



The effect of 7, 12-dimethylbenz(a)anthracene and 3-methylcholanthrene upon osteogenic sarcomas induced in mice by the FBJ virus
by Alice Louis Crouch Huston

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirement for the degree of
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Abstract:

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The virus was injected intraperitoneally into 36 hour or younger mice and DMBA or 3-MC was applied in an acetone solution at weekly intervals:to the skin on the backs of the animals. Weekly weights, latency and survival times, and the results of the histologic examination of the tumors, were recorded and used to determine any possible effect. Virus, chemical, and negative controls were also observed in the same manner.

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THE EFFECT OF 7,12-DIMETHYLBENZ(A)ANTHRACENE AND 3-METHYLCHOLANTHRENE
UPON OSTEOGENIC SARCOMAS INDUCED IN MICE BY THE FBJ VIRUS

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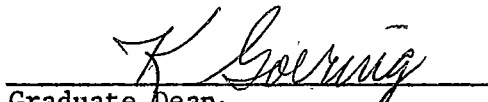
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ABSTRACT

An inhibitory effect produced by two chemical carcinogens upon murine leukemias induced by Friend and Rauscher viruses has previously been reported. To determine a probable explanation for this observation the effects of the same two carcinogens upon an osteosarcoma induced by FBJ virus were studied.

The virus was injected intraperitoneally into 36 hour or younger mice and DMBA or 3-MC was applied in an acetone solution at weekly intervals to the skin on the backs of the animals. Weekly weights, latency and survival times, and the results of the histologic examination of the tumors were recorded and used to determine any possible effect. Virus, chemical, and negative controls were also observed in the same manner.

INTRODUCTION

Ever since the carcinogenic effect of chemicals as well as viruses has been demonstrated, the interaction between the two has been studied. In nearly all in vivo studies the results have shown varying degrees of enhancement. However, in 1967, Fiscus et al. observed an inhibitory effect of the chemical carcinogens 7,12-dimethylbenz(a)anthracene (DMBA) and 3-methylcholanthrene (3-MC) upon splenomegaly of Friend and Rauscher viral leukemias. In addition to markedly reduced splenomegaly, prolonged latent periods and survival times were noted in the test animals. A single dose of Friend or Rauscher virus was injected intraperitoneally into adult female BALB/c mice. The chemicals were administered to one group by subcutaneous injections at weekly intervals and to the other by skin paintings, also at weekly intervals. In all test animals results indicated a significant inhibition of the murine leukemia, in contrast to the control animals.

Fiscus and his co-workers proposed four possible explanations for their results:

- (1) The carcinogen in some way interacted directly with the virus.
- (2) The leukemogenic process itself was inhibited.
- (3) The somatic growth, and thus the tumor growth, was inhibited.
- (4) The carcinogen produced a hormone-mimetic effect.

The first and the fourth possible explanations proposed by the investigators were at the same time tentatively eliminated by them.

The possibility of the carcinogens acting directly upon the virus was, at that time, an unlikely possibility since there was no available evidence to support such a mechanism. Since both DMBA and 3-MC are considered as having progesterone-mimetic effects, and since estradiol has been shown to inhibit splenomegaly, the possibility of hormone-mimetic effects was eliminated on the logical speculation that a progesterone-mimetic substance would enhance rather than inhibit the neoplasm.

In 1970, Elliot et al. reported results of a follow-up investigation conducted to discover a possible explanation for the results obtained by Fiscus et al. (1967). The test procedure was carried out in exactly the same manner with the exception that only Friend virus and DMBA were used. Splenectomy, weekly virus titers, and blood were studied. From the results obtained, Elliot et al. (1970) concluded that DMBA interferes with the direct neoplastic effect of the Friend virus rather than with indirect neoplastic effects as postulated earlier (Fiscus, 1967). It was concluded that, "In Friend virus leukemia, DMBA delays the neoplastic transformation of the reticulum cells in the spleen and suppresses their maturation to erythroblastic cells as shown by the absence of erythroblasts in the peripheral blood," (Elliot et al., 1970).

