained with hematoxylin and eosin (H & E). Multiple sections from the osteosarcomas (day 1: 10 tumors; day 4: 6 tumors) of killed rats were minced immediately under fixative into 1 mm³ blocks, fixed in cold 3% glutaraldehyde with 0.1 M sodium cacodylate buffered at pH 7.4, post-fixed in 1% osmium tetroxide in s-collidine, dehydrated in graded ethanols, transferred to propylene oxide, and embedded in "hard" Epon (Shell Chemical Company, New York,
N.Y.). Thin sections were cut with a diamond knife on an LKB ultramicrotome and floated on a water bath buffered at pH 7.4 to prevent demineralization. Sections were stained with uranyl acetate and lead citrate and were examined with a Philips 200 electron microscope.

Results

Pathogenesis Experiment

Initiation and development of osteosarcomas in rats inoculated with the SVP at 1 day of age (<24 hours old) were observed radiographically as early as 10 to 12 days pi (11/12 rats). A small radiodense zone was observed with focal lysis of the tibial diaphysis (Figure 1). Neoplastic cells appeared to arise from the endosteal surface proliferating within the metaphysis and diaphysis at day 14 pi with infiltration and disruption of the tibial cortex (Figure 2). Many osteosarcomas grew rapidly, producing disruption of the tibial cortex and compression of the adjacent musculature. At day 20 pi, the osteosarcomas had attained an average diameter of more than 1 cm. Radiographs showed both radiolucent zones and irregular dense spicules (Figure 3) in the osteosarcomas. The densely cellular sarcomatous proliferation was surrounded by a prominent chondroid border (Figure 4). Not all osteosarcomas developed this rapidly in rats inoculated at day 1 of age. Daily palpation and periodic radiographs of many inoculated litters of rats indicated that the latent period was occasionally greater than 12 days, and sometimes palpable tumors were not observed until day 23 pi (6% of inoculated rats).
Rats inoculated at day 4 after birth developed palpable and/or radiographic evidence of tumor development as early as day 12 pi. Most tumors were palpable by day 15 pi (6/8 rats) but some tumors did not appear until as late as day 24 pi (3% of inoculated rats). Radiographically, the osteosarcomas that developed early were similar to those described following inoculation at day 1 of age, but at day 20 pi they were histologically more differentiated neoplasms with considerable mineralized matrix.

Survival and Tumor Development Study

Tumor-bearing rats inoculated at 4 days of age lived significantly longer than rats inoculated at 1 day of age (Figure 5). Litters of rats inoculated at 1 day (4 litters) and 4 days (5 litters) of age were observed for 100 days pi. By 25 days pi, 25% of tumor-bearing rats inoculated at 1 day of age had died, whereas the earliest death observed in rats inoculated at 4 days of age was at day 60 pi. By day 100 pi, 95% of rats inoculated at 1 day of age had died, compared with 64% of rats inoculated at 4 days of age. No control rats of either group died during the same periods of observation. Tumor growth was rapid during the early weeks in both groups of inoculated rats (Figure 6). The degree of variability in tumor size was considerable in both groups of rats. Nonetheless, the mean diameters of osteosarcomas in rats inoculated at day 1 was consistently greater than for rats inoculated at day 4. The difference was significant at days 30 and 60 pi. Striking differences were also observed in the radiodensity and gross consistency of the tumors in the two groups. Osteosarcomas in rats inoculated on day 4 of age were radiodense, firm, and contained
abundant mineralized stroma. Conversely, rats inoculated on day 1 of age developed osteosarcomas with large radiolucent zones that were fleshy and contained extensive areas of necrosis.

**Tumor Associated Cachexia**

The development of rapidly growing osteosarcomas was commonly accompanied by progressive wasting. Tumor-bearing rats failed to achieve the weight gains of their respective controls (Figure 7). Male \( (n = 10) \) and female \( (n = 17) \) tumor-bearing rats inoculated at 1 day of age had significant weight loss compared to their respective controls on days 60 and 70 pi, and tumor bearing males at day 80 pi. At days 80 and 90 pi, the mean weight of female rats with tumors was less than control female rats, although the differences were not significant. Few rats of either sex with tumors lived beyond day 80 pi.

Figure 8 shows the cachexia developing in tumor-bearing male \( (n = 13) \) and female \( (n = 12) \) rats inoculated 4 days after birth compared to controls. Female rats with osteosarcoma developed a significant loss in body weight by 40 days pi. Thereafter, both male and female tumor-bearing rats consistently failed to match the weight gains of their respective controls throughout the time period evaluated (up to day 90 pi).

**Radiographic and Light and Fine Structural Features of Osteosarcomas**

Radiographic and histologic evaluation of osteosarcomas produced after inoculation of rats at 1 day and 4 days of age revealed the development of markedly different bone neoplasms in these two groups. Neoplasms in rats inoculated with the SVP on day 1 were radiolucent
tumors with fine interspersed radiodense spicules. They were densely cellular and composed of pleomorphic tumor cells with large, prominent (occasionally multiple) nuclei with coarsely clumped chromatin and a vacuolated cytoplasm (Figure 9). Smaller isomorphic cells resembling osteoblasts and fibroblasts were interspersed throughout the neoplasm. Occasional fine spicules of osteoid or chondroid matrix were observed in the tumors (Figure 10) as well as broad zones of central necrosis. In some areas of the osteosarcoma large giant cells, containing 20 or more evenly distributed nuclei, were observed either in association with bone matrix or located within the densely cellular areas of the tumor. Areas of chondroid differentiation (Figures 4 and 10) were most often seen at the periphery of the rapidly growing neoplasms during the early weeks of development.

Rats inoculated with MSV on day 4 after birth developed more uniformly radiodense osteosarcomas with only small lucent zones. These tumors were characterized histologically by the presence of an abundant osteoid stroma. Some areas of chondroid were present, but osteoid differentiation was more commonly observed. Pleomorphic tumor cells were interspersed between well differentiated osteoblasts lining the osteoid stroma (Figure 11). Multinucleated osteoclasts also were observed in the osteosarcomas that developed in rats inoculated at 4 days of age.

Histologic evaluation of sublumbar lymph nodes and lungs revealed 94% (17/18) of rats inoculated at day 1 and 93% (13/14) of tumor-bearing rats inoculated at day 4 had metastatic lesions. Although metastases were composed of similar types of cells as in the primary neoplasm, they infrequently produced an osteoid matrix. Tumor cells were
observed within vascular channels in the lungs and occasionally large metastatic nodules compressed adjacent pulmonary tissue (Figure 12). Most metastatic lesions were occult depending upon microscopic examination for detection; however, sublumbar lymph nodes with metastases were sometimes grossly enlarged. When osteosarcomas developed unilaterally, metastatic lesions were present only in the ipsilateral sublumbar node. Evaluation of other major organs in tumor-bearing rats revealed no evidence of metastases, except in two rats where histologically benign proliferative lesions were present in the mandible and ribs. In addition, no other neoplasms developed in any control or MSV-inoculated rats during the period of observation (until death or day 150 pi).

Ultrastructural evaluation of osteosarcomas from rats inoculated at days 1 and 4 after birth revealed a spectrum of tumor cell types. Many neoplastic cells were poorly organized with an irregular villous cell membrane and one or more irregular, eccentrically located nuclei. Most of the cells had scattered mitochondria, dilated rough endoplasmic reticulum, a large Golgi apparatus, and clusters of free ribosomes (Figure 13). These cells were most frequently observed in osteosarcomas from rats inoculated at 1 day of age. Other tumor cells resembling osteoblasts had a large nucleus with condensed nuclear chromatin and an irregular cytoplasmic outline (Figure 14). Their abundant cytoplasmic area was filled with rough endoplasmic reticulum, mitochondria, and a few homogeneous electron-dense bodies. These osteoblastic tumor cells were surrounded by osteoid (Figure 14). Many neoplastic cells had prominent dilated endoplasmic reticulum filled with a finely granular proteinaceous material. In some neoplastic cells, this proteinic
material was condensed in a circular or branching pattern in the
dilated cisternae (Figure 15). Occasional tumor cells in all neo-
plasms evaluated by electron microscopy had intercellular tight junc-
tions or zonula occludens (Figure 16). Microtubules and microfilaments
(Figure 17) were seen frequently interspersed throughout the cytoplasm
in tumor cells.

Ultrastructural evaluation of osteosarcomas from rats inoculated
at 4 days of age revealed many well-differentiated osteoblasts sur-
rounded by a fibrillar osteoid stroma with a mineralization front
(Figure 18). The majority of cells observed in these tumors were ovoid
or fusiform with abundant profiles of endoplasmic reticulum, mitochon-
dria and lysosomal bodies within the cytoplasm (Figure 18). The nuclei
had a smooth nuclear membrane and a single prominent nucleolus. Cyto-
plasmic processes extended into the adjacent osteoid stroma, which con-
tained foci of mineralization (Figure 18). Giant cells with multiple
nuclei distributed throughout the cell were observed in tumors from
both groups of rats. They contained abundant mitochondria interspersed
throughout the cytoplasm, and perinuclear Golgi apparatus. Most giant
cells observed ultrastructurally were not associated with mineralized
matrix.

C-type viral particles were frequently observed budding from the
plasma membrane of all types of cells within these osteosarcomas (Fig-
ure 19). Virus particles often were observed between the cytoplasmic
interdigitations of tumor cells. Viral budding also was observed from
the plasma membrane of superficial osteocytes (Figure 20) into the la-
cunar space, which contained numerous mature C-type particles. A-type
virus particles were seen frequently within dilated cisternae of rough endoplasmic reticulum in neoplastic cells.

**Biochemical Investigations**

Rats inoculated with the SVP on day 4 of age developed significant elevations of serum calcium (Figure 21) and alkaline phosphatase (Figure 22) \((n = 9)\) when compared to controls \((n = 7)\). Conversely, significant elevations in both parameters were observed infrequently in rats inoculated 1 day after birth \((n = 7)\) (Figures 21 and 22). On days 40, 50 and 70 pi both serum calcium and alkaline phosphatase in rats inoculated on day 4 were significantly elevated over corresponding control values, and alkaline phosphatase was also significantly elevated at day 60 pi. Serum calcium but not alkaline phosphatase was significantly elevated at day 80 pi in rats inoculated on day 4. At day 60 pi, the mean serum calcium value from tumor bearing rats inoculated on day 1 was greater than corresponding control values, resulting from hypercalcemia as high as 15.7 mg/100 ml serum in one rat. These differences were not significantly different from control rats evaluated at day 60 pi.

Urinary hydroxyproline excretion expressed as HOP/CR was significantly elevated in both groups of tumor-bearing rats compared to controls at days 25 and 30 pi, and again at day 70 pi (Figure 23). Sustained high levels of hydroxyproline excretion were observed for rats inoculated at day 4 of age \((n = 9)\) compared to controls \((n = 5)\) at all time points evaluated. For tumor bearing rats inoculated at day 1 of age \((n = 10)\), no other data points were significantly different from corresponding controls \((n = 5)\).
Discussion

In this investigation, we report the pathogenesis and the morphological, biochemical and tumor-associated characteristics of the intratibial MSV-induced osteosarcoma in the rat. As opposed to other published models of osteosarcoma, either spontaneous or induced, this animal model combines the advantages of reproducibility and high tumor incidence with a brief latent period. These discrete osteosarcomas can be subjected to numerous evaluation procedures including palpation, measurement, biopsy or surgical excision. In addition, other neoplasms arising in conjunction with or as a result of the inoculation of MSV were not observed, thus providing an uncomplicated in vivo tumor system.

In previous communications we reported an age-related tumor incidence of MSV-induced murine osteosarcoma. Age susceptibility to viral transformation also has been demonstrated with Gross virus-induced lymphoma in rats. While osteosarcomas in our studies occurred with a similar high incidence in rats inoculated at 4 days of age compared to rats inoculated at day 1, the former group had consistently more osteoproliferative neoplasms on the basis of radiographic, gross and microscopic evaluation. These findings suggested that the age of the rat at inoculation of the MSV may be significant in the subsequent development of different types of osteogenic sarcomas. The exposure of a more differentiated population of bone cells in older rats (day 4 of age) to the MSV may be related to the subsequent development of more osteoproliferative bone tumors compared to rats inoculated at 1 day of age.
The results of the pathogenesis study indicated an average latent period of 10 to 12 days in rats inoculated on 1 day of age. These findings are compatible with results recently reported by Friedlaender et al. In observations of rats up to 150 days pi, we have noted that rats inoculated intratibially either develop palpable tumors by 30 days pi or do not develop osteosarcomas at all. We have further defined the neoplasm as arising from the endosteal surface of bone following inoculation of MSV in the marrow space. Fujinaga et al. reported the periosteal proliferation of multicentric MSV-induced osteosarcomas in hamsters and rats following intraperitoneal inoculation. This may be explained on the basis of a vascular distribution following inoculation of the virus. We hypothesize that the viral transformation of osteoprogenitor cells in rats inoculated on day 1 gives rise to poorly differentiated osteosarcomas with minimal osteoid formation. Rats inoculated at 4 days of age had a slightly longer latent period (10-15 days) and developed more typical osteoproliferative bone tumors. This may be the result of MSV-induced transformation and subsequent proliferation of more differentiated, osteoblastic cells capable of forming abundant osteoid.

