The alkaline phosphatase level in mice with experimentally induced osteosarcomas
by Janice Marie Bailey

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Microbiology
Montana State University
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Abstract:
Experimentally induced osteosarcomas are detected roentgenographically in mice. In order to develop a more practical and earlier means of osteosarcoma detection the alkaline phosphatase level of CFl/Anl mice was studied. Plasma samples obtained from the retro-orbital sinus were assayed using the Bessey-Lowry-Brock procedure. Osteosarcomas were induced in 55 mice with FBJ virus and in 26 mice with strontium-90. In the FBJ virus study group weekly enzyme assays were begun on mice 4 weeks of age and followed over an 8 week period. No rise in alkaline phosphatase values occurred in mice with FBJ virus induced osteosarcomas. Enzyme assays of the strontium-90 test group were begun on mice near 3 months of age and were continued for 8 months. Twenty-one of the strontium-90 injected mice which developed osteosarcomas had significantly elevated alkaline phosphatase levels compared with normal controls. Elevation of values occurred as much as 2 to 8 weeks prior to roentgenographic tumor detection. Five other mice with strontium-90 induced osteosarcomas failed to show elevated alkaline phosphatase levels until 2 to 6 weeks after radiographic detection of tumors.

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Date December 1, 1971

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JANICE MARIE BAILEY

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree

of

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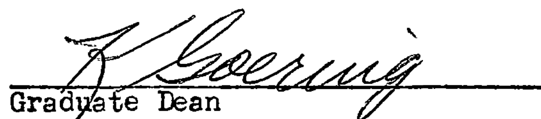
Approved:

Head, Major Department

Chairman, Examining Committee



Graduate Dean

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ABSTRACT

Experimentally induced osteosarcomas are detected roentgenographically in mice. In order to develop a more practical and earlier means of osteosarcoma detection the alkaline phosphatase level of CF1/Anl mice was studied. Plasma samples obtained from the retro-orbital sinus were assayed using the Bessey-Lowry-Brock procedure. Osteosarcomas were induced in 55 mice with FBJ virus and in 26 mice with strontium-90. In the FBJ virus study group weekly enzyme assays were begun on mice 4 weeks of age and followed over an 8 week period. No rise in alkaline phosphatase values occurred in mice with FBJ virus induced osteosarcomas. Enzyme assays of the strontium-90 test group were begun on mice near 3 months of age and were continued for 8 months. Twenty-one of the strontium-90 injected mice which developed osteosarcomas had significantly elevated alkaline phosphatase levels compared with normal controls. Elevation of values occurred as much as 2 to 8 weeks prior to roentgenographic tumor detection. Five other mice with strontium-90 induced osteosarcomas failed to show elevated alkaline phosphatase levels until 2 to 6 weeks after radiographic detection of tumors.

INTRODUCTION

The only chemical analysis of blood pertinent to osteosarcomas is the serum alkaline phosphatase level (Jaffe, 1959). Values from 2 to 40 times the upper normal limit have been reported in humans with osteogenic sarcomas (Schwartz, et al., 1969). In mice a strong histochemical activity of alkaline phosphatase has been noted with osteosarcomas, although blood levels of alkaline phosphatase have not been correlated with the presence of osteosarcomas (Timmer, et al., 1968).

A comparison of plasma alkaline phosphatase levels and osteosarcoma development has been made in this study to provide a more practical and earlier means of tumor detection in experimental mice than is possible by the use of roentgenographs. In terms of expense alkaline phosphatase tests are advantageous, for they are one-third the cost of x-rays. For example, materials for 500 alkaline phosphatase assays cost approximately $23 compared with $69 for 500 x-rays.

Osteosarcomas in CF1/Anl mice were induced with FBJ virus or strontium-90. Alkaline phosphatase levels were determined using the Bessey-Lowry-Brock method. Tumors were detected radiographically and their appearance was correlated with alkaline phosphatase levels. Uninjected animals were tested concurrently to establish a base line of normal values.