The effect of DMBA and 3-MC upon osteogenic sarcomas induced in mice by the Finkel-Biskis-Jenkins (FBJ) virus is the subject of this

paper. The project was undertaken to elicit a possible explanation for the results obtained by Fiscus et al. in 1967.

The FBJ virus, first isolated in 1966 by Finkel, Biskis, and Jenkins (1966), was chosen for this investigation for two reasons. First, it was selected because of its consistency in producing only malignant bone tumors (Kelloff et al., 1969; Finkel et al., 1966(a)). In conjunction with this, it provided a useful test system for this study since the target tissue, the periosteum, is entirely different from that of the Friend and Rauscher leukemia viruses.

The first attempt to show an interaction between a virus and a chemical carcinogen was made in 1923 by Teague and Goodpasture with Herpes simplex virus and tar. Since that time many investigations into cocarcinogenesis have been completed with different viruses, both oncogenic and non-oncogenic, and chemical carcinogens.

Teague and Goodpasture (1923), in the first known attempt to demonstrate interaction between a virus and a chemical carcinogen, painted the flanks of guinea pigs and rabbits with tar and simultaneously injected the Herpes simplex virus into the same area. In both guinea pigs and rabbits, severe herpetic lesions developed at the site of inoculation with tar and virus; eventual central nervous system infection and death of the animals occurred in 8 to 14 days. The Herpes simplex control animals, however, developed only microscopic lesions.

Rous and Kidd (1936, 1938) in a series of experiments demonstrated

a pronounced difference in the effect of Shope papilloma virus upon rabbits ears which had been previously painted with tar and ears which were untreated. Approximately three weeks after intravenous injection of the papilloma virus many neoplasms were evident on the tarred ears. The untarred ears, even though injected with virus, never developed lesions.

In 1938, Ahlstrom and Andrews investigated the interaction of tar with Shope fibroma virus. The tar was injected subcutaneously or intramuscularly and, at the same time, the fibroma virus was injected intraperitoneally, intravenously, or intracutaneously into domestic rabbits. The animals receiving the virus by the latter two routes in conjunction with the tar developed large neoplasms which eventually progressed to generalized fibromatosis and death. Similar results were observed when the virus was injected intraperitoneally and 3-methylcholanthrene and benzo(a)pyrene were used instead of tar.

Carr (1942) injected Rous sarcoma virus into the breast and 3-methylcholanthrene into the leg of resistant inbred chickens. Only small, slow-growing tumors developed in those receiving virus alone, while small neoplasms which regressed developed in those receiving the combination. In addition, swellings which turned to tumors and then regressed appeared in the legs of test birds injected with both the 3-MC and virus. No neoplasms developed in birds treated only with 3-MC.

Friedwald (1942) and Rous and Friedwald (1944) observed a ten-to one hundred-fold greater infective titer, shortened latent periods, accelerated neoplastic growth, and an increased number of neoplasms in rabbits which had received Shope papilloma virus and either tar or 3-methylcholanthrene by scarification of the skin. Benzene, a mixture of turpentine and acetone, combinations of 3-MC and scarlet red, and 3-MC and tar, X-rays, and ultraviolet light were also used in conjunction with the virus. All produced varying degrees of enhancement with 3-MC exhibiting the most dramatic effect and X-rays the least.

Virus activation by means of a chemical carcinogen was investigated by F. Duran-Reynals and Bryan (1952) using fowl pox virus and 3-MC. Naturally infected chickens were treated with 3-MC; malignant and benign neoplasms developed in the area of skin painting with the chemical. For up to fifteen months following initial treatment, the virus could be isolated from the neoplasms, and after this period could be reactivated by further paintings with 3-MC.

Shope fibroma virus was injected into the skin and testes of rabbits by Harel and Constantin (1954). The injection was followed by massive doses of cortisone and the development of large neoplasms, some of which were invasive, in 11 to 22 days.