Osteosarcomas in both groups of rats were very rapidly growing. Despite some variability in tumor size within each group, the mean tumor diameter of rats inoculated at 1 day of age was consistently greater than that of rats inoculated at 4 days of age. This suggests that osteosarcomas may grow more rapidly in rats inoculated at an earlier age. In a previous report, we indicated that 14% of rats inoculated with 0.05 ml of MSV at 10 days of age developed palpable
osteosarcomas. These tumors averaged only 0.8 cm. in diameter at 50 days pi. We observed that by day 60 pi, many rats inoculated with MSV at day 1 of age had large osteosarcomas which impeded functional utilization of the limb, compared to the smaller neoplasms in the rats inoculated at 4 days of age. In addition, tumor necrosis was frequently observed in the group inoculated at 1 day of age by 60 days pi but was rarely seen in tumors of rats inoculated on day 4.

Tumor associated cachexia, as evaluated by significant weight loss in comparison to age and sex-matched controls, was observed in both groups of rats at several time points in both sexes. Patients with osteosarcoma and other neoplastic diseases often have cachexia which is hypothesized to be related to anorexia, tumor necrosis, water and electrolyte abnormalities, increased basal metabolic rate, and severe derangement of host metabolic patterns.30

Light microscopic study of osteosarcomas in rats inoculated at 1 day of age was similar to previously published investigations of virus,9-12,16,28,29 chemical,13,14 and radiation-induced12,15 experimental osteosarcomas. We did observe a consistently high incidence (93%) of metastases either within sublumbar lymph nodes, lungs or both in tumor-bearing rats. Those rats in which no microscopic evidence of metastatic disease was observed usually had small or very slow growing neoplasms, and may have been "regressors"28 or rats in which osteosarcoma growth ceased at some point as a result of either cell-mediated or humoral immune response against the neoplasm. A recent study investigating the immunologic responsiveness of rats to osteosarcomas induced by intratibial inoculation of MSV reported both cell-mediated
immunity (CMI) and serum factors capable of augmenting or decreasing the in vitro CMI response. In another investigation the humoral immunologic response was greatest in rats with regressing neoplasms. These findings are significant in the light of recent reports of a tumor-specific immune response in patients with osteosarcoma, and suggest a basic immunologic similarity between this model and the natural human neoplasm.

Recent reports of human osteosarcoma described the spectrum of cell types in these neoplasms and evaluated their fine structural features. In our investigations, tumors from rats inoculated at day 1 of age were composed predominately of poorly differentiated bone cells. Large cells with poorly differentiated cytoplasmic organelles resembled osteoprogenitor cells. In more differentiated osteoblast-like cells, an extensive network of endoplasmic reticulum was seen, which often contained finely granular or condensed flocculent material in dilated cisternae, similar to that described in human osteosarcomas. The nature of the branched material is not known, although the production of an abnormal matrix protein has been postulated.

Intercellular tight junctions were frequently seen between well-differentiated neoplastic cells, similar to those described between osteoblasts and osteocytes in normal bone. These zonula occludens in normal bone are considered to be associated with the intercellular transport of nutrients and electrolytes, and have been described both in human osteosarcoma and in a recent ultrastructural study of canine osteosarcoma.
Osteosarcomas in rats inoculated at day 4 of age were composed predominately of well-differentiated osteoblastic cells, frequently surrounded by partially mineralized osteoid matrix, similar to human osteogenic sarcomas.\textsuperscript{33,34} Primitive cells and bone cells in varying stages of differentiation also were present, but were considerably less numerous than in tumors from the day 1 group. Budding and mature C-type virus particles were observed frequently and were similar to those described in other MSV-induced osteosarcomas.\textsuperscript{10,29} Viral particles were seen between interdigitating plasma membranes of anaplastic tumor cells, as well as along plasma membranes of neoplastic osteoblasts and osteocytes in both groups of rats. Microfilaments and microtubules were observed within the cytoplasm of neoplastic cells from all tumors, and appeared similar to those described in human osteosarcoma cells.\textsuperscript{33,36}

Additional evidence for the development of two types of osteosarcomas with different biologic characteristics is indicated by the sustained increased excretion of urinary hydroxyproline (HOP:CR) only in tumor-bearing rats inoculated at day 4. This indicates a more rapid turnover of bone matrix\textsuperscript{,40} compared to both tumor-bearing rats (day 1 group) and to controls. Elevated HOP excretion has been reported in the majority of human osteosarcoma patients.\textsuperscript{41} This is in agreement with our histologic and ultrastructural observation that tumors in rats inoculated on day 4 are primarily osteoproliferative, with production and breakdown of osteoid matrix. Further support for these differences between osteosarcomas in rats inoculated at day 1 and 4 of age was seen with the elevated serum alkaline phosphatase and calcium values. Alkaline phosphatase was elevated in rats inoculated at 4 days
of age with more osteoblastic neoplasms that released this enzyme into the serum. Murine osteosarcomas have been reported to elaborate alkaline phosphatase, which is released into the culture medium. Preliminary findings in our laboratory demonstrate that the bone isoenzyme constitutes the major fraction in rats in the day 4 group with marked serum alkaline phosphatase elevations.

Hypercalcemia associated with malignancy has been described in numerous neoplastic disorders, but is not consistently observed in human osteosarcoma. The hypercalcemia in this model is sporadic, but occurs in some rats of both groups at certain time points. Many factors may be responsible for the hypercalcemia of malignancy and its control may have a direct bearing on prognosis of the osteosarcoma.

There has been considerable interest recently in the development of animal models for osteosarcoma, since this is a serious disease of young adults and current therapeutic measures to manage its progression have been only moderately successful. This report presents a murine model of osteosarcoma which features a discrete, manipulable neoplasm produced with a high incidence after a short latent period with significant differences in biological activity and effects on the host depending upon time of viral inoculation. The MSV-induced osteosarcoma should be a valuable animal model to investigate the biologic behavior of osteosarcoma and to evaluate new therapeutic regimes.
Summary

Osteosarcomas were produced by the intratibial inoculation of New Zealand black rats with Moloney sarcoma virus (MSV) at 1 day and 4 days of age. Radiographic evidence of osteosarcoma development was first demonstrated at 10 to 15 days post-inoculation (pi) in both groups. Subsequent radiographic, light and electron microscopic evaluation of tumor-bearing rats demonstrated that osteosarcomas in rats inoculated at day 4 of age were more osteoproliferative osteosarcomas than in rats inoculated on day 1. Rats inoculated at 4 days of age lived longer, had more slowly growing osteosarcomas, and developed a consistent tumor-associated cachexia compared to tumor-bearing rats inoculated at day 1. Both groups of rats had a 93% metastasis rate involving either sublumbar lymph nodes, lungs, or both. Tumor-bearing rats inoculated at 4 days of age had consistent elevations in both urinary hydroxyproline excretion (HOP:CR) and serum alkaline phosphatase, and in serum calcium at some time points. The high tumor incidence after a short latent period and the morphologic and biochemical similarities between the MSV-induced murine osteosarcoma and the osteosarcoma in human beings makes this discrete tumor a valuable animal model for the evaluation of new therapeutic regimens.
Figures 1-4. Radiographic and macroscopic features of developing MSV-induced osteosarcoma in young rats inoculated 1 day after birth. Figure 1. Radiodense zone with lysis of left tibial cortex (white arrow) at 12 days pl (X 2.5).
Figure 2. Metaphyseal proliferation of neoplastic cells (arrow) at 14 days pi. There is also neoplastic infiltration of adjacent cortex (H & E, X 13).
Figure 3. Lytic destruction of right tibia and irregular radiodense spicules (white arrow) at 20 days pi (X 2.5).
Figure 4. Disruption of tibial cortex (arrow) by osteosarcoma at 20 days pi. Note prominent chondroid border (arrowheads) (H & E, X 13).
Figure 5. Cumulative mortality data for tumor-bearing rats inoculated intratibially with MSV on day 1 (open circles) and day 4 (open triangles) of age. The mortality incidence of rats inoculated at day 1 \( (n = 18) \) is significantly greater (at \( p < .05 \) level or greater) than for rats inoculated at day 4 \( (n = 14) \) at all time points after day 20 pi, except at 90 days pi \( (p > .05) \).
CUMULATIVE MORTALITY OF RATS WITH OSTEOSARCOMA
Figure 6. Rate of tumor growth in rats inoculated with MSV on day 1 (open circles) and day 4 (closed circles) of age. The bars indicate standard error of the mean and the asterisks indicate significant difference of means at p < .05 or greater.
RATE OF TUMOR GROWTH IN RATS INOCULATED WITH MSV

**FIG 6**

TUMOR DIAMETER (cm)

DAYS POST INOCULATION
Figure 7. Tumor-associated cachexia in tumor-bearing male (solid circles) and female (open circles) rats inoculated with MSV < 24 hours after birth. Age-matched control male (closed squares) and female rat (open squares) mean weights are shown. The bars represent standard error of the mean and the asterisks indicate significant difference of means at p < .05 or greater.
TUMOR-ASSOCIATED CACHEXIA IN RATS INOCULATED WITH MSV <24 HOURS AFTER BIRTH
Figure 8. Tumor-associated cachexia in tumor-bearing male (solid circles) and female (open circles) rats inoculated with MSV at 4 days of age. Age-matched control male (closed squares) and female rat (open squares) mean weights are shown. The bars represent standard error of the mean and the asterisks indicate significant difference of means at $p < .05$ or greater.
TUMOR-ASSOCIATED CACHEXIA IN RATS INOCULATED WITH MSV AT 4 DAYS AFTER BIRTH

FIG 8
Figure 9. Osteosarcoma from rat inoculated at 1 day of age, day 60 pi. Pleomorphic ovoid neoplastic cells with single or multiple nuclei (arrows) and multinucleated giant cells, and a smaller, isomorphic cell population (arrowheads) (H & E, X 735).
Figure 10. Osteosarcoma from rat inoculated at 1 day of age, day 30 pi. Broad zones of chondroid matrix (C) and osteoid spicules (arrowhead) adjacent to cellular area (H & E, X 300).
Figure 11. Osteosarcoma from rat inoculated at 4 days of age, day 50 pi. An area of fusiform cells separates osteoblastic cells lining osteoid matrix (arrow) from pleomorphic tumor cells with prominent nuclear chromatin (arrowhead) (H & E, X 700).
FIG 11
Figure 12. A nodule of neoplastic cells within a vascular space in the lung of a rat inoculated with MSV on day 4 of age, day 70 pi. Tumor cells with large prominent nuclei are compressing the adjacent pulmonary parenchyma (H & E, X 700).
Figure 13. Electronmicrograph of a portion of a large, poorly organized neoplastic cell from osteosarcoma (inoculated day 1 of age, day 60 pi). There is an irregular convoluted nuclear membrane, and the cytoplasm is composed of a homogeneous granular matrix with mitochondria, free ribosomes and dilated profiles of endoplasmic reticulum (X 6500).
Figure 14. Osteoblast-like cell from osteosarcoma in a rat inoculated at 1 day of age, day 60 pi. There is a prominent irregular nucleus, several electron-dense bodies (B) and mitochondria, dilated profiles of endoplasmic reticulum (ER) and a convoluted plasma membrane. An abundant fibrillar matrix (M) with collagen fibers surrounds the cell (X 10,600).
Figure 15. Portion of an osteoblast-like cell from osteosarcoma in rat inoculated at 1 day of age, day 60 pi. Cisternae of dilated endoplasmic reticulum filled with a finely granular matrix (arrows) and a branched and circular electron-dense material (arrowheads) are seen (X 27,500).
Figure 16. Zonula occludens between tumor cells in an osteosarcoma from rat inoculated at 1 day of age, day 30 pi (X 22,000).
Figure 17. Microtubules (arrowheads) and microfilaments (arrows) scattered throughout the cytoplasm of a neoplastic cell. Nucleus (N) (X 32,000).
Figure 18. Osteosarcoma from rat inoculated at 4 days after birth, day 37 pi. Two well-differentiated osteoblasts with prominent arrays of endoplasmic reticulum, lysosomal bodies, mitochondria, and a prominent ovoid nucleus. Cytoplasmic extensions (arrows) are surrounded by fibrillar osteoid matrix, with adjacent foci of mineralization (M) (X 11,500).
Figure 19. Osteocyte with virus particles (arrow) budding into lacunar space in an osteosarcoma from a rat inoculated at 1 day of age, day 30 pi (X 18,300).
Figure 20. Typical morphology of C-type virus particles is seen along plasma membrane of osteocyte in an osteosarcoma from a rat inoculated at 1 day of age, day 30 pi (X 95,000).
Figure 21. Total serum calcium in control (closed circles) rats, and tumor-bearing rats inoculated at 1 day of age (open circles) and 4 days of age (open triangles). Bars indicate standard error of the mean, and the asterisks indicate significant difference of means at $p < .05$ or greater.
Figure 22. Serum alkaline phosphatase in control (closed circles) rats, and tumor-bearing rats inoculated at 1 day of age (open circles) and 4 days of age (open triangles). Bars indicate standard error of the mean, and the asterisks indicate significant difference of means at \( p < .05 \) or greater.
FIG 22

SERUM ALKALINE PHOSPHATASE (I.U./L)

DAYS POST INOCULATION

F I G  2 2
Figure 23. Urinary hydroxyproline excretion (HOP:CR) in control (closed circles) rats, and tumor-bearing rats inoculated at 1 day of age (open circles) and 4 days of age (open triangles). Bars indicate standard error of the mean, and the asterisks indicate significant difference of means at p < .05 or greater.
CHAPTER II

INTRATIBIAL MOLONEY SARCOMA VIRUS-INDUCED OSTEOSARCOMA IN THE RAT: TUMOR INCIDENCE AND PATHOLOGIC EVALUATION

Introduction

Osteosarcoma is a neoplasm in young adults characterized by early pulmonary metastases and a low survival rate. It has been experimentally produced in rats, mice, hamsters and rabbits by intravenous or intraperitoneal instillation of viruses, heavy metals, and radioisotopes. Tumors produced experimentally were multicentric and often included sarcomas of tissues other than bone, and in some cases developed only after latent periods of several months. In addition, these models of osteosarcoma have not been characterized with regard to clinicopathologic parameters often altered in osteosarcomas of man including elevated serum calcium, alkaline phosphatase and urinary hydroxyproline excretion.