REVIEW OF LITERATURE

Neoplastic processes are found in all vertebrates (Schlumberger and Lucké, 1948). The most common primary malignant neoplasm of bone is the osteosarcoma, which is fatal in most instances, irrespective of treatment (Bennett, 1961). Osteogenic sarcomas are highly malignant tumors of osteoblast cells and usually arise in the periosteum (Moulton, 1961; Reifenstein, 1962). Most tumors originate from osseous tissue directly, although some may begin in soft tissue by metaplastic differentation of mesenchymal tissue to produce an osteoid (Robbins, 1964).

An osteosarcoma usually forms a large white fibrous mass with variable areas resembling cartilage or bone (Cotchin, 1957). The neoplasms are round, ovoid, or spindle-shaped and have intimate association with the bones from which they arise. From one-fourth to one-half of the affected bone may be replaced by neoplastic tissue (Moulton, 1961).

RADIONUCLIDE INDUCTION OF OSTEOSARCOMAS

Radioactive substances with metabolic activity similar to calcium are extremely effective in producing bone tumors (Brues, 1956; Biskis and Finkel, 1970). One of the first elements to be implicated in the production of bone tumors was radium (Bennett, 1961). Strontium-90,

another bone-seeking radionuclide, has come into prominence due to its presence in radioactive fallout (McLean, 1964).

Carcinogenicity of radionuclides is related to their physical and chemical properties (Finkel, et al., 1964). The emission of high-energy beta particles and a long half-life of 27.7 years are two features which make strontium-90 an excellent carcinogen (McLean, 1964). The solubility of strontium enhances its absorption from the gastro-intestinal tract and entry into the blood stream (Petrov, et al., 1966). Strontium locates in areas where bone is being formed or reconstructed (McLean, 1964). The incidence of bone tumors caused by radiostrontium can approach 100% (Ito, et al., 1969).

Chronic oral exposure to strontium-90 gives a more uniform distribution of radiation in the bone than a single dose (Howard, et al., 1969; McLean, 1964). Deposition of strontium occurs mainly in the mineral phase of bone through diffusion exchange, recrystallization, and new crystal formation (Brues, 1956). Results from experiments using intravenous injections of strontium-90 show that a single dose is more carcinogenic than fractionated amounts of the same dose given over a number of days (Finkel, et al., 1964). A single intravenous injection yields an uneven skeletal deposition and may lead to tumors in one or more bones. Hot spots in the bone result in foci of severe necrosis while other regions of the same bone are relatively unaffected.

Sublethal amounts of irradiation are necessary for the inducement of neoplastic changes, since animals die before tumor appearance with high dosages (Brues, 1956).

A latency time has been noted between the administration of a radioactive material and the appearance of tumors. Experimental evidence indicates that tumor latency time is the same for all ages of mice receiving strontium-90 injections. The earliest radiographic identification of tumors was 98 days after the optimum carcinogenic amount of strontium-90 had been injected (Finkel, et al., 1966a). Within the sublethal range the incidence of sarcoma formation increases with the amount of radioactive material administered. There is usually an abrupt rise in the incidence of bone sarcomas among animals surviving longest with relatively high doses (Jaffe, 1959). The incidence of bone tumors in mice is reduced as smaller doses of strontium are administered (Brues, 1956). There appears to be a threshold below which sarcomas are not induced since no malignant bone tumors have been produced by a dose of 8.9 micro Curies (μCi)/kg body weight (Finkel, et al., 1959). An intravenous injection of 1.0 μCi/g body weight appears to be an optimum dose of strontium-90 for tumor induction (Finkel, et al., 1966a).

Typical radiation-induced osteosarcomas usually appear first as areas of increased or decreased density within bone in the metaphyseal

or epiphyseal region. The tumor breaks through the bone cortex and spreads by direct extension and invasion of adjacent tissue (Finkel, et al., 1966a; Finkel and Biskis, 1969). Radiographically the lesions are quite osteolytic, resulting in extensive areas of bone destruction (Howard, et al., 1969). Streaks, patches, or even large blotchy areas of radiopacity reflect the presence of bone necrosis and its consequences. Scattered areas of radiolucency are also present (Jaffe, 1959; Dunlap, 1966).