In 1957, F. Duran-Reynals again studied the interaction between fowl pox virus and 3-MC. In this investigation the virus was inoculated into fifteen-day embryos of a breed of chickens in which no

fowl pox virus could be activated by 3-methylcholanthrene painting. Typical fowl pox lesions were evident when the birds hatched. The lesions regressed, and following regression fowl pox virus could no longer be isolated. Four months after tumor regression, these same birds were painted with 3-methylcholanthrene. Neoplasms developed at the site of skin painting and the virus was reisolated.

Imagawa, Yorkimori, and Adams in 1957 infected mice intranasally with influenza virus and intraperitoneally with urethane. A higher incidence of lung tumors was observed in those animals receiving both agents than in those receiving either agent alone.

F. Duran-Reynals (1957a) injected cortisone and vaccinia virus into areas of the skin of mice which had been previously painted with 3-MC. Ulcers formed in approximately seven days and healed leaving scar tissue. Later, in 66 per cent of the mice, neoplasms appeared at the site of scar tissue formation; one half of these became malignant.

M. L. Duran-Reynals (1961) demonstrated that tumor formation by vaccinia virus and 3-methylcholanthrene was more dependent upon the sequence of application rather than the quantity of either agent. When the chemical was applied after virus injection, incidence of tumor formation was much greater with a smaller dose than when it was applied before virus injection.

C57BL mice, which are known to have a low incidence of spontaneous

lung tumors, were used by Wisely et al. (1961) to determine the interaction of influenza virus with "ozonized gasoline," a chemical carcinogen. Significantly more lung tumors were observed in those mice injected with the virus and exposed to the carcinogen through inhalation than in any of the control animals.

Rowson et al. (1961) injected newly born C3H mice with polyoma virus and applied to the skin DMBA alone or followed by fifteen paintings with croton oil. Benzo(a)pyrene was also tested by painting animals injected with polyoma virus. The results indicated a substantial increase in the number of tumors and the rate of their development in the test animals.

Martin et al., (1961) tested the interaction of vaccinia with DMBA, 2-aminofluorene, and dibenz(a,h)anthracene. The results obtained showed a significant enhancement of tumor formation in all animals which received both the virus and a chemical.

West Nile virus in combination with skin painting with 3-MC or benzo(a)pyrene was investigated in 1962 by Tanaka and Southam. The virus was injected into mice previously painted three times with one of the chemicals. A series of seven additional skin paintings followed virus injection and resulted in the enhancement of tumor formation as manifested by shortened latent periods and increased numbers.

In 1963, Kotin and Wisely reported the interaction of influenza

virus with hydrocarbons, using the same procedure of virus injection and "ozonized gasoline" inhalation as before. The purpose of this second investigation was to observe pathologic changes brought about by the pulmonary tumor. The data substantiated the results of the earlier investigation of Wisely et al. in 1961. The pathologic studies showed development of squamous cell cancer exclusively in those animals receiving both agents.

Salaman and Roe (1964) reported work done by Chieco-Bianchi (1963) in which Graffi leukemogenic virus was injected into C57BL mice followed by four injections of urethane. The incidence of leukemia was much greater than the sum of the incidences in control groups receiving each agent alone. It is interesting to note that the investigators also tested mice first injected with urethane and secondly inoculated with the virus and observed no greater effect than the sum of the two agents alone.

In 1964, Martin reported experiments using vaccinia, Coxsackie B4, ECHO 9, and poliovirus 2 in combination with DMBA, 2-aminofluorene, and dibenz(a,h)anthracene. Following simultaneous injection with a single dose of virus and a subthreshold dose of one of the carcinogens, Swiss mice developed malignant lymphomas, granulocytic leukemias, a reticulum cell sarcoma, and a subcutaneous fibrosarcoma - malignant neoplasms which have never been reported to arise de novo in Swiss mice.

Hamberg and Svet-Moldavsky (1967) injected newborn hamsters simultaneously with 7,12-dimethylbenz(a)anthracene and Simian Virus 40. The results showed a marked stimulation of carcinogenesis in comparison with the control animals. The enhancement was manifested by a greater number of tumors and by considerably shortened latency periods.