In this communication we present a model of osteosarcoma induced by the intratibial instillation of Moloney sarcoma virus (MSV) in rats from 24 hours through 10 days after birth, resulting in discrete osteosarcomas arising after a brief latent period. The model is characterized by tumor-incidence studies, histopathologic, ultrastructural and selected biochemical parameters. This animal model is highly reproducible and appears to be useful for future investigations on new therapeutic regimens for osteosarcoma.
Materials and Methods

Treatment of Animals

NBR Pl/Cr young New Zealand black (NZB) rats were from a breeding colony kindly supplied by Mr. Clarence Reeder, Drug Research and Development Branch, National Cancer Institute. They were inoculated <24 hours (1 day) to 20 days of age by direct intratibial inoculation of a partially purified preparation of MSV\(^8\) that was kindly supplied by Dr.'s L.1. Sekely and D. Howell, Viral Oncology, National Cancer Institute. Our standard virus preparation (SVP) consisted of the original MSV (Lot MSV-B-77, from BALB/C mice, titered at 5.7-6.5 log focus forming units/ml), diluted 1:7 with a diluent consisting of 2% inactivated fetal calf serum and 1% antibiotics without mycostatin in sterile physiological saline. Rats received a constant volume (either 0.025 or 0.05 ml) of SVP in one or both tibias. Control rats received an equal volume of diluent with citrate buffer approximating the concentration in the SVP.

Experimental Studies

To evaluate tumorigenicity, 1 day old rats received 0.025 ml SVP in both tibias and log2 dilutions of this preparation at constant volume until no osteosarcomas developed. In a second experiment, rats received the SVP bilaterally at sequential days after birth beginning at day 1 to evaluate tumor incidence compared to age. All rats were palpated for tumors at weekly intervals following weaning at 21 days after birth.
Samples for Biochemical and Morphologic Evaluation

In tumor-bearing rats inoculated with SVP at 1 day and 4 days after birth and corresponding diluent-inoculated control rats, twenty-four hour urine samples were collected under toluene at day 30 post-inoculation (pi) and at 10 day intervals thereafter up to day 60 pi. Aliquots of urine were assayed for total urinary hydroxyproline (HOP) and creatinine (CR). Final values were expressed as a HOP to CR ratio to correct for variations in urine volume.

Serum samples were collected at 10 day intervals from tumor-bearing and control rats at day 30 to day 60 pi under light ether anesthesia from the retro-orbital sinus or the abdominal aorta. Serum calcium was determined by atomic absorption spectrophotometry (Perkin-Elmer Model 303) and alkaline phosphatase by the method of Hausamen et al. Statistical analyses were performed using Student's t test.

Live rats were radiographed at 10 day intervals with a General Electric Model 11 AA-3A X-ray unit (30 KV, 5 ma, 12 sec/exposure) and Kodak no-screen medical X-ray film (Ready Pack NS 2T). At necropsy, sections of osteosarcoma were fixed in 10% neutral buffered formalin, decalcified for 7 days in 10% buffered EDTA, embedded in paraffin, sectioned at 7μ and stained with hematoxylin and eosin (H & E). Multiple sections from the osteosarcomas were minced under fixative into 1 mm³ blocks, fixed in cold 3% glutaraldehyde with 0.1 M sodium cacodylate buffered at pH 7.4, post-fixed in 1% osmium tetroxide in s-collidine, dehydrated in graded ethanols, transferred to propylene oxide, and embedded in "hard" Epon (Shell Chemical Company, New York, N.Y.). Thin sections were cut with a diamond knife on an LKB ultra-
microtome and floated on a water bath buffered at pH 7.4 to prevent
demineralization. Sections were stained with uranyl acetate and lead
citrate and were examined with a Philips 200 electron microscope.

Results and Discussion

Figure 1 shows that 83% of rats bilaterally inoculated at 1 day of
age with SVP developed osteosarcomas. There was a linear dose response
relationship between log2 virus dilution and tumor incidence until only
11% of inoculated rats developed tumors at a 1:16 dilution. No rats
developed tumors at a 1:32 dilution of the SVP. Therefore, the SVP has
a calculated 50% tumor producing capacity at a 1:2 dilution (Figure 1).
Up to 92% of rats inoculated with SVP at 1 day of age developed osteo-
sarcomas following a latent period of 10 to 12 days (Table 1). Dilution
of the SVP had no effect on the latent period.

Figure 2 shows the osteosarcoma incidence at day 50 pi in rats
inoculated from 1 day through 20 days after birth with either 0.025 or
0.050 ml of SVP. All tumor-bearing rats in Figure 2 had developed
palpable osteosarcomas by weaning at 21 days of age. For rats re-
ceiving 0.025 ml, there was a similar high incidence of osteosarcoma
following inoculation during the first five days after birth. However,
at day 7 significantly fewer rats developed tumors compared to the pre-
ceding groups. Doubling the volume of inoculum did not result in
greater numbers of osteosarcomas at days 4, 5, or 7 after birth, but
inoculation of 10 day old rats with 0.05 ml SVP resulted in 14% (2/14)
tumor incidence by 50 days pi as compared to the development of no
tumors in rats receiving 0.025 ml. These results suggest an age-re­
lated susceptibility to MSV-induced osteosarcomas in NZB rats. Rats
inoculated with SVP at day 15 or 20 after birth did not develop osteo­
sarcomas within 50 days pi.

Evaluation of tumors from rats receiving SVP as shown in Figure 2
revealed considerable histologic variability depending on the age of
the rat at the time of inoculation. While the tumor incidence was not
significantly different, rats inoculated on day 4 after birth developed
consistently more densely mineralized osteosarcomas following a latent
period of 10 to 15 days (Table 1) than did rats inoculated 1 day after
birth (Figures 3 and 6). Rats inoculated on day 1 developed more
radiolucent bone tumors (Figure 3) that were composed histologically
of fine spicules of osteoid surrounded by large numbers of pleomorphic
tumor cells (Figure 4) with prominent nuclei and large multinucleated
giant cells (Figure 5).

Bone tumors from rats inoculated 4 days after birth were more
osteogenic radiographically (Figure 6). Histologically, they had large
areas of mineralized bone and abundant osteoid (Figure 7) with both
pleomorphic and well-differentiated bone cells interspersed between
the osteoid matrix (Figure 8). The occurrence of more osteogenic osteo­
sarcomas in rats inoculated at 4 days of age may be related to a more
differentiated population of bone cells in the older rats exposed to
MSV. Histologic evaluation of osteosarcomas in rats inoculated at
day 2 after birth revealed predominately cellular neoplasms similar to
those induced at day 1. Osteosarcomas in rats inoculated at 3 days of
age generally contained more stroma and appeared similar to that
described for rats inoculated on day 4. Similarly, osteosarcomas in rats inoculated on days 5 and 7 after birth had wide-spread areas of mineralized osteoid. Both tumor-bearing rats receiving 0.05 ml SVP on day 10 of age developed small neoplasms containing primarily well-differentiated osteoblasts and densely mineralized osteoid matrix.

Ultrastructural studies on the osteosarcomas revealed many similarities to fine structural investigations of human osteosarcomas. Osteosarcomas from rats inoculated at both 1 and 4 days of age were composed of many polyhedral, osteoblast-like cells. They had irregular nuclei, an abundant network of dilated endoplasmic reticulum, intracytoplasmic microfilaments, and were surrounded by osteoid with interlacing collagen fibers (Figure 9 and 10). Large undifferentiated cells with prominent nuclei and poorly developed cytoplasmic organelles were often noted in rats inoculated at day 1, but were rarely seen in rats inoculated at day 4 after birth. In both groups, viral particles were present within the cytoplasm of tumor cells and were observed budding from the plasma membrane (Figure 11). Multinucleated giant cells resembling osteoclasts were observed occasionally in the MSV-induced osteosarcomas. They had multiple nuclei, a sparse endoplasmic reticulum, numerous mitochondria, and appeared similar to osteoclasts described in previous reports of osteosarcoma.

In tumor-bearing rats inoculated on day 4 after birth, significant elevations in serum calcium and alkaline phosphatase (n = 4-12 determinations/mean ± SEM) were observed on days 40 and 50 pi, and alkaline phosphatase continued to be elevated on day 60 pi, compared to diluent inoculated control rats (Table 2). Corresponding data from tumor-
bearing groups on day 30 pi, and also in tumor-bearing rats inoculated on day 4 after birth at days 40 and 60 pi. These findings support the histologic and ultrastructural evidence of rapid turnover of osteoid and mineralized matrix by the osteoblastic cells in the tumor, particularly in rats inoculated 4 days after birth. The elevation of serum alkaline phosphatase in this group is consistent with the rapid proliferation of osteoblastic tumor cells. Isoenzyme determination of serum to identify the tissue origin of alkaline phosphatase that is elevated is presently in progress.

These results indicate that young NZB rats inoculated intratibially one to five days after birth with MSV develop a high incidence of osteosarcomas with a short latent period. The high tumor incidence and consistent reproducibility as well as the production of only osteosarcomas following intratibial inoculation of MSV in young rats compare favorably with other viral-induced models of osteosarcoma. It has the distinct advantage of a brief latent period and a discrete nature confined to the tibia compared to other experimental models of osteosarcoma. Rats inoculated 4 days after birth develop more densely mineralized osteosarcomas, elevated serum calcium and alkaline phosphatase, and increased urinary hydroxyproline excretion compared to control rats and rats inoculated at day 1. The MSV-induced osteosarcoma in rats characterized in this report fulfills many of the essential criteria for osteosarcoma in human patients and should be a valuable animal model to investigate new therapeutic regimens for osteosarcoma.
Summary

Osteosarcomas were induced in approximately 80% of young New Zealand black rats by the intratibial inoculation of Moloney sarcoma virus (MSV) from day 1 to day 5 after birth. The neoplasms were composed of a spectrum of well to poorly differentiated osteoblasts, osteocytes and osteoclasts. Budding of C-type viral particles was associated with tumor induction. Compared to rats inoculated on day 1 after birth, rats inoculated at 4 days of age developed consistently more osteogenic bone tumors that often were associated with hypercalcemia, increased serum alkaline phosphatase and elevated urinary hydroxyproline.
Table 1. Latent Period\textsuperscript{+} for Rats Inoculated 1 Day and 4 Days After Birth

<table>
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<tr>
<th>Time of Inoculation</th>
<th>Days Postinoculation</th>
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<tr>
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<tr>
<td>Day 1</td>
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</tr>
<tr>
<td>Day 4</td>
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</tr>
</tbody>
</table>

\textsuperscript{+} Determined by radiography of rats at indicated days pi

\textsuperscript{*} No. of rats with tumors/No. of rats inoculated
Table 2. Biochemical Parameters in Tumor Bearing and Control Rats

<table>
<thead>
<tr>
<th>Days Postinoculation</th>
<th>Alk. Phos. (IU/1)</th>
<th>Calcium (mg/100 ml)</th>
<th>HOP:CR</th>
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<tr>
<td></td>
<td>Serum</td>
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<tr>
<td></td>
<td>Alk. Phos. (IU/1)</td>
<td>Calcium (mg/100 ml)</td>
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<tr>
<td></td>
<td>Day 1</td>
<td>Day 4</td>
<td>Control</td>
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</tr>
<tr>
<td></td>
<td>+72</td>
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<td>+10</td>
</tr>
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</table>

+ Mean value ± SEM
a P<.05 compared to controls
b P<.01 compared to controls
c P<.005 compared to controls
Figure 1. Linear decrement in tumor incidence with log_2 dilutions of SVP in 1 day old rats. Each point represents % incidence with 9 to 14 rats per point. The 50% tumor producing capacity is at a 1:2 dilution of SVP.
PERCENT OF RATS DEVELOPING TUMORS

FIG 1

LOG₂ VIRUS DILUTION

0  1  2  3  4

0  1  2  3  4  5  6  7  8  9  10
Figure 2. Osteosarcoma incidence in NZB rats inoculated intratibially at 24 hours (1 day) to 20 days old with SVP. Numbers at base of columns indicate rats with osteosarcoma/total number of rats inoculated.
INOCULATION TIME AFTER BIRTH