It has been suggested that radiostrontium usually induces osteogenic sarcomas by inactivating a viral inhibitor (Finkel and Biskis, 1969). There is evidence that a virus may reside in the mouse at the time of irradiation (Huebner and Todaro, 1969). Germ-free mice are rendered leukemic by irradiation in the absence of contamination by microorganisms from the environment. Tissues of germ-free mice with x-ray induced leukemia as well as normal germ-free mice contain leukemia virus-like particles (Kajima and Pollard, 1965). All strontium-90 induced tumors examined in thin section have contained virus-like particles (Finkel and Biskis, 1969).

FBJ VIRUS INDUCTION OF OSTEOSARCOMAS

In 1966 a unique viral agent was isolated from a spontaneous osteosarcoma of a CF1/Anl mouse by Finkel, Biskis, and Jinkins. Tumor extracts injected into newborn mice caused the development of

osteosarcomas. This agent has been named the FBJ virus. It differs from other oncogenic viruses in that it produces only osteogenic sarcomas (Finkel, et al., 1966b). It is a Type C RNA virus with a complement-fixing antigen characteristic of the murine leukemia-sarcoma viruses (Finkel, et al., 1970). Electron microscopy demonstrates Type C viral particles both in cell-free extracts and in ultrathin sections of osteosarcomas induced by the FBJ virus (Finkel, et al., 1966a).

Newborn mice are more susceptible to FBJ virus-induced tumors than older animals (Gross, 1970). The appearance of an osteosarcoma in one mouse is usually followed shortly by the appearance of tumors among other members of the injected litter. However, animals in the same litter may not be equally sensitive to the virus (Finkel, et al., 1966b). In 1969 Kelloff and his coworkers found that CFW and NIH Swiss mice were highly susceptible (40-45%), and $C_{57}Bl$ was weakly susceptible (10%) to the FBJ virus. No tumors were induced in newborn NIH hamsters or newborn Fisher rats (Kelloff, et al., 1969).

The ribs were the most frequent site of tumor formation with FBJ virus-induced osteosarcomas (Finkel, et al., 1966a). Tumors appeared subcutaneously at or near the site of inoculation (Kelloff, et al, 1969). The tibia had tumor growth primarily in the periosteum and the marrow cavity was infrequently involved, and then only slightly

(Finkel, et al., 1966a). Osteosarcomas may occur anywhere along the bone, first as cortical thickening and as small areas of increased density in soft tissues adjacent to the bone (Finkel, et al., 1966b), The principle growth is periosteal, and as the tumor enlarges, it appears to evoke periosteal growth in nearby bone (Finkel, et al., 1966a). Growth proceeds peripherally with delayed involvement of deep cortical bone (Finkel, et al., 1966b). Induction time for tumor appearance after virus injection decreased from 280 days in the first passage of the virus to 21 days after subsequent passages (Finkel, et al., 1966a).

The roentgenographic appearance of virus-induced tumors is somewhat different from osteosarcomas induced with strontium-90. In contrast to radionuclide-induced lesions, FBJ virus tumors first appear as areas of periosteal growth or as regions of bone formation in soft tissue adjacent to the bone. They may occur anywhere along the bone surfaces. Radiographs may show a sunburst pattern with older lesions due to an invasion of soft tissue adjacent to the bone and subsequent calcification of the surrounding tissue. Successive passages of the virus have not changed the radiographic appearance of tumors (Finkel, et al., 1966b).

THE EFFECT OF OSTEOSARCOMAS ON ALKALINE PHOSPHATASE ACTIVITY

Histochemical analysis of osteosarcomas regularly shows a high

concentration of alkaline phosphatase, with the enzyme activity being greater in the more rapidly growing portions of the tumor. The concentration of alkaline phosphatase is greatest in the vicinity of the blood vessels. The blood level of alkaline phosphatase parallels the course of the tumor, being reduced by tumor excision and increased by extension or metastasis to other tissues (Reifenstein, 1962).