Engle and Groupe (1969) injected Rous sarcoma virus subcutaneously into the wing web of four day old Leghorn chicks. Either 24 hours prior to or 24 or 72 hours following virus injection, a suspension of chemical carcinogen was injected into the breast muscle of the chicks. The six carcinogens used were 1,2,5,6,-dibenzanthracene, 9,10-dimethyl-1,2,-benzanthracene, 20-methylcholanthrene, DMBA, N-2-fluorenylacetamide, and benzo(a)pyrene. The results obtained indicated a greater incidence of tumor development and an increase in size of the tumors in birds tested with both virus and carcinogen.

Hook, Chirigos, and Chan (1969) investigated the cocarcinogenic interaction between murine sarcoma virus (Maloney) and Rauscher virus, two oncogenic viruses. Male BALB/c mice four to six week old were injected intraperitoneally with Rauscher virus and five days later were injected with murine sarcoma virus (Maloney) by the same route. Those animals receiving both viruses developed rapidly growing tumors with metastases and early death. These were contrasted to the controls in which tumor regression was observed.

One study has been completed which shows inhibition of the virus by a chemical carcinogen. Engle and Groupe (1969) reported an investigation conducted by DeSousa, Boyland, and Nery in 1965 in which the carcinogen N-hydroxyurethan interfered with tumorigenesis produced by Shope fibroma virus both in vivo and in vitro.

From the preceding survey of the literature it is apparent that a relationship exists between viruses and chemical carcinogens. The exact nature of this interaction has long been the subject of controversy and continues to remain so.

MATERIALS AND METHODS

TEST ANIMALS

The test animals used throughout this investigation were inbred CF1/ANL mice obtained from Argonne National Laboratory, Argonne, Illinois. The strain was developed at Argonne National Laboratory from the Carworth Farms #1 Strain (CF1).

The mice were weaned and males and females separated not earlier than 21 days and not later than 25 days after birth. The number of mice per cage after weaning and sex separation ranged from one to eight with an average of five per cage. Cage mates were at all times members of the same test or control group, and were often littermates.

The animals were fed Purina Mouse Chow pellets, and received water ad libitum.

VIRUS

The virus was obtained from Dr. C. A. Reilly, Jr. of Argonne National Laboratory in Argonne, Illinois. The specimen was preserved in a Revco freezer at -70°C . Pools of the virus were prepared by repeated mouse inoculations followed by excision and grinding of the resulting tumor. The pooling procedure follows.

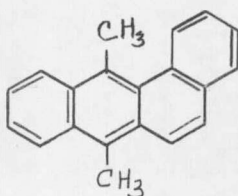
The frozen viral specimen was rapidly thawed by agitation in a 37°C water bath. A 1:3 dilution of the virus with cold sterile phosphate buffered saline was made and 0.1 ml of the resulting

suspension was injected intraperitoneally into newborn CF1/ANL mice (Reilly, 1970). When the animals began to waste due to neoplastic growth, they were sacrificed and their tumors removed. The tumors were ground into a fine paste with a mortar and pestle and Hank's balanced salt solution was added on a 1:1 (weight to volume) basis. The pooled preparation was made cell free by differential centrifugation in a refrigerated centrifuge (Reilly, 1970):

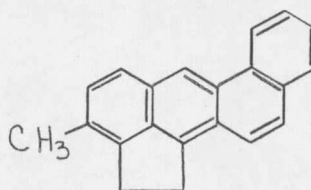
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| 1) ground tissue tumor including virus | 10 min. | 2000 rpm |
| 2) supernatant fluid | 15 min. | 5000 rpm |
| 3) supernatant fluid | 30 min. | 5000 rpm |

The final supernatant containing the virus was put into sterile rubber stoppered serum vials in 1.0 ml volumes and stored at -70°C .