- 0.025ml INTO EACH TIBIA
- 0.050ml INTO EACH TIBIA

PERCENT RATS DEVELOPING OSSEOUS SARCOMA

HOURS
- <24
- 24-48
- 48-72
- 4
- 5
- 7

DAYS
- 10
- 15
- 20

TIME AFTER BIRTH
Figures 3-8. Radiographic and histologic features of MSV-induced osteosarcomas in rats inoculated 1 day (3-5) and 4 days (6-8) after birth.
Figure 3. Osteosarcoma (50 days pi) with large radiolucent zones (arrow). (X 2.5).
Figure 4. Densely cellular osteosarcoma from rat inoculated 1 day after birth. (X 215)
Figure 5. Cells from osteosarcoma with prominent nuclei and occasional multinucleated giant cells (arrow) from rat inoculated 1 day after birth. (X 440)
Figure 6. Osteosarcoma (50 days pi) from rat inoculated 4 days after birth with a high degree of osteogenic activity. (X 2.5)
Figure 7. Abundant osteoid stroma (arrowhead) and mineralized bone in an osteosarcoma from rat inoculated 4 days after birth. (X 215)
Figure 8. Osteosarcoma with both pleomorphic (arrow) and well-differentiated tumor cells from rat inoculated 4 days after birth. (X 440)
Figure 9. Well-differentiated osteoblasts in an osteosarcoma that developed after inoculation of SVP at 4 days of age. Note the fibrillar osteoid matrix (arrow) with a mineralization front (M). (X 12,600)
Figure 10. Osteoblastic cells (N, nuclei) comprising an osteosarcoma induced after inoculation of SVP at 4 days of age. The cytoplasm has an abundant network of dilated rough endoplasmic reticulum filled with a moderately electron-dense material (arrow). (X 14,200)
Figure 11. C-type viral particles budding (arrows) from plasma membrane of a neoplastic cell in an osteosarcoma induced after inoculation of SVP at 1 day of age. (X 62,000).
CHAPTER III
SUBACUTE CARDIOTOXICITY OF ADRIAMYCIN IN THE RAT:
BIOCHEMICAL AND ULTRASTRUCTURAL INVESTIGATIONS

Introduction
Adriamycin (ADR) is an anthraquinone antitumor antibiotic derived from Streptomyces peucetius var caesius with a high chemotherapeutic effectiveness against many human neoplastic diseases. ADR also has been effective in the treatment of many experimental solid neoplasms and leukemias. However, the clinical usefulness of ADR has been limited by the development of a total dose-dependent cardiomyopathy in human patients. Investigations in animals, including the rabbit and mouse, indicate that subacute and chronic administration of ADR to non-tumor-bearing animals may produce a total dose-dependent cardiotoxicity similar to that described in treated patients with neoplastic disease.

This study was designed to evaluate the cardiotoxicity of single and divided dosages of ADR in the rat over a 4 week observation period. Compared to the rabbit, the rat should require less ADR to produce cardiotoxicity. In addition, the rat and mouse are used extensively as model systems for the study of experimental neoplasms, thus permitting the concomitant evaluation of ADR analogues for both oncolytic activity and cardiotoxicity. The New Zealand Black rat (NBR Pl/Cr)
has been used in the development of experimental osteosarcomas, \(^{20,21}\) which determined the selection of rats of this strain to evaluate the cardiotoxicity of ADR as a baseline for future chemotherapeutic investigations.

**Materials and Methods**

**Animals and Chemotherapeutic Agent**

Thirty-four day old male and female NZB PI/Cr (New Zealand black) rats were injected intravenously in the lateral tail vein with a lyophilized preparation of Adriamycin (ADR) hydrochloride (bulk drug kindly provided by Adrialabs, Wilmington, DL). ADR was weighed in 10 mg aliquots on an electrobalance, placed in small glass vials and stored in the dark under refrigeration. At the time of injection, ADR was reconstituted with 0.85% sterile saline at 2 mg/ml and given by either single bolus or divided doses (at 48 hour intervals) in groups as indicated in Figure 1. Twelve to 34 rats per group were observed daily for evidence of toxicity. All rats were weighed weekly and mean body weights were tabulated as percent of initial weight (Figure 2).

**Experimental Design and Sample Preparation**

Based on results of the initial toxicity study in ADR-treated rats, (Figure 1), groups of rats were injected with ADR at dosages that demonstrated mortality. Following injection with ADR (experimental) or saline (control) rats were killed at 6 and 24 hours, and days 2, 4, 6, and 8 after injection. At each time point, 3 to 5 rats were lightly anesthetized with ether and bled from the abdominal aorta. Serum was
collected and frozen for subsequent analysis. The heart was immediately removed from each rat and the apex was sliced (2 mm) free and prepared for electron microscopic evaluation. Another slice (2 mm) was made through the ventricular myocardium and placed in a vial of 10% neutral buffered formalin and subsequently prepared for light microscopic evaluation. The remainder of the myocardium was washed in cold 0.15 M KCl, atria were dissected free, and the ventricular myocardium was weighed (fresh tissue weight) and frozen for subsequent mineral analysis. Other tissues collected for light microscopic examination included duodenum, spleen, liver, kidney and proximal tibia.

Following fixation, myocardium for light microscopy was embedded in paraffin, sectioned at 6μm, and stained with hematoxylin and eosin (H & E), periodic acid-Schiff with diastase digestion (PAS-D), and Gomori's aldehyde fuchsin. The apex of the myocardium was minced immediately under fixative into 1 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 Epon (Shell Chemical Company, New York, NY). 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 200 electron microscope.

Ventricular myocardial tissue, H2O2 and calcium determinations were performed as described previously.19 Total calcium analysis of both myocardial tissue and serum was performed by atomic absorption spectrophotometry using a Perkin-Elmer model 303. Serum samples also were
assayed for creatine phosphokinase (CPK)\textsuperscript{25,26} and lactic dehydrogenase (LDH).\textsuperscript{31} Statistical analyses were performed using Student's T test.\textsuperscript{29}

Results

A dose-response relationship was observed in toxicity studies of rats treated with single or divided high dosages of ADR (Figure 1). A total dosage of 20 mg/kg given either as a single bolus or divided into two 10 mg/kg injections (48 hours apart) produced 100% mortality within 10 days. In subsequent studies of ADR at 10 mg/kg X 2, some rats lived as long as 2 weeks. Dividing 20 mg/kg into 10 mg/kg X 2 had the effect of extending mortality over a greater time period (Figure 1). A 10 mg/kg single bolus produced a substantially lower mortality rate (26%) which extended over a greater portion of the period of observation, with the last death observed 21 days after injection. Rats in this group first began dying 4 days after injection, the earliest of any group observed. The single dose LD\textsubscript{50} in the rat was approximately 13 mg/kg (Figure 1). Deaths in this group also occurred throughout the period of observation, with the last rat dying 25 days after ADR injection.

An important feature of mortality in rats injected intravenously with ADR is that deaths were delayed as no rats died before 4 days after injection. The greatest mortality was observed at or following 6 days after ADR injection in all groups except 10 mg/kg given as a single bolus. Rats injected at 5 mg/kg X 2 or 5 mg/kg X 1 did not die during the period of observation.
Figure 2 shows the effect of different dosages of ADR on mean body weight during weekly intervals following injection. Rats injected at very high dose levels (20 mg/kg, 10 mg/kg X 2) had rapid weight loss compared to their initial weight. The data point at 14 days after administration of 10 mg/kg X 2 ADR represents rats surviving treatment that were weighed and killed for tissue collections and not included in the toxicity study (Figure 1). Rats receiving lower or divided dosages of ADR lived longer and had a progressive increase in body weight by 28 days after injection (Figure 2). Intermediate dosages (13 mg/kg and 10 mg/kg) produced an initial rapid drop in body weight (80% of initial weight at 7 days), with a slow subsequent increase in weight during the next 3 weeks. Dosages of ADR associated with 0% mortality (5 mg/kg X 2 and 5 mg/kg) produced an attenuating effect on rate of growth compared to controls (Figure 2).

Rats that received 13 mg/kg ADR (LD$_{50}$) had lesions in the duodenum and tibial bone marrow and epiphysis-metaphysis regions. The duodenum demonstrated foci of epithelial regeneration. The tibial marrow was severely depleted of hematopoietic cells which were replaced by mesenchymal cells and stroma. Trabeculae in the metaphysis were characterized by a deficiency of osteoblasts and osteoclasts, and the epiphyseal plate thickness was attenuated. There was no evidence of these lesions in tissues from rats killed 28 days after injection of 13 mg/kg ADR. Rats that were administered 10 mg/kg ADR had less severe myeloid depletion. All other tissues examined in these rats appeared normal.
Toxic dosages of ADR produced a significant hypocalcemia in rats at 6 hours after injection compared to control rats (Table 1). At 24 hours after injection significant hypocalcemia was observed in rats receiving 10 and 13 mg/kg ADR while serum calcium levels had returned to normal following a dosage of 20 mg/kg ADR. All serum calcium values at 48 hours after ADR injection were not significantly different from saline-treated control rats, although control data was inexplicably low (Table 1).

Gross appearance of rat hearts at the time of death was unremarkable. Evaluation of rat myocardium by light microscopy did not consistently demonstrate definitive evidence of cardiomyopathy. Small intramyocytic vacuoles and increased intermyocytic fibrosis were observed in Gomori aldehyde fuchsin-stained sections of myocardium from rats 8 days after receiving 10 mg/kg × 2 ADR. However, degenerating myofibers could not be consistently identified with the PAS-D. Evidence of cardiotoxicity by light microscopic evaluation of myocardium at other dosages could not be conclusively demonstrated.

The fine structural detail of typical control ventricular myocardium from saline-injected rats is shown in Figure 3. Determination of ADR-induced fine structural myocardial lesions was made by comparison with tissue from saline-injected controls collected at the same time intervals.

Electron microscopic examination revealed a progression of myocardial lesions that were dependent both upon dose of ADR and time following injection. However, the most severe alterations were not always observed at the highest dosages of ADR. The most frequently observed
and earliest fine structural alterations in rat myocardium were intra-cellular edema with mitochondrial swelling and degeneration. At 4 days following injection of 20 mg/kg there were swollen mitochondria and numerous myelin figures suggesting early degenerative changes in myocytes (Figure 4). Nuclear pyknosis was also observed frequently. An early myocardial lesion observed in nearly all rats receiving cardiotoxic dosages of ADR was dilatation of the sarcoplasmic reticulum in association with early mitochondrial degeneration (Figure 5). Myelin figures often were observed and the sarcoplasmic reticulum contained a flocculent precipitate within its lumen (Figure 5).

The myocardium of rats receiving 10 mg/kg ADR (lowest mortality-producing dosage) was characterized by swelling of mitochondria and separation of myofibrils at 4 days post injection (Figure 6). Z-bands were observed to be out of register as a result of the mitochondrial swelling and the nuclei occasionally were pyknotic with an irregular nuclear membrane.

Another lesion observed in rats at 4 days after receiving 10 mg/kg ADR was separation of intercalated disks (Figure 7). This focal intercellular change was noted only at this dosage and time point. It was characterized by the presence of a thin convoluted membrane interposed between the separated intermyocytic junction of the fasciae adherentes. The gap junctions appeared to be intact. There was no myocyte damage associated with the separation except for the mitochondrial edema noted above.

Frequent severe focal myofiber degeneration was observed in rats 8 days after administration of 10 mg/kg X 2 ADR (Figure 8). Affected
myocytes were more electron-dense and contracted compared to adjacent unaffected myocytes (Figure 8). Nuclear pyknosis often was observed in affected myocytes. At this early degenerative stage (Figure 8), mitochondria appeared more swollen with separation of cristae in damaged myocytes compared to surrounding mitochondria. Average sarcomere length in these affected myocytes was 2.2μ compared to 2.8μ in unaffected myocytes and control myocardium. As degeneration of myocytes became more severe the entire cell became uniformly electron-dense and Z-bands were difficult to identify (Figure 9). Many mitochondria were completely disrupted with only a fine filamentous membrane remaining (Figure 9). There was sharp demarcation between the electron-dense degenerate myocyte and more normal appearing adjacent myocytes at the intercalated disk (Figure 9). Similar degenerate, contracted myofibers with severely damaged mitochondria were seen in the myocardium of rats 6 days after receiving 20 mg/kg ADR.

The results of ventricular myocardial calcium determinations are presented in Table 2. Tissue calcium levels were significantly increased over control myocardium at all time points measured up to 8 days after injection of rats with 20 and 13 mg/kg ADR. Rats receiving 10 mg/kg ADR had mean tissue calcium levels that were consistently greater than control myocardium, although the increase was significant (P<.025) only at day 4 after injection. Analyses of tissue water content for all groups revealed that there was no significant difference in myocardial H2O in ADR-treated rats compared to saline-treated controls.
Assay of serum creatine phosphokinase (CPK) and lactate dehydrogenase (LDH) showed significant increases in all ADR-treated groups compared to saline-treated control rats. Serum CPK (Figure 10) reached highest significant levels ($P < .005$) at 2 days following injection (values for 10 mg/kg X 2 indicate time following injection of the second dose) of 20, 10 X 2, and 13 mg/kg ADR. Rats administered 10 mg/kg X 1 ADR achieved highest significant CPK levels at 4 days after injection ($P < .025$). Other groups that had survivors to 4 days also had significantly elevated CPK values ($P < .025$) compared to controls. All CPK values at 6 or 8 days after injection were either less than or not significantly different from control rats.