ADDITIONAL FACTORS WHICH AFFECT ALKALINE PHOSPHATASE ACTIVITY

Bone alkaline phosphatase, first discovered in 1923 by Robison, is highly concentrated in the osteoblasts with microsome fractions yielding the greatest amount of the enzyme (Vaes and Jacques, 1965). The elevation of alkaline phosphatase is associated with a number of bone diseases other than osteosarcoma. Such conditions may include: rickets, Paget's disease, primary hyperparathyroidism, osteomalasia, Von Recklinghausen's disease with bone involvement, osteitis deformans juvenilia due to vitamin D deficiency, malabsorption of calcium, or renal tubular dystrophies (Damm, 1965, Davidsohn and Wells, 1963; Kay, 1930).

Under normal physiologic conditions the blood level of bone alkaline phosphatase may fluctuate (Fennelly, et al., 1969). Kuan and his associates (1966) noted a change in the alkaline phosphatase level of developing chicks. Others have also noted a variation of

alkaline phosphatase levels in humans of different ages. In neonates and children, normal values range from upper adult levels to approximately two times the upper normal levels of adults. In the 3 to 10 year old group, a steady decline occurs. With the onset of puberty values begin to rise again, with a return to normal adult levels after puberty (Davidsohn and Wells, 1963). Experimental evidence indicates that these physiological variations are due to the osteoblastic activity of bones (Eisenberg, 1970). Genetically endowed variations may also occur. Rats have a high alkaline phosphatase activity compared to that of other species (Saini and Posen, 1969). In inbred strains of mice, alkaline phosphatase levels vary as much as 12 King-Armstrong units among different mouse strains (Yuhas, et al., 1967).

Other tissues besides bone which contain a high level of alkaline phosphatase include the intestinal mucosa, placenta, kidney, and liver (Posen, et al., 1967; Damm 1965; Fishman, et al., 1962). Even within the same tissue multiple forms of alkaline phosphatase may be produced. Three different isoenzymes have been detected in the mouse duodenum at different stages of maturity (Etzler and Moog, 1968; Moog, et al., 1969).

Alkaline phosphatase contributed by the intestine may cause a variation of the serum phosphatase level (Madsen and Tuba, 1952). Differences in the circulating level of intestinal alkaline

phosphatase may be mediated by changes in diet (Tadayyon and Lutwak, 1969; Keeling, 1969). Fasting rats showed lower levels than normal (Bodansky and Jaffe, 1932) and rats on fat diets showed higher levels than normal (Saini and Posen, 1969; Jackson, 1952).

In humans the alkaline phosphatase level may be raised by a placental isoenzyme. During the third trimester of pregnancy, a spill over into the blood stream occurs (Kitchener, et al., 1965). No comparable spill over occurs in rats (Posen, et al., 1969; Manning, et al., 1969).

Abnormal conditions of the liver which may contribute to an elevated blood alkaline phosphatase level include biliary tract obstruction and hepatic infiltrations with neoplastic or granulomatous processes (Ticktin and Trujillo, 1970; Taswell and Jeffers, 1963). Extra hepatic bile obstruction associated with elevated alkaline phosphatase may be due to impaired excretion of products. In biliary tract diseases an increased alkaline phosphatase may be due to overproduction or impairment of secretory function of the liver (Damm, 1965). Through experiments of bile duct ligation, it was concluded that increased alkaline phosphatase values in obstructive liver disease were the result of de novo synthesis of alkaline phosphatase in the liver and subsequent leakage of this induced enzyme into the serum (Kaplan and Righetti, 1969).

The proliferation of malignant tumors may also contribute to an increased serum phosphatase activity (Annotation, 1969). The Regan isoenzyme, found in the serum of patients with various malignant tumors, is biochemically and immunologically indistinguishable from placental alkaline phosphatase (Fishman, 1969; Ghosh, 1969). It also occurs in tumor tissue and malignant effusion fluids (Kellen, 1970). Quantitative analysis of serum levels is useful in monitoring the progression or regression of tumors (Stolbach, et al., 1969; Nathanson, and Fishman, 1971). Types of alkaline phosphatase other than Regan isoenzyme may be produced by tumors (Stolbach, et al., 1969).