CHEMICAL CARCINOGENS



7,12-Dimethylbenz(a)anthracene
(DMBA)



3-Methylcholanthrene
(3-MC)

The chemical carcinogens (illustrated above) were obtained from Eastman Organic Chemicals, Rochester 3, New York. They were prepared as needed for application to the skin by dissolving 0.05 grams in 12.5 ml of reagent grade acetone. The DMBA solution was maintained at $2-5^{\circ}\text{C}$ in a tightly stoppered bottle; the 3-MC solution, however,

was kept in a tightly stoppered bottle at room temperature because of its tendency to precipitate when stored at 2-5°C.

STUDY GROUPS

The mice were divided into ten test and control groups.

<u>Group No.</u>	<u>Treatment</u>	<u>No. of Animals</u>
1	DMBA + X-ray	13
2	Virus + X-ray	11
3	3-MC + X-ray	12
4	Virus + 3-MC + X-ray	10
5	Virus + DMBA + X-ray	11
6	Neg. Control + X-ray	10
7	Virus + DMBA	7
8	Neg. Control	9
9	Virus + 3-MC	12
10	Virus	9

METHODS TO STUDY THE INTERACTION OF FBJ VIRUS WITH DMBA AND 3-MC

Virus Injection: The frozen viral suspensions were quick thawed in a 37°C water bath. A two-fold dilution in phosphate buffered saline was made and 0.1 ml of the resulting viral suspension was injected intraperitoneally through the left hind leg muscles of 36 hours old or

younger CF1/ANL mice using a sterile 1.0 ml tuberculin syringe with a 25 gauge, 5/8 inch needle.

Chemical Application. A 1.0 ml tuberculin syringe with an 18 gauge needle was used to apply 0.05 ml of the DMBA or 3-MC solution (200 µgms of chemical) to the skin on the backs of 36 hour or younger mice. In those groups also receiving virus, the chemicals were applied immediately following injection of the virus. The painting of the chemicals was repeated at weekly intervals for 133 days.

As the mice began to develop a coat of hair, it became necessary to remove the hair from an area on the back each week. A commercial depilatory ("Nair") was used.

Animal Observations. All test and control animals were observed individually by number; they were numbered by clipping the appropriate toes (front feet, equalling ten through 100; hind feet, one through ten). Each animal was weighed beginning the day of virus injection and/or skin painting with one of the chemicals and following every week thereafter until the death of the mouse. The animals inoculated with virus were, in addition, palpated at two and three day intervals for the presence of tumor. The date of the first appearance of a neoplasm was recorded although palpation continued until the death of the mouse in order to determine the occurrence of additional tumors.

In addition to being weighed and palpated, all mice from groups one through six were x-rayed each week. Roentgenograms were taken of all mice inoculated with virus (groups 2, 4, and 5) to ascertain the earliest indications of tumor development. The negative control and chemically treated mice (groups 1, 3, and 6), however, were only shammed in order to save time and film costs.

A portable Picker x-ray machine with filters removed was used at the following settings:

small focus

KV 34

MA 65

Exposure time. 0.3 second

Distance (from mouse to x-ray tube) 9 inches

The unanesthetized mice were held in a supine position by one inch padded alligator clamps fastened with wires to screws set in a 3/4 inch thick plywood frame containing a 4 x 5 inch cutout. The axis of the mouse was aligned parallel to the x-ray tube. Redipac Kodak AA-2 industrial type x-ray film was used and the roentgenograms were developed by standard procedures immediately after exposure.

Histological Preparations. After the death of each mouse, all tumors were excised and fixed in 70-80 ml. of 10% formalin for not less than 24 hours or more than four weeks. Small central sections of the

tumors were removed and decalcified for 45 to 60 minutes in approximately 100 ml of RDO, a rapid bone decalcifier.* Immediately following decalcification the tumor sections were placed in metal crickets in a container of 10% formalin - the first container of the series in the Autotechnicon Model 2A, an automatic tissue processor. Upon completion of the processing, the tissues were removed, embedded in melted Paraplast Tissue Embedding Medium, and cooled in an ice bath. The blocks were cut with a microtome and the slide sections stained with hematoxylin-eosin for observation.