Serum LDH values for all ADR-treated groups were significantly increased ($P < .025$) over saline-treated controls as soon as 24 hours after injection (Figure 11). Elevation of this enzyme was sustained at significant levels ($P < .025$) at 2 days after ADR injection for rats receiving 20, 10 X 2 and 13 mg/kg ADR, but not 10 X 1 ADR. At 4 days rats receiving 10 mg/kg ADR reached the highest significant level ($P < .005$) and rats receiving 20 mg/kg ADR were still significantly elevated ($P < .025$) compared to control serum LDH. One serum sample at day 4 after injection from a rat receiving 13 mg/kg was 1380 m U/ml (Figure 11). All LDH values at days 6 and 8 post injection were not significantly different from controls.

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Discussion

The results of this study show that the NZB rat is sensitive to the cardiotoxic effects of single or divided high dosages of ADR given by intravenous injection. Fatal dosages of ADR either produced acute weight loss or attenuation of weight gain, acute reversible hypocalcemia and fine structural alterations compatible with severe myocardial damage. Significant increases of myocardial tissue calcium and serum enzymes (CPK, LDH) in association with myocardial damage were found to precede the onset of highest mortality. The most severe myocardial ultrastructural alterations were seen in rats that received dosages of ADR that produced high mortality (10 mg/kg X 2) at 8 days following treatment.

There are several recently published investigations that delineate the cardiotoxic effects of the anthraquinone anticancer antibiotics ADR or Daunomycin (DNR) in the rat.3,5,10,35 Recent studies in the mouse indicate that the BDF1 strain is also sensitive to the cardiotoxic effects of ADR and DNR.18,27,28 The rat appears also to be a sensitive, short-term predictor for cardiotoxicity of these drugs in
man, and may be a useful screening animal both for new ADR analogues, and, as is the mouse, for therapeutic intervention to bypass cardiotoxicity.18

ADR dosages of 20, 10 X 2 and 13 mg/kg are highly toxic in the rat and produced ≥50% mortality during the 28-day observation period. Rats receiving these dosages developed initial rapid weight loss followed by gradual increments of body weight in surviving rats (13 mg/kg). The decrease in mortality and concimitant increase in body weight gains with decreasing dosages of ADR suggest that the pathogenic mechanisms related to morbidity and mortality are both dose- and time-dependent. These effects also are dependent upon scheduling of ADR treatment, since dividing the total dosage into two boluses given at 48 hour intervals consistently results in prolongation of the interval from dosing until death. The debilitation and death associated with high dose ADR treatment suggests a multifactorial process including anorexia and 'wasting' associated with gastrointestinal toxicity and bone marrow suppression.23

A previous study in rats reported subtle cardiotoxicity, including fine structural alterations of mitochondria with membraneous whorls, and swelling of capillary endothelium in ventricular myocardium of Wistar rats treated with 25 mg/kg DNR.5 Another report showed this cardiotoxic effect in Sprague Dawley rats to be dose-dependent.1 DNR and ADR have been shown to inhibit the ATP production and O2 consumption, respectively, of rat mitochondria both in vivo and in vitro.3 Other investigators report the effect of ADR and related compounds as uncouplers of mitochondrial oxidative phosphorylation.10 In this
study, one of the earliest and most consistent lesions was a spectrum of alterations in myocardial mitochondria. A sequence of lesions was observed in the present investigation including intramitochondrial swelling, condensation of mitochondria, and advanced degeneration with dissolution of mitochondrial cristae leaving disorganized membraneous whorls (myelin figures). Swelling and separation of myofibrils and large vacuoles representing dilated sarcoplasmic reticulum were present in severe myocardial lesions of ADR-treated rats. These ultrastructural alterations are similar to those described in canine myocardium following acute ischemic injury with varying periods of blood reflow and in rats after large doses of sympathomimetic amines. The similarity of the lesions in myocardium of ADR-treated rats to vascular-associated injury or the metabolic effects of catecholamines on the myocardium suggests that an alteration of cell membrane permeability, accompanied by an influx of calcium, may be associated with the fine structural cardiomyopathic alterations. A marked increase in intracellular calcium produces damaging effects on mitochondrial metabolism and excitation-contraction coupling in heart muscle. In addition, the focal nature of the lesion and the severity of the associated mitochondrial changes suggests that some myocytes may be more susceptible than others to the cardiotoxic effects of ADR.

Separation of intercalated disks was observed only in rats receiving 10 mg/kg at day 4. There was no consistent degeneration of adjacent myocytes in association with separation of the intercalated disks, although some cells had separation of myofibrils and swelling of mitochondria. Poche described similar separation, referred to as
"dehiscences of the intercalated disks" with myogenic dilatation. Other investigators produced similar separation of intercalated disks experimentally with EDTA treatment or perfusion with low calcium solutions to be associated with known chelating effects of EDTA for ionic calcium (Ca++) at the intercellular junction. Either or both events may explain this lesion with ADR although chelation after such a brief period and at only this dosage would be unexpected. ADR has been shown to be a chelator for a variety of cations in vitro and has an affinity for mineralized bone surfaces. This chelating property may explain the acute reversible hypocalcemia that was observed following administration of ADR. However, we cannot explain why separation of intercalated disks was seen only at this dose of ADR and at this time point.

Serum CPK and LDH determinations were used as possible biochemical parameters of myocardial damage with the presumption that elevation of CPK and LDH reflects myocardial muscle damage. Presently, we are evaluating LDH isoenzymes more specific for cardiac muscle at selected dosages and time points after injection. Significant elevations in myocardial enzyme levels as soon as 24 hours following injection (LDH) with apparent peaks for all dosages at 2 days after administration except 10 mg/kg ADR, indicates the early onset of myocardial damage. For rats receiving 10 mg/kg ADR, the peak of serum enzyme activity correlated with earliest deaths at 4 days after injection. We suggest that enzyme activity reflects myocardial damage and that this clearly precedes the earliest detectable ultrastructural evidence of myocyte damage. Earlier findings indicate a similar peak in CPK and LDH values
in BDF1 mice before onset of fatalities with 20 and 10 mg/kg of ADR given by intraperitoneal injection.\textsuperscript{22} We do not know whether the NZB rats are dying from cardiotoxicity, although previous reports in mice show that ADR does not produce marked suppression of bone marrow or damage to the gastrointestinal mucosa.\textsuperscript{28}

In this study, we demonstrated that the New Zealand Black rat, a strain in which experimentally induced osteosarcomas have been produced,\textsuperscript{20,21} is sensitive to the cardiotoxic effects of high single and divided dosages of ADR. Recent reports suggest that the murine may be a useful screening model for the study of anthracycline antibiotic cardiotoxicity\textsuperscript{3,5,10,27,28,35} as well as chemical intervention to obviate this cardiotoxic problem.\textsuperscript{34} Further investigations in our laboratory and the work of others\textsuperscript{15} suggest that the rat may develop a cardiomyopathy similar to that produced in man by the repeated intravenous or subcutaneous administration of ADR at 1 or 2 mg/kg weekly for 10 to 14 weeks. Therefore, NZB rats bearing viral-induced osteosarcomas may provide investigators interested in the chemotherapeutic effects of ADR on experimental neoplasms with an animal species that is also sensitive to the cardiotoxic effects of this drug.
Summary

The toxicologic and cardiotoxic effects of single and divided high dosages of adriamycin (ADR) given by intravenous injection were evaluated in 34 day-old New Zealand Black rats. ADR at dosages of 20 mg/kg, 10 mg/kg \times 2, 13 mg/kg, and 10 mg/kg produced fatality and rapid weight loss followed by gradual weight gain in groups of rats that survived the 28 day observation period. ADR at dosages of 5 mg/kg \times 2 and 5 mg/kg produced no mortality but rats had attenuated weight gain compared to saline-injected controls. The earliest myocardial fine structural alterations included swelling and degeneration of mitochondria and dilation of sarcoplasmic reticulum at all dosages of ADR. More advanced myocardial lesions included separation of myofibrils and the fasciae adherens of intercalated disks in rats 4 days after administration of 10 mg/kg ADR.

The most severe lesions were observed in rats 8 days after receiving 10 mg/kg \times 2 ADR and included focal myocyte degeneration with increased electron-density and contraction of the sarcomeres, nuclear pyknosis and mitochondrial degeneration. Toxic dosages of ADR produced an acute, reversible hypocalcemia in rats. Significant increases of serum creatine phosphokinase and lactic dehydrogenase preceded the onset of highest mortality following ADR administration. Ventricular tissue calcium concentration was significantly increased in groups of rats receiving toxic dosages of ADR. This investigation demonstrates that the rat is sensitive to the cardiotoxic effects of single or divided high dosages of ADR. Investigations with ADR in rats bearing Moloney sarcoma...
virus-induced osteosarcomas will permit the study of chemoresponse of an experimental neoplasm in an animal species that is also sensitive to the cardiotoxic effects of this drug.
Table 1. Serum Calcium Concentrations from ADR and Saline-Treated Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>6</th>
<th>24</th>
<th>48</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mg/kg ADR</td>
<td>9.5a</td>
<td>10.3</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>±0.2</td>
<td>±0.3</td>
<td>±0.2</td>
</tr>
<tr>
<td>10 mg/kg X 2 ADR</td>
<td>7.5b</td>
<td></td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>±0.1</td>
<td></td>
<td>±0.1</td>
</tr>
<tr>
<td>13 mg/kg ADR</td>
<td>9.0b</td>
<td>9.2a</td>
<td>8.6</td>
</tr>
<tr>
<td></td>
<td>±0.1</td>
<td>±0.3</td>
<td>±0.1</td>
</tr>
<tr>
<td>10 mg/kg ADR</td>
<td>9.3a</td>
<td>9.0b</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>±0.4</td>
<td>±0.2</td>
<td>±0.1</td>
</tr>
<tr>
<td>Saline</td>
<td>10.4</td>
<td>10.2</td>
<td>8.8</td>
</tr>
<tr>
<td></td>
<td>±0.1</td>
<td>±0.2</td>
<td>±0.2</td>
</tr>
</tbody>
</table>

+ All values are mean ± SE (n = 3 to 5 samples per point) expressed as mg calcium/100 ml serum; tests of significance indicate experimental groups vs control.

a \( p < .05 \)

b \( p < .01 \)
Table 2. Calcium Content of Ventricular Myocardium from ADR and Saline-Treated Rats

<table>
<thead>
<tr>
<th>Group</th>
<th>Time After Injection</th>
<th></th>
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<th></th>
<th></th>
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<tbody>
<tr>
<td></td>
<td>Hours</td>
<td>Days</td>
<td>Days</td>
<td>Days</td>
<td>Days</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>8</td>
</tr>
<tr>
<td>20 mg/kg ADR</td>
<td>295.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>272.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>406&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+58.3</td>
<td>+5.6</td>
<td>+60</td>
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</tr>
<tr>
<td>13 mg/kg ADR</td>
<td>125.0</td>
<td>346.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>428.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>398.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>224.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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<td></td>
<td>+27.8</td>
<td>+28.8</td>
<td>+43.2</td>
<td>+82.7</td>
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</tr>
<tr>
<td>10 mg/kg ADR</td>
<td>196.2</td>
<td>224.5</td>
<td>300.9&lt;sup&gt;b&lt;/sup&gt;</td>
<td>346.4</td>
<td>272.6</td>
</tr>
<tr>
<td></td>
<td>+45.6</td>
<td>+41.3</td>
<td>+13.8</td>
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<tr>
<td>Saline</td>
<td>133.9</td>
<td>192.7</td>
<td>227.5</td>
<td>190.2</td>
<td>152.9</td>
</tr>
<tr>
<td></td>
<td>±8.4</td>
<td>±20.0</td>
<td>±22.2</td>
<td>±44.7</td>
<td>±22.4</td>
</tr>
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</table>

*All values are mean ± SE (n = 3 to 5 heart samples per point) expressed as µg calcium/g tissue wet weight; tests of significance indicate experimental group vs control.