CHARACTERISTICS OF ALKALINE PHOSPHATASE

Alkaline phosphatase is a zinc metalloenzyme (Posen, et al., 1969) with a molecular weight ranging from 86,000 to 200,000 (Scutt and Moss, 1968; Schlesinger, et al., 1969). Experiments with bacteria and tissue cultures have shown alkaline phosphatase to be a constitutive enzyme in some instances and an inducible enzyme in others (Schlesinger, et al., 1969; Griffin and Bottomley, 1969; Martin, et al., 1969). The natural substrate of alkaline phosphatase and its physiological role are unknown (Kachmar, 1970). Optimal activity occurs around a pH of 9 (Damm, 1965). A number of reliable procedures have been developed for the assay of alkaline phosphatase

of blood (Nathanson and Fishman, 1971).

Most tissue forms of alkaline phosphatase may be differentiated by their individual physical and chemical properties (Kellen, 1970). Electrophoretic migration patterns show liver in the first position followed by bone, placental, and intestinal phosphatase, respectively (Suzuki, et al., 1969). Heat lability is detected by heating serum to 55° C for 16 minutes. Liver phosphatase is inactivated 50-70 percent, intestinal phosphatase 50-60 percent, and bone 90-100 percent, while the placental isoenzyme is not affected. Five minutes exposure to a temperature of 65° C causes complete inhibition of bone, liver, and intestinal alkaline phosphatase. No inhibition of the placental isoenzyme occurs. Isoenzyme exposure to L-penylalanine causes minimal inhibition of liver and bone phosphatase while intestinal and placental phosphatase show greater inhibition (Stolbach, 1969). Exposure to various concentrations of urea at 37° C for 30 minutes is another differential method. An irreversible inactivation concentration is 8 molar (M) urea for placental, 6-7 M for intestinal, 3 M for liver, and less than 3 M for bone phosphatase (Moss, 1969). Urea experiments have also shown a difference in 3-D structure for placental and Regan isoenzymes which otherwise are indistinguishable (Fishman, 1969).

MATERIALS AND METHODS

Source and Maintenance of Animals

CF1/Anl mice were obtained from Argonne National Laboratory. Purina Mouse Chow and water were supplied *ad libitum* to the animals.

FBJ VIRUS STUDY GROUPS

Uninjected animals. Testing was begun at 28 days of age on female CF1/Anl mice. Blood was drawn from the retro-orbital sinus by means of heparinized capillary tubes. The tubes were sealed with clay and plasma was separated from the cells in a Clay-Adams microcentrifuge. Weekly assays were continued for 3 months. Mice were palpated weekly for the presence of tumors.

Uninjected animals with x-ray exposure. At 28 days of age, 10 CF1/Anl female mice were bled and total body x-rays were taken. Weekly sampling and x-ray exposure was continued for 3 months. Mice were also palpated weekly to detect tumors.

FBJ virus injected animals. Newborn CF1/Anl mice from 12 litters were injected intraperitoneally with 0.1 ml of FBJ virus via the leg muscles. At 28 days of age, 55 female mice were sampled. Weekly samples were taken and testing was extended for 2 months on surviving animals. Total body x-rays were taken weekly to detect tumors. In addition, palpations for tumors were performed twice weekly.

STRONTIUM-90 STUDY GROUPS

Uninjected animals with minimal x-ray exposure. A control group of 15 female CF1/Anl mice from 77 to 127 days of age (mean age 97 days) were bled once a month for 3 months, then biweekly. Testing was continued for 8 months. Total body x-rays were taken at the beginning of testing, then once every 4 months.

Uninjected animals with maximal x-ray exposure. Fifteen female CF1/Anl mice from 77 to 127 days of age (mean age 97 days) were bled once a month for 3 months, then biweekly. The mice were exposed to total body x-rays at the time of each bleeding.