* Du Page Kinetic Laboratories, Inc., P.O. Box 416, Downers Grove, Illinois.

RESULTS

Tumor development in those animals infected with FBJ virus was identical to that described earlier by Finkel, Biskis, and Jenkins (1966), Finkel et al. (1966a), and by Kelloff et al. (1969). The tumors were palpably hard - the consistency of bone tissue - and nearly always located at or near the site of injection. In this investigation, with the exception of three (two in the pelvis and one in a humerus), all tumors developed in the bones of the hind legs and rib cage. One hundred per cent of the animals infected with virus developed at least one tumor.

In situ the tumors appeared as white compact masses varying considerably in size. Figure 1 illustrates the typical appearance of the neoplasms; the tumor is located on the internal surface of the ribcage of the mouse.

Three parameters were used to study the effect of DMBA and 3-MC upon osteosarcomas induced by the FBJ virus. These included latency and survival times of virus-infected mice, average weekly weights of all groups, and the microscopic appearance of the tumors.

Latency and survival times of virus-infected mice. Originally the x-ray procedure was developed for latency period determinations; however, it was evident by the third week that the earliest signs of tumor development were not detectable by this method. Thus the latency period was determined by palpation rather than by initial

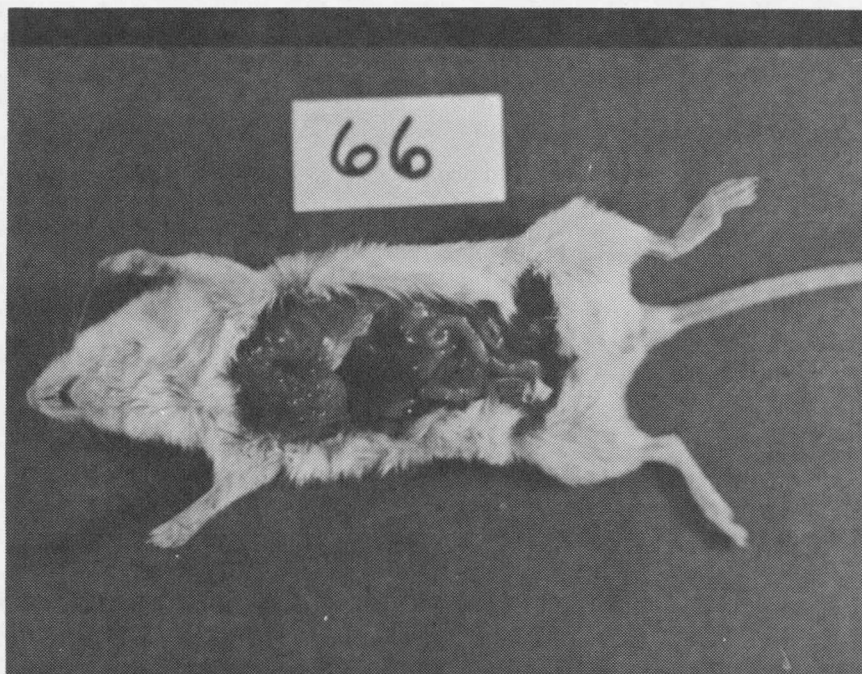


Figure 1. The in situ appearance of an osteosarcoma produced in the ribs by FBJ virus

tumor appearance in the roentgenogram.

Considerable variation in latency and survival times was observed among the mice in each group. Figures 2 through 7 illustrate the latency and survival time, in days, for each mouse infected with virus alone or infected with virus and painted with either DMBA or 3-MC. Tables 1 through 6 contain the data collected on each group in tabulated form. In addition to enumerating the location of each tumor, the day of initial tumor palpation, the day of death, and the number of days survived with the tumor(s) are listed for each mouse.

In order to discern possible significant differences among the groups, average latency and survival times were calculated for each. Figure 8 illustrates the relationships among the groups when the averages are compared and table 7 presents