<sup>a</sup> P < .05

<sup>b</sup> P < .025
Figure 1. Mortality of NZB rats during 4 week period following intravenous injection of ADR. Rats were 34 days old at the time of injection.
# Adriamycin 28 Day Toxicity Study

<table>
<thead>
<tr>
<th>Dosage (mg/kg)</th>
<th>Rats Group</th>
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<th>8</th>
<th>12</th>
<th>16</th>
<th>20</th>
<th>24</th>
<th>28</th>
<th>% Mortality</th>
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<tbody>
<tr>
<td>20x1</td>
<td>12</td>
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<td></td>
<td></td>
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<td>0</td>
</tr>
</tbody>
</table>
Figure 2. Weekly mean body weight changes expressed as percent of initial weight of rats following ADR injection.
Figure 3. Electron micrograph of myocardium from a rat 20 days after receiving an injection of saline. A capillary (C) is present. N, nucleus of myocyte. Intercalated disk (arrows). X 11,250
Figure 4. Electron micrograph of myocardium from a rat receiving 20 mg/kg ADR at day 6 after injection. Mitochondria (M) are swollen with separation of cristae, condensation of membranes, and numerous myelin figures (arrows). The nucleus of the myocyte is irregularly shrunken (N) with condensed chromatin. X 20,300
Figure 5. Dilated sarcoplasmic reticulum (arrows) and a myelin figure in myocyte from a rat 8 days after receiving 10 mg/kg x 2 ADR. X 36,800
Figure 6. Myocardium from a rat 4 days after receiving 10 mg/kg ADR. Myofibrils are widely separated and Z-bands are out of register. Mitochondria are moderately vacuolated. N, nucleus of myocyte. X 13,000
Figure 7. Myocardium from a rat 6 days after administration of 10 mg/kg ADR. There is separation of the fasciae adherens of the intercalated disk between myocytes. Gap junctions appear intact. A convoluted membrane (arrows) is observed in the separated junction. X 30,000
Figure 8. Focal myofiber degeneration in myocardium from a rat 8 days after receiving 10 mg/kg x 2 ADR. The degenerate myocyte is more electron-dense and contracted with a pyknotic nucleus (arrow) and vacuolated mitochondria compared to adjacent myocytes. The nucleus of an adjacent myocyte is present at the upper left. X 4500
Figure 9. Advanced myocyte degeneration in myocardium from a rat 8 days after administration of 10 mg/kg x 2 ADR. Degenerate myocyte is extremely dense and contracted with extensive mitochondrial vacuolization. There are remnants of mitochondria in myocytes with only fine filamentous membranes (arrow) remaining. Z-bands cannot be identified with certainty in the degenerate myocytes. Intercalated disks (white arrowheads). X 6500
Figure 10. Serum creatine phosphokinase (mU/ml) in control (solid circles) rats and rats injected with ADR at 20 mg/kg (open circles), 13 mg/kg (closed triangles), 10 mg/kg x 2 (open triangles), and 10 mg/kg (closed squares). Bars indicate standard error of the mean, and the asterisks indicate significant difference of means at P<0.05 or greater.
FIG 10
Figure 11. Serum lactic dehydrogenase (mU/ml) in control (solid circles) rats and rats injected with ADR at 20 mg/kg (open circles), 13 mg/kg (closed triangles), 10 mg/kg x 2 (open triangles), and 10 mg/kg (closed squares). Bars indicate standard error of the mean, and the asterisks indicate significant difference of means at $P<0.05$ or greater.
FIG 11

mU/ml LDH vs TIME POST INJECTION

HOURS: 6, 24, 2, 4, DAYS: 6, 8
CHAPTER IV

CHRONIC CARDIOTOXICITY OF ADRIAMYCIN IN THE RAT:
MORPHOLOGIC AND BIOCHEMICAL INVESTIGATIONS

Introduction

Adriamycin (ADR)-induced cardiotoxicity has been characterized in human patients (Lefrak et al., 1973; Buja et al., 1973) and in several animal species (Jaenke, 1974; Young, 1975; Rosenoff et al., 1975) as a total dose-dependent, multifocal, myocardial degeneration that occurs following either acute or chronic administration. The effectiveness of this anthracycline antibiotic in the treatment of a broad spectrum of neoplastic diseases (Blum and Carter, 1974) has resulted in investigations to maintain oncolytic activity of ADR while ameliorating cardiotoxicity, including the development of new analogues of ADR (e.g., rubidazone, carminomycin) and improved schedules of treatment (Weiss et al., 1976). Other studies have reported the effects of digitalis (Arena, 1972) and α-tocopherol (Myers et al., 1977) given with ADR in experimental animals in efforts to ameliorate cardiotoxicity. Although the results in some studies have been promising, they have not demonstrated a consistent protective effect (Philips et al., 1975; Young et al., 1976).

Reports from several laboratories (Rosenoff et al., 1975; Lambertenghi-Deliliers et al., 1976; Chalcroft et al., 1973) have shown that the rat and mouse are both sensitive to the acute cardiotoxic
effects of ADR. Recent results from our laboratory have shown that high dosages of ADR produce an acute toxic effect on rat myocardium that includes swelling and degeneration of mitochondria, dilation of sarcoplasmic reticulum, separation of myofibrils and fasciae adherens of intercalated disks, and focal myocyte degeneration (Olson and Capen, 1977a). Mettler et al. (1977) have shown that the weekly administration of ADR to Fischer rats at 1 and 2 mg/kg over 10 to 14 weeks produces a fatal cardiotoxicity similar to that described in man. The objectives of this investigation were to elucidate the cardiotoxic effects of chronic ADR administration in the New Zealand black rat by relating accumulative mortality incidence with histopathologic, ultrastructural and biochemical evidence of cardiomyopathy. The relationship of ADR scheduling to the incidence of cardiotoxicity was also examined.

Materials and Methods

Experimental Animals and Adriamycin

Thirty-four (34)-day-old male and female NZB Pl/Cr (New Zealand black) rats, kindly supplied by Mr. Clarence Reeder, Drug Research and Development Branch, National Cancer Institute, were injected intravenously (I.V.) in the lateral tail vein or by the intraperitoneal (I.P.) route with a lyophilized preparation of Adriamycin (ADR) hydrochloride (bulk drug kindly provided by Adrialabs, Wilmington, DL). An additional ten rats received ADR by subcutaneous (S.Q.) injection. ADR was weighed in 10 mg and 5 mg aliquots on an electrobalance, placed in small glass vials and stored in the dark at 1.1°C. The ADR for injection was reconstituted with 0.85% sterile saline at 1 mg/ml and given immediately.
Experimental Design and Sample Preparation

A total of 45 rats were injected I.V. with ADR or saline (controls) using three treatment schedules. Eleven rats received ADR at 2 mg/kg/week, 10 rats received 1 mg/kg/week ADR, and 12 rats received 1 mg/kg on Monday, Wednesday, and Friday (MWF) followed by one week of rest, then another MWF schedule followed by a week of rest up to a total dose of 10 mg/kg. Twelve rats received an equivalent volume of saline by I.V. injection on a weekly basis for 10 weeks. All animals received a total of 10 injections. Careful injection of ADR permitted most to be given I.V., but perivasculitis was severe in some rats resulting in necrosis of the tail. Therefore, injection of ADR by the I.P. route was selected as the alternative route of administration.

All rats in each group were observed daily for evidence of toxicity (terminal cachexia or death). Rats were weighed at weekly intervals from the initiation to the completion of injections (up to 63 days) and thereafter at 20 day intervals.

A group of 10 rats was injected S.Q. at 2 mg/kg/week for up to 12 weeks. Serum samples were collected at time zero under light ether anesthesia from the retroorbital sinus. Groups of 3 or 4 rats were killed at 4 week intervals and samples of heart and serum were collected for microscopic evaluation and chemical analysis. Terminal serum samples were collected from the abdominal aorta. Serum calcium was determined by atomic absorption spectrophotometry (Perkin-Elmer model 303). Serum creatine phosphokinase (CPK) and lactic dehydrogenase (LDH) (Calbiochem Stat-Pack™, La Jolla, CA) assays were performed on all rats. Statistical analyses of data were performed using
Student's $t$ test (Steel and Torrie, 1960) and 2 x 2 contingency tables (Finney et al., 1963).

Animals receiving ADR by I.V. administration were necropsied at the time of death or at 150 days after the first injection (termination of the experiment). Sections of heart, lung, liver, kidney, spleen, duodenum and tibial epiphyseal plate were collected for histopathologic examination. The hearts from 22 rats (3 to 6 samples per group) were examined ultrastructurally. Portions of the apex, right and left ventricular free walls and interventricular septum were sliced (1-2 mm) and prepared for electron microscopic evaluation. An adjacent section (2 mm) was taken through the ventricular myocardium and fixed in 10% neutral buffered formalin for light microscopic evaluation. Myocardium for light microscopy was embedded in paraffin, sectioned at 6 μ, and stained with hematoxylin and eosin (H & E) and periodic acid-Schiff with diastase digestion (PAS-D). The myocardium collected for fine structural evaluation was 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 gradedethanols, transferred to propylene oxide, and embedded in Epon 812 (Shell Chemical Company, New York, N.Y.). 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 200 electron microscope.

Results

The accumulative mortality data during the 150 day observation period for all groups of rats is presented in Figure 1. For comparison
of acute versus chronic ADR administration, the mortality data of rats receiving a single I.V. injection of 10 mg/kg ADR is given. Deaths following this single bolus of 10 mg/kg plateaued first at 30 days after the injection, and then rose sharply at 70 days to plateau at 100 days with 86% total mortality (12/14 rats) at 150 days. Rats receiving 2 mg/kg/week ADR for 10 injections had significantly fewer deaths (p<0.05 at 150 days compared to 10 mg/kg x 1) during the observation period. One rat died 20 days after receiving 3 injections of ADR. No further deaths occurred until 80 days (last injection given at 63 days) when rats began dying over a subsequent 50 day period to achieve a total mortality of 45% (5/11 rats). Rats receiving 1 mg/kg/week for 10 weeks had one death at 120 days for an accumulative mortality of 10% (p<0.005 at 150 days compared to 10 mg/kg x 1 ADR). No deaths were observed in the group of rats receiving 1 mg/kg MWF with a one week rest between ADR administration to a total of 10 mg/kg, or in the saline control group.

There was an attenuation in mean weight gain in rats receiving 2 mg/kg/week ADR compared to saline-injected controls after the third week of treatment (Figure 2). Other schedules of ADR administration produced a less inhibiting effect on mean weight gain compared to controls. There was little difference in body weight gains between groups of rats that received a total dosage of 10 mg/kg (either by weekly administration or the MWF schedule). The rates of growth in all groups except 2 mg/kg/week ADR were comparable to controls at and after 100 days (Figure 2).
Congestive heart failure (CHF) was identified grossly by the presence of ascites, pleural effusion and/or cardiac enlargement. Forty-five percent (5/11) of rats receiving 2 mg/kg/week ADR developed CHF, while only 10% (1/10) receiving 1 mg/kg/week were similarly affected (Table 1). Animals receiving 1 mg/kg ADR on the MWF schedule developed no gross evidence of CHF (Table 1).

Histologic evidence of myocardial damage was evident in rats that died during or after the sequence of ADR injections (2 and 1 mg/kg/week). Ventricular myocardium from a control rat is shown in Figure 3. Focal myocyte degeneration was identified by the presence of densely eosinophilic cells lacking cross-striations that gave a positive PAS reaction following diastase digestion (Figure 4). Degenerating muscle cells were observed throughout the myocardium in affected rats. Other myocytes were atrophic or vacuolated and interstitial edema was noted frequently.

Another lesion observed by light microscopy in myocardium of rats receiving the weekly schedules of ADR and developing cardiomyopathy was intermyocytic vacuolization (Figure 5). Muscle cells were separated by clear spaces often containing only membrane-lined vacuoles. This feature often was observed in areas of myocardium adjacent to vessels, but was not restricted to this location. Intermyocytic vacuolization was occasionally seen in association with focal muscle cell degeneration (Figure 4).

Cardiomyopathy in ADR-treated rats was defined by the presence of focal degenerating myocytes, interstitial and intramyocytic vacuolization and edema (Table 1). Ninety-one percent (10/11) of rats receiving
2 mg/kg/week x 10 ADR and 40 percent (4/10) of rats administered 1 mg/kg/week x 10 ADR had evidence of cardiomyopathy (Table 1). Only 1 rat receiving 1 mg/kg ADR by the MWF schedule had histologic evidence of cardiomyopathy. Saline-injected rat myocardial tissue was unaffected.

Fine structural evaluation of myocardium conclusively demonstrated the perivascular distribution of myocyte and intermyocytic vacuolization. Large membrane-lined spaces were observed frequently with a centripetal distribution around small capillaries (Figure 6). The interstitial vacuolization often extended into the intermyocytic spaces. Adjacent myocytes also had dilated sarcotubular spaces (Figure 7), although the severity of interstitial vacuolization and intramyocytic sarcotubular dilation were not consistently related. Degenerating muscle cells were characterized by a decrease in the number and disorganization of contractile elements, severe sarcotubular dilatation frequently with a perinuclear distribution, and often an increase in lysosomal bodies (Figure 8). Occasional muscle cells had an increased electron-density and were contracted with a loss of identifiable sarcomeres and extensive vacuolization, while adjacent myocytes appeared normal (Figure 9). Other lesions observed infrequently in myocardium of chronically treated ADR rats included nuclear pyknosis, contraction bands, endothelial cell swelling, and separation of intercalated disks (fasciae adherens). The nuclei and mitochondria of most degenerating myocytes were not affected (Figures 6 through 9).

Rats receiving ADR at 2 mg/kg/week x 12 S.Q. had fine structural evidence of subtle cardiomyopathy at 4 weeks (Figure 8). Mean serum CPK and LDH levels from these rats were increased at 4 weeks after the
beginning of ADR injections, but significant elevations were not observed until 8 weeks (Table 2). The elevations in CPK and LDH continued to be elevated significantly at 12 weeks. No deaths occurred in NZB rats receiving ADR by S.Q. administration up to 12 weeks of age. Serum calcium values were decreased at 8 and 12 weeks after initiation of ADR injections (Table 2); however, these changes were not significant compared to baseline levels.

Extracardiac lesions were detected by light microscopy in kidney, liver, tibial epiphyseal plate, spleen and small intestine. Glomerular vacuolization and thickening of Bowman's capsule were observed consistently. Many rats with hepatomegaly had periacinar necrosis. There was an attenuation in the thickness of epiphyseal growth plates suggesting an arrest of growth of long bones. Some rats (4/11) receiving 2 mg/kg/week x 10 ADR had mild lymphoid depletion of the spleen. Subtle epithelial regeneration in intestinal crypts was observed in all groups of rats receiving ADR.