Strontium-90 injected animals. A test group of 30 female CF1/Anl mice from 77 to 127 days of age (mean age 97 days) were bled. The following day the mice were injected with strontium-90 (1 μCi/g body weight) via the tail vein. The mice were bled at 30 day intervals for 3 months, then biweekly. Total body x-rays were performed at each sampling to detect the presence of tumors.

SOURCE AND PREPARATION OF FBJ VIRUS

FBJ virus packed in dry ice was received from Argonne National Laboratory. The specimen was preserved in a REVCO freezer at -70° C.

At the time of mouse inoculations, specimen vials were agitated in a 37° C water bath for 5 minutes. A 1:3 dilution of the virus was made with sterile phosphate buffered saline and 0.1 ml of the solution was injected intraperitoneally via the leg muscles into newborn CFl/Anl mice.

Tumors were excised from dead or moribund animals. After weighing, tumors were ground into a fine paste, and Hank's Balanced Salt Solution added on a 1:1 (volume to weight) basis. The suspension was then made cell-free by differential centrifugation under refrigeration, using the following schedule (Argonne National Laboratory, 1970):

1) Tumor paste including virus - 10 minutes, 2,000 rpm (approximately 450 g)

2) Supernatant fluid - 15 minutes, 5,000 rpm (approximately 2,800 g)

3) Supernatant fluid - 30 minutes, 5,000 rpm (approximately 2,800 g)

4) Final supernatant fluid containing virus was stored at -70° C.

X-RAY PROCEDURE

Unanesthetized mice were attached in the supine position to a 3/4 inch thick plywood frame, containing a 4 x 5 inch cutout. Attachment was made with 1 inch padded alligator clamps fastened with wires to screws set in the frame. The axis of the mouse was aligned parallel

to the x-ray tube. Redipac Kodak AA-2 industrial type x-ray film was used (Argonne National Laboratory, 1970). Roentgenographs were taken with a portable Picker x-ray machine with filters removed. The following settings were used:

Small focus
KV - 34
MA - 65
Time exposure - 3/10 second
Distance from mouse to x-ray tube - 9 inches.

ALKALINE PHOSPHATASE ANALYSIS

Preparation of reagents and standardization of test. An alkaline buffer was prepared by dissolving 7.50 g glycine (anhydrous) and 0.995 g $MgCl_2$ or (0.203 g $MgCl_2.6H_2O$) in approximately 750 ml of distilled water. To bring the pH to 10.5, 85 ml of 1 N NaOH was added. The reagent was then diluted to 1,000 ml with distilled water and four drops of chloroform added. To make the buffered substrate, 0.2 g of p-nitrophenylphosphate was dissolved in about 40 ml of distilled water in a 100 ml volumetric flask. An alkaline buffer of 50 ml was added and the solution brought to 100 ml. After mixing, the substrate solution was tubed in 1 ml amounts. Substrate tubes were covered with parafilm and stored at -70° C. A stock standard was prepared by dissolving 1.3911 g of p-nitrophenol in a liter volumetric flask (10 mM/liter). The standard remained stable for 1 year at 4° C. To

make a working standard, 5.0 ml of the p-nitrophenol stock standard was pipetted into a 1-liter volumetric flask. The solution was then diluted to 1 liter. The working standard remained stable one day (Davidsohn and Wells, 1963).

The following dilutions were made to establish a standard curve:

Tube No.	Working Standard (ml)	Water (ml)	0.02N NaOH (ml)	IU/liter Alkaline Phosphatase
1	1	9	1.1	33
2	2	8	1.1	66
3	4	6	1.1	133
4	6	4	1.1	200
5	8	2	1.1	266
6	10	0	1.1	334

The absorbance of each of the above mixtures was read at 410 mμ, using 0.02 N NaOH in the reference tubes and the resulting values were plotted on a curve. Alkaline phosphatase values were read from the curve (Davidsohn and Wells, 1963).