Discussion

The results of this investigation show that the NZB rat is sensitive to the cardiotoxic effects of repeated I.V. (I.P.) injections of low dosages of ADR. The cardiotoxic effect of ADR in the NZB rat with development of ascites and pleural effusion and light and fine structural features of cardiomyopathy, is apparently total dose-dependent. Cardiotoxicity also was schedule-dependent, as indicated by the low incidence of cardiomyopathy (CMY) in rats on the 1 mg/kg MWF schedule compared to weekly administration. The accumulative dosage of 20 mg/kg (2 mg/kg/
week x 10) produced high mortality, an attenuation of weight gain, increased serum CPK and LDH values. The mild alterations observed in gastrointestinal mucosa, tibial epiphyseal plate, hematopoietic and lymphoid organs and kidney are similar to those described by Mettler et al. (1977) and did not appear to be a cause of death in the experimental rats.

Previous investigations have shown that chronic I.V. administration of ADR to healthy rabbits results in a total dose-dependent cardiomyopathy similar to that occurring in human patients receiving ADR therapy (Jaenke, 1974; Olson et al., 1974; Young, 1975). Several reports of ADR-induced cardiotoxicity in mice (Rosenoff et al., 1975; Lambertenghi-Delliliers et al., 1976) and rats (Chalcroft et al., 1973; Zbinden and Brandle, 1975; Olson and Capen, 1977a) have been reported following single or multiple high dosages of ADR. Myocardial lesions in rats were not detectable by light microscopic examination (Chalcroft et al., 1973; Philips et al., 1975) and depended upon ultrastructural evaluation for identification (Olson and Capen, 1977a). In addition, the lesions described in these subacute studies were not identical to those occurring in patients or in healthy rabbits following chronic ADR administration. Nonetheless, subacute studies of analogues of ADR in rats may be of value as an initial screening procedure for determining the qualitative capacity of these drugs to produce cardiotoxicity. Chronic investigations of ADR analogues in rats will permit the evaluation of cardiotoxic potential versus concomitant therapy to obviate cardiotoxicity (e.g., α-tocopherol; Myers et al., 1977) or cardiotoxic potential versus oncolytic activity in tumor-bearing rats (Olson and Capen, 1977b).
Light and electron microscopic examination of myocardium from ADR-injected rats suggested a vascular distribution of early lesions with a progression to an interstitial vacuolization and sarcotubular dilatation. The perivascular distribution of lesions is similar to earlier investigations in the rabbit (Olson et al., 1974). The absence of severe and generalized nuclear and mitochondrial lesions in most rats examined ultrastructurally differs from acute ADR investigations in rats (Chalcroft et al., 1973; Olson and Capen, 1977a) and probably reflects the variable effects of differing ADR schedules (acute high dosage versus chronic low dosage). The myocardial lesions described in this investigation are similar to those occurring with chronic ADR administration in humans (Buja et al., 1973), rabbits (Jaenke, 1974; Olson et al., 1974) and rats (Mettler et al., 1977).

Serum CPK and LDH values during chronic S.Q. ADR administration demonstrated that significant elevations occur following the earliest detectable ultrastructural lesions (sarcotubular dilatation) at 4 weeks. At 4 weeks, muscle cell membrane damage with concomitant release of enzymes was not extensive. We attribute the increase in serum enzymes largely to heart muscle damage, although ADR may have a damaging effect on skeletal muscle as well. Histopathologic evaluation of diaphragm from the same rats in this study did not show evidence of vacuolization or degeneration of skeletal muscle.

The administration of ADR on the MWF schedule appeared to reduce cardiotoxicity compared to the weekly schedule. Weiss et al. (1976) have suggested that scheduling of ADR treatments may play an important role in the development of CHF. Studies are presently planned to repeat
this schedule at 2 mg/kg MWF to compare mortality and development of CMY with rats on the 2 mg/kg/week schedule.

A decrease in serum calcium values also has been described by Mettler et al. (1977) following chronic ADR administration. Previous investigations have demonstrated a significant but reversible hypocalcemia in rats at 24 hours following acute high dosages of ADR (Olson and Capen, 1977a) which was attributed to the chelating capacity of ADR (Yesair et al., 1974). It is not known whether ADR has any long-term effect on calcium homeostatic mechanisms which might help explain the decrease in mean serum calcium of experimental rats following ADR administration.

The gross lesions of congestive heart failure (ascites, pleural effusion, cardiomegaly) in association with light and fine structural evidence of cardiomyopathy (focal myocytic degeneration, interstitial and intramyocytic vacuolization, loss and disorganization of contractile elements) in the NZB rat demonstrates the rat to be a reproducible model for large scale testing of the cardiotoxic potential of ADR analogues. In addition, the sensitivity of this strain of rat to the cardiotoxic effects of ADR provides a model system in which to evaluate simultaneously the chemotherapeutic effects of ADR on an experimental osteosarcoma (Olson and Capen, 1977) and new therapeutic measures (e.g., α-tocopherol) to ameliorate cardiotoxicity.
Summary

A cardiotoxic response similar to that occurring with chronic adriamycin (ADR) administration in man and rabbits has been produced in the New Zealand black (NZB) rat. ADR was given by the intravenous (I.V.) or intraperitoneal (I.P.) route in groups of rats at 2 mg/kg/week for 10 weeks, 1 mg/kg/week for 10 weeks, and 1 mg/kg on Monday, Wednesday and Friday with a week of rest then repeating to a total of 10 injections. Rats on the weekly ADR schedule developed gross (ascites, pleural effusion, cardiomegaly) and microscopic (focal myocytic degeneration, interstitial and myocytic vacuolization, loss and disorganization of sarcomeres) evidence of cardiotoxicity during the 150 day period of observation. A dosage of 2 mg/kg/week I.V. in rats produced high mortality (45%) and attenuation in mean weight gains compared to controls. This dosage given subcutaneously (S.Q.) resulted in significant elevations in serum creatine phosphokinase (CPK) and lactic dehydrogenase (LDH) suggestive of a progressive cardiotoxicity. Preliminary evidence suggests that cardiotoxicity may be diminished by ADR administration on a MWF-rest-repeat schedule. The sensitivity of this strain of rat to the cardiotoxic effects of ADR provides a model system in which to evaluate simultaneously the chemotherapeutic effects of ADR on an experimental osteosarcoma and new therapeutic measures to ameliorate cardiotoxicity.
Acknowledgments

The authors acknowledge the excellent technical assistance of Mr. Charles Pendley, Mr. Mike Placke, Mr. Robert Ashleman, Mr. Owen Kindig and Mr. Mike Ott.

This work was supported in part by Grants GM 1052 and RR 05463 from the National Institutes of Health, Fellowship (to HMO) CA 00549 from the National Cancer Institute, and Grant IN 160 from the American Cancer Society.
<table>
<thead>
<tr>
<th>ADR Dosage Schedule</th>
<th>No. of Rats</th>
<th>Total ADR Dose (mg/m²)</th>
<th>CHF Cardiotoxicity</th>
<th>Cardiomyopathy</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mg/kg q7d X 10, I.V.</td>
<td>11</td>
<td>118</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>1 mg/kg q7d X 10, I.V.</td>
<td>10</td>
<td>59</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>1 mg/kg MWF, 1 wk rest, repeat to 10 mg/kg total dose, I.V.</td>
<td>12</td>
<td>59</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Saline q7d X 10, I.V.</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1 Congestive heart failure (ascites, pleural effusion, cardiomegaly).

2 Determined by histopathologic examination.
Table 2 — Serum CPK and LDH and Calcium Values in NZB Rats Receiving 2 mg/kg/week ADR, S.Q. X 12

<table>
<thead>
<tr>
<th>Time (Weeks) After Start of ADR Injections</th>
<th>0</th>
<th>4</th>
<th>8</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPK</td>
<td>78.0</td>
<td>84.0</td>
<td>271.0</td>
<td>275.0</td>
</tr>
<tr>
<td>mU/ml serum</td>
<td>± 7.1</td>
<td>± 35.3</td>
<td>± 44.3</td>
<td>± 72.0</td>
</tr>
<tr>
<td>LDH-L</td>
<td>189.6</td>
<td>314.7</td>
<td>523.7</td>
<td>692.8</td>
</tr>
<tr>
<td>mU/ml serum</td>
<td>± 23.5</td>
<td>± 124.3</td>
<td>± 58.2</td>
<td>± 81.2</td>
</tr>
<tr>
<td>Calcium</td>
<td>10.14</td>
<td>10.28</td>
<td>9.62</td>
<td>9.48</td>
</tr>
<tr>
<td>(mg/100 ml)</td>
<td>± 0.24</td>
<td>± 0.28</td>
<td>± 0.30</td>
<td>± 0.39</td>
</tr>
</tbody>
</table>

All values are mean ± SE (n = 3 to 4 samples per point).

ap < 0.05, compared to time 0.

bp < 0.01, compared to time 0.
Figure 1. Accumulative mortality data for NZB rats receiving 10 mg/kg x 1 (single dose) ADR (X-X), 2 mg/kg/wk x 10 ADR (Δ-Δ), 1 mg/kg/wk x 10 ADR (□-□) and 1 mg/kg ADR on MWF with a one week rest period, repeating to 10 injections (O-O). No deaths were observed in saline-injected control rats (not shown).
PERCENT MORTALITY

DAYS AFTER START OF ADR INJECTIONS

FIG 1

PERCENT MORTALITY

DAYS AFTER START OF ADR INJECTIONS
Figure 2. Mean weight gain, expressed as percent of initial weight, for NZB rats receiving 2 mg/kg/wk x 10 ADR (△-△), 1 mg/kg/wk x 10 ADR (□-□), 1 mg/kg MWF ADR (○-○), and saline controls (●-●).
PERCENT OF INITIAL WEIGHT

SALINE (12)
ADR 2mg/kg q7d x 100
ADR 1mg/kg q7d x 100
ADR 1mg/kg MWF x 10

DAYS AFTER START OF ADR INJECTIONS

PERCENT OF INITIAL WEIGHT
Figure 3. Light microscopic appearance of rat ventricular myocardium. Myocardium from a saline-injected control rat killed at 150 days after the first injection; H & E, X 900
Figure 4. Degenerating muscle cell in myocardium from a rat receiving 2 mg/kg/wk ADR that died 107 days after the first injection; PAS-D, X 900.
Figure 5. Extensive intermyocytic vacuolization in myocardium of a rat receiving 2 mg/kg/wk ADR that died 106 days after the first injection; H & E, X 900.
Figure 6. Electron micrograph of a degenerating capillary (C) with multiple perivascular vacuoles in ventricular myocardium of a rat receiving 1 mg/kg/wk ADR at 110 days. There is mild edema of adjacent myocytes. X 5600
Figure 7. Electron micrograph illustrating vacuolated interstitial spaces with a large dilated intramyocytic sarcotubule (S). X 9000
Figure 8. Perinuclear vacuoles (V) representing dilated sarco-tubules and numerous lysosomal bodies (arrows). Myocardium from rat killed at 4 weeks after receiving ADR at 2 mg/kg/wk, S.Q. Nucleus (N) and most mitochondria are normal, but there are occasional myelin figures (arrowhead). X 10,300
Figure 9. Electron micrograph illustrating focal myocyte degeneration with loss of identifiable sarcomeres and extensive intracellular vacuolization (arrows). Adjacent myocyte appears normal. X 8,500
CHAPTER V

CHEMoresponsiveness of Moloney Sarcoma Virus-Induced Osteosarcoma to Adriamycin in the Rat

Introduction

Of primary malignancies of bone in man, osteosarcoma occurs most frequently, and has a poor prognosis. The five year survival rate with surgery and radiotherapy remains at or below 20% (8). Initial reports with immunotherapy and surgery in the treatment of osteosarcoma show no improvement over surgery alone (14). However, recent results with chemotherapy coupled with surgical ablation of the primary neoplasm have been encouraging (2,3,10,19,20). Methotrexate with citrovorum factor rescue, and adriamycin presently show the most encouraging responses (10).

Although a viral etiology for human osteosarcoma has not been conclusively demonstrated, bone tumors characterized as osteosarcomas have been produced by viruses (4,5,21) and other agents (11,25) in several animal species. Recent reports with immunotherapy of experimental osteosarcomas have demonstrated the responsiveness of these tumors to cell-mediated and humoral immune mechanisms (7,24). The chemoresponsive effect of experimental osteosarcomas to ADR and other chemicals appears to be variable and may not predict well for human studies (1,23).

We have recently described an experimental osteosarcoma produced by inoculation of young New Zealand black (NZB) rats with Moloney sarcoma virus (MSV) (15,16). 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 (15). This investigation was designed to evaluate the chemoresponsiveness of this osteosarcoma model to adriamycin (ADR), including (1) prolongation of life-span, (2) reduction in tumor size and incidence of metastatic disease, and (3) morphological alterations of the osteosarcoma with chemotherapy.

Materials and Methods

All New Zealand black (NZB) rats in this study were inoculated at 4 days of age by intratibial instillation of a partially purified preparation of Moloney sarcoma virus (MSV), as described previously (9, 15, 16). Rats were palpated at weaning (21 days of age) and those without palpable tumors were eliminated from the study.

Adriamycin (ADR) chemotherapy of rats with osteosarcomas were begun at 34 days of age (30 days post-inoculation). Rats were injected intravenously (i.v.) in the lateral tail vein or by the intraperitoneal (i.p.) route with a lyophilized preparation of ADR hydrochloride (bulk drug kindly provided by Adrialabs, Wilmington, DL). ADR was weighed in 10 and 5 mg aliquots on an electrobalance, placed in small glass vials and stored in the dark at 1°C. At the time of injection, the ADR was reconstituted with 0.85% sterile saline at 1 mg/ml and given immediately.

A total of 54 rats were injected with ADR or saline (placebo) using three treatment schedules. Fifteen rats received ADR at 2 mg/kg/week, 14 rats received 1 mg/kg/week, and 13 rats received 1 mg/kg on Monday, Wednesday and Friday (MWF) followed by one week of rest then another MWF schedule followed by a week of rest up to a total dose of 10 mg/kg. Twelve control rats received i.v. saline injections on a weekly basis for 10 weeks. All
animals received a total of 10 injections. Careful technique in the injection of ADR permitted most injections to be given I.V., but perivasculitis was severe in some rats resulting in necrosis of the tail. Injection of ADR by the I.P. route was selected as the alternative route of administration.

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 Hellos calipers in three different dimensions at weekly intervals (15) 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 data were performed using Student's t test (22) and 2 x 2 contingency tables (6).

Animals receiving ADR by I.V. administration 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 epiphyseal plate in controls were collected in 10% neutral buffered formalin for histopathologic examination. The hearts from 10 rats and bone tumors 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. Bone was decalcified for 7 days in 10% buffered EDTA. All tissues for light microscopy were embedded in paraffin, sectioned at 6 u, and stained with hematoxylin and eosin (H & E). Selected sections of myocardium also were stained with periodic acid-Schiff with diastase digestion (PAS-D). Myocardium and osteosarcoma collected for fine structural evaluation was 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 (Shell Chemical Company, 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 200 electron microscope.

Results

The accumulative mortality of tumor-bearing rats on ADR therapy or receiving saline placebo is presented in Chart 1. The administration of ADR in all regimens initially had an attenuating effect on mortality rate compared to control rats. The mortality incidence in all ADR treatment groups was significantly less than controls (p<.05) from 40 to 50 days after initiation of treatment. Rats receiving 2 mg/kg/week ADR had significantly fewer deaths (p<.01) at 35 days after start of treatment. The mortality rate for rats receiving 1 mg/kg/week ADR was significantly less (p<.025) from 40 through 95 days. At all points after 95 days from the start of treatment, there was no significant difference in 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. There was no significant effect of any regimen of ADR therapy on rate of weight gain in tumor-bearing rats compared to saline controls. At 150 days after initiation of therapy, both groups of rats receiving 10 mg/kg total dose ADR had weight percentage increases above the placebo group while the group administered a total dosage of 20 mg/kg (2 mg/kg/week) was below the placebo group.
Chart 3 illustrates the results of chronic ADR therapy regimens on mean tumor diameter compared to saline controls. There was a progressive increase of mean tumor diameter 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 1 mg/kg/week ADR (p<.05 from 7 days through 50 days after start of ADR treatment). Rats treated at 2 mg/kg/week had significant (p<.05) regression in tumor size at 14 days through 50 days, except at day 28 compared to controls. For rats receiving 1 mg/kg ADR on a MWF schedule, the duration of significant (p<.05) reduction in tumor size was from 28 days through 50 days. Most rats (9/12) with osteosarcomas receiving saline injections had rapidly-growing neoplasms. The sharp decline in mean tumor diameter after 50 days from the start of treatment in the placebo group reflects the spontaneous tumor regression rate in 25% (3/12) of rats surviving the period of observation (Chart I).

Radiographic evaluation of tumor-bearing rats receiving 2 mg/kg/week ADR illustrated evidence of tumor regression from 7 days through 50 days after the start of ADR therapy (Figs. 1-4). By comparison, radiographic evaluation of an osteosarcoma in the placebo group at the beginning (Fig. 5) and end (Fig. 6) of this evaluation period demonstrated progressive tumor growth and lysis of pre-existing bone of the tibia. Not only did many of the regressing tumors under ADR therapy become progressively more radiodense, but there was a reduction in total tumor size to the point that only a very small, barely palpable enlargement remained (Fig. 4). 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 morphologic features of an osteosarcoma from the placebo group is illustrated in Figure 7. 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. 8). Many areas of the osteosarcomas were replaced by collagen-producing fibroblasts (Fig. 9). Extensive mineralization of osteoid matrix was observed in tumors of varying size in rats receiving both 1 and 2 mg/kg/week (Figure 10). There were broad zones of osteoid matrix with mature osteoblasts aligned along the surface 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. 13).

Metastatic disease was identified in all groups of rats and included gross and/or histopathologic lesions in sublumbar lymph nodes and/or lungs (Table 1). The incidence of metastases did not appear to be affected by any schedule of ADR chemotherapy. Many of the metastatic lesions in the lung of rats receiving 2 mg/kg/wk were very small and appeared to be undergoing degeneration (Fig. 11). One enlarged sublumbar lymph node in a rat receiving 1 mg/kg/wk had a focus of mineralization of osteoid matrix (Fig. 12). Similar lesions were not observed in metastases from the placebo group.

Cardiotoxicity of ADR was defined by two parameters: gross evidence of congestive heart failure (e.g., ascites, pleural effusion and/or cardiomegaly) and histopathologic evidence of cardiomyopathy, including presence of focal degenerating myocytes by the PAS-D stain, 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. Fewer rats developed gross evidence of congestive heart failure at accumulative dosages of 10 mg/kg (4/14 at 1 mg/kg/week; 3/13 at 1 mg/kg MWF) compared to 20 mg/kg (8/15 at 2 mg/kg/week) but the difference was not significant (p<.05).

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. The greatest response to ADR chemotherapy was observed with rats receiving 1 mg/kg/week ADR. Chronic ADR administration had no effect on body weight gains compared to placebo. ADR therapy regimens all had a significant effect in reducing the size of primary osteosarcomas at certain time points, but 1 mg/kg/week gave the most consistent effect in reducing the size of the primary tumor. Preliminary data suggest that ADR-induced cardiotoxicity may be an important factor in causing death of osteosarcoma-bearing rats at an accumulative dosage of 20 mg/kg.

Recent reports using ADR chemotherapy in human osteosarcoma have been encouraging. Many of the studies have employed multidrug chemotherapy, including both ADR and methotrexate to effect regression of metastases (10, 19, 20). One investigation recommends aggressive presurgical chemotherapy to induce primary osteosarcoma regression followed by on bloc resection of the neoplasm with total knee prosthetic replacement (19). These studies demonstrate the sensitivity of osteogenic sarcoma to ADR and the drug's potential usefulness in combination with surgery and additional chemotherapy to provide 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 (17) and chronic low dose ADR (18) given I.V. Rats with osteosarcomas in this investigation receiving either 10 mg or 20 mg total dose ADR had a similar sensitivity to this important toxic side effect. This observation makes this animal model particularly useful as a system in which to monitor objective tumor response as well as the development of cardiotoxicity. Recent studies (12,13) have shown that the ADR-induced cardiotoxicity in mice may be related to the production of free radicals which suggests that pretreatment of tumor-bearing rats with alpha-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 cell with mineralization. This is similar to the response that has been described when aggressive chemotherapy preceded subtotal en bloc resection of the primary osteosarcoma (19). 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 explain the ultimate similar mortality in our treatment groups in spite of a lesser total dosage of ADR (10 mg/kg).

This study has demonstrated the usefulness of this experimental model of osteosarcoma regarding its sensitivity to ADR chemotherapy. No attempt was made to reduce the initial tumor load by subtotal or total resection. Even though ADR therapy was instituted well after the primary osteosarcoma was palpable, objective chemoresponsiveness has been demonstrated with this model.
MSV-induced osteosarcoma in NZB rats has considerable potential as an animal model system to investigate different chemotherapeutic regimens for osteosarcoma. Total resection before and after instituting chemotherapy may be useful in determining which approach best prolongs lifespan. The use of younger tumor-bearing rats may enhance chemoresponsiveness. Finally, the sensitivity of the NZB rat to the cardiotoxic effects of ADR provides an animal model system in which to evaluate the oncolytic activity of new analogues as well as their cardiotoxic potential.
Summary

The chemoresponsiveness of a Moloney sarcoma virus-induced osteosarcoma in the rat to intravenous adriamycin (ADR) therapy was determined by (1) prolongation of lifespan, (2) reduction in tumor size, and (3) morphological alterations of the neoplasm. ADR was given by the intravenous route or intraperitoneal route in groups of osteosarcoma-bearing rats at 2 mg/kg/week for 10 weeks, 1 mg/kg/week for 10 weeks, and 1 mg/kg on MWF with a week of rest, repeating to a total of 10 injections. Rats receiving 1 mg/kg/week had the most consistent response to ADR treatment with a significant prolongation of lifespan from 40 through 95 days and a significant decrease in tumor diameter from 7 days through 50 days after the start of ADR treatment. Other ADR regimens also produced a significant increase in lifespan and a reduction in tumor size compared to rats in the placebo group. Radiographic evaluation of osteosarcomas demonstrated an increased radiodensity with ADR therapy compared to a rapid proliferation and destruction of pre-existing bone in the placebo group. Microscopic and ultrastructural evaluation of osteosarcomas following various intervals of ADR revealed necrosis of tumor cells, fibroblastic proliferation, and mineralization of osteoid matrix. Metastatic lesions in lung and sublumbar lymph node also were sensitive to ADR therapy as reflected by necrosis of tumor cells. Evidence of congestive heart failure and cardiomyopathy were observed in all groups of rats on ADR chemotherapy. The sensitivity of the osteosarcoma-bearing NZB rat to the antitumor and cardiomyopathic effects of ADR provides an animal system in which to evaluate the oncolytic activity of new analogues as well as their cardiotoxic potential.
Table 1. ADR-induced chemoresponsiveness and cardiotoxicity in osteosarcoma-bearing NZB rats.

<table>
<thead>
<tr>
<th>Intravenous Dose of ADR</th>
<th>No. of Rats</th>
<th>% Rats with Metastatic Disease</th>
<th>Cardiotoxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CHF(^1) (%)</td>
</tr>
<tr>
<td>2 mg/kg q7d x 10</td>
<td>15</td>
<td>57 (4/7)</td>
<td>53</td>
</tr>
<tr>
<td>1 mg/kg q7d x 10</td>
<td>14</td>
<td>75 (6/8)</td>
<td>28</td>
</tr>
<tr>
<td>1 mg/kg MWF, 1 wk. rest, repeat to 10 mg/kg total dose</td>
<td>13</td>
<td>50 (4/8)</td>
<td>23</td>
</tr>
<tr>
<td>Saline q7d x 10</td>
<td>12</td>
<td>75 (9/12)</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^1\)Congestive heart failure (e.g., ascites, pleural effusion, cardiomegaly).

\(^2\)Percent incidence based on number of hearts with histopathologic evidence of cardiomyopathy.
Chart 1. The accumulative mortality of groups of osteosarcoma-bearing rats receiving three schedules of ADR chemotherapy or saline placebo. Numbers of rats in each group are indicated in parentheses.
Chart 2. The weight gain in all groups of tumor-bearing rats receiving three schedules of ADR chemotherapy or saline placebo.
Chart 3. Mean tumor diameter of groups of rats receiving three schedules of ADR chemotherapy or saline placebo. Osteosarcoma of each rat was measured in three dimensions (length, width, and thickness) with calipers and the average tumor diameter computed.
Figures 1-4. Radiographs illustrating sequential regression of osteosarcoma in rat receiving 2 mg/kg/week ADR.
Figure 1. Osteosarcoma with osteogenic activity at 7 days after start of ADR injections. X 2.5
Figure 2. Osteosarcoma is smaller and more radiodense after 21 days ADR.

X 2.3
Figure 3. Small osteosarcoma elevated above the diaphyseal area (arrow) after 50 days ADR. X 2.4
Figure 4. Regression of osteosarcoma to small, radiodense mass after 80 days ADR. X 2.2
Figures 5 and 6. Radiographs illustrating the appearance of an osteosarcoma in a rat from the placebo group.
Figure 5. Osteosarcoma with considerable osteogenic activity at 7 days after start of saline injections. X 2.5
Figure 6. Progressive growth of osteoproliferative osteosarcoma with destruction of pre-existing tibial bone after 50 days of saline injections. X 1.7
Figure 7. Osteosarcoma from placebo group composed of large pleomorphic and small fusiform tumor cells with an abundant osteoid stroma. X 700
FIG 7
Figure 8. Extensive area of coagulation necrosis in an osteosarcoma at 26 days after start of ADR treatment. X 300
Figure 9. Scattered neoplastic cells (arrow) with marked proliferation of fibroblasts in an osteosarcoma from a rat 21 days after start of 2 mg/kg/week ADR. X 700
Figure 10. Mineralization of osteoid matrix in an osteosarcoma of a rat receiving 2 mg/kg/week ADR. X 300
Figure 11. Vacuolization and pyknosis of tumor cells in a pulmonary metastasis from an osteosarcoma in a rat receiving 2 mg/kg/week ADR. X 700
Figure 12. Mineralization of osteoid matrix (0) in a metastatic focus of osteosarcoma in the sublumbar lymph node from a tumor-bearing rat treated with 1 mg/kg/week ADR. X 700
Figure 13. Electron micrograph of cells in an osteosarcoma from a rat receiving 1 mg/kg/week ADR. Degeneration of neoplastic cells includes cytoplasmic vacuolization, mitochondrial degeneration, myelin figure formation, nuclear degeneration and cell lysis. X 3200
BIBLIOGRAPHY

Chapter 1


Chapter II


Chapter III


34. Young DM, National Cancer Institute: Personal communication.

Chapter IV


Chapter V