Principle and performance of test. The phosphate group of p-nitrophenyl phosphate is split off by alkaline phosphatase at an alkaline pH to yield yellow-colored p-nitrophenol. The amount of p-nitrophenol released is directly proportional to the amount of alkaline phosphatase present in the plasma (Davidsohn and Wells, 1963). Results are reported in International Units (IU), defined as the number

of micro molecules of substrate hydrolyzed per minute by one liter of serum at 37° C (Richterich, 1969). To 1 ml of an alkaline buffered solution of p-nitrophenyl phosphate 0.05 ml of plasma was added (Sigma 104). After tubes were incubated at 37° C for 30 minutes, 10 ml of 0.02 N NaOH was added to inactivate the enzyme and dilute the substrate for reading. A Coleman 124 Spectrophotometer set at 410 mu was used to read the tests. Ten ml of NaOH was added to a substrate tube to serve as a reagent blank for each test. To each tube 0.1 ml of concentrated hydrochloric acid was added after the first reading to reduce p-nitrophenol to a colorless state for use as a serum color blank. A Versatol E control was run with each group of tests.

Results greater than 160 IU/liter were repeated using 0.025 ml of plasma. In some cases, in addition to diluting the plasma volume, it was necessary to reduce the incubation time to 10 minutes. Results were then multiplied times 6. Incubation time could be reduced since the alkaline phosphatase activity is linear (Richterich, 1969).

Isoenzyme differentation. Heat lability was used to tentatively identify alkaline phosphatase isoenzymes in 10 plasma specimens with elevated levels. The plasma was incubated at 55° C for 16 minutes, then cooled immediately. Results of the Bessey, Lowry, Brock analysis on the heat-treated specimens was compared with unheated specimens. A 90 to 100 percent loss of activity occurs in the human isoenzyme produced through osteoblastic activity (Stolbach, 1969).

RESULTS

A "t" test was used to determine the significance of mean differences in alkaline phosphatase levels of the various treatment groups. No pooled error was used, error being calculated separately for each group. The essential nature and variation of control animals were estimated. In both treatment and control groups, mean alkaline phosphatase levels over time were examined along with variation among individual animals.

Alkaline Phosphatase Levels in Uninjected Groups. The alkaline phosphatase levels of uninjected animals in the group beginning at 28 days of age showed a significant difference from animals beginning at mean age of 97 days. The mean alkaline phosphatase values of the younger animals dropped from 145 IU to 53 IU over a 12 week period, whereas the mean values for mice beginning at mean age of 97 days dropped from 65 IU to 34 IU over an 8 month period. Therefore, the two age groups were treated separately.

FBJ Virus Base Line Group. The weekly alkaline phosphatase values of non x-rayed and x-rayed animals without virus injection are given in Tables I and II, respectively. The alkaline phosphatase levels showed a large animal to animal variation in uninjected, non x-rayed animals starting at 28 days of age as illustrated in Table III. However, the variance dropped with age. Animal to animal variation

Table I. Weekly alkaline phosphatase levels in IU of uninjected mice beginning at 4 weeks of age.

Mouse Number	Week of Assay											
	1	2	3	4	5	6	7	8	9	10	11	12
1	114	147	80	95	64	88	90	66	49	47	48	55
2	148	177	132	125	91	111	130	76	80	74	68	56
3	129	102	133	115	79	77	75	75	58	55	56	27
4	122	205	103	94	66	57	77	80	67	56	58	65
5	193	242	187	237	122	102	120	107	104	51	65	100
6	141	176	150	139	90	91	114	85	78	87	70	76
7	147	176	87	114	90	100	133	85	93	61	53	32
8	146	127	133	120	102	127	102	83	86	80	77	31
9	175	166	133	131	112	81	56	75	82	66	65	41
10	117	62	143	152	140	129	174	145	113	63	65	57
11	201	252	77	137	127	132	110	71	85	77	75	60
12	137	122	99	113	88	72	97	53	63	51	51	43
13	192	134	126	104	117	100	75	90	100	82	81	88
14	114	101	101	107	65	65	124	61	39	47	47	57
15	147	126	110	127	79	87	90	79	53	26	58	42
16	135	90	84	106	82	72	58	71	63	66	63	57
17	118	151	94	114	69	103	75	66	75	55	57	48
18	144	138	91	82	82	76	107	48	32	49	52	43
19	128	65	120	79	60	54	55	47	49	47	38	46
20	146	119	46	122	65	74	90	72	73	73	63	45
21	145	98	110	105	70	84	77	74	79	72	57	47
22	138	145	104	109	86	108	73	81	81	75	54	47
23	145	116	105	129	78	54	103	54	41	49	47	55
24	186	194	127	103	109	81	90	96	79	67	70	57
25	156	213	140	107	104	121	106	106	88	58	64	51
26	186	133	122	100	80	107	87	87	70	59	50	61
27	161	195	102	119	79	92	107	86	83	54	70	56
28	109	224	133	115	105	93	86	81	67	60	58	29
29	154	122	108	101	84	84	68	70	59	59	57	52
30	149	113	103	100	77	87	79	79	80	69	43	63
31	123	253	94	85	109	117	82	95	77	70	61	49

TABLE I. (Continued)

Mouse Number	Week of Assay 1	2	3	4	5	6	7	8	9	10	11	12
32	93	153	125	92	90	80	71	--*	46	39	65	55
33	100	152	102	102	86	86	66	65	62	78	34	66
34	146	157	126	96	90	85	85	84	77	54	67	43
35	187	171	120	86	91	65	70	77	77	44	61	55
36	130	130	98	82	85	86	19	54	68	65	55	51
37	104	133	106	83	75	55	70	62	55	41	27	52
38	189	157	115	102	77	78	80	51	78	41	58	53

* Insufficient quantity of plasma available for testing.

TABLE II. Weekly alkaline phosphatase levels in IU of uninjected mice with x-ray exposure beginning at 4 weeks of age.

Mouse Number	Week of Assay 1	2	3	4	5	6	7	8	9	10	11	12
39	162	116	94	113	85	100	90	79	64	57	62	48
40	117	131	110	87	137	131	121	77	69	77	80	52
41	84	171	90	101	123	63	112	68	52	53	55	57
42	92	128	106	65	115	76	90	80	70	80	76	57
43	91	123	102	98	108	81	81	83	67	63	52	57
44	108	140	96	115	100	81	119	67	55	65	53	45
45	165	148	84	82	107	63	77	60	55	64	91	67
46	114	148	128	118	141	110	89	90	85	83	86	59
47	100	147	123	96	78	70	74	58	54	65	47	43
48	140	120	122	92	115	122	158	59	56	50	50	79

TABLE III. Pattern of mean alkaline phosphatase variance in 8 uninjected mice beginning at 4 weeks of age with no x-ray exposure.

Sampling Week	Mean	Variance
1	144	391
2	128	1,481
3	100	615
4	105	310
5	77	239
6	79	390
7	84	293
8	67	292
9	64	388
10	61	145
11	55	94
12	48	24

was greater in uninjected animals which received x-ray exposure (Table IV). A comparison of untreated animals without x-ray exposure and untreated animals with x-ray exposure showed significant differences in mean alkaline phosphatase values in 5 out of 12 assay periods (Table V).

FBJ Virus Injected Group. The tumor incidence of mice injected with FBJ virus was 100 percent. Osteosarcomas appeared from 18 to 98 days after injection, with the majority of tumors appearing on the 19th day. The tibia was the most frequent site of primary tumors, occurring as the first tumor in 47 percent of the test animals. Deaths were distributed throughout the testing period, the first death occurring on day one of testing. By the 13th day there was a mortality of 52 percent. At the last bleeding 5 animals remained alive.

Table VI contains alkaline phosphatase values for the 55 mice injected with FBJ virus at birth. A comparison of mean values beginning at 4 weeks of age and continuing over an 8 week period showed no significant difference in FBJ injected mice and uninjected, non x-rayed animals (Table VII). The mean alkaline phosphatase level of animals at the time of tumor appearance on roentgenographs was not significantly different from the values of uninjected non x-rayed animals at the same age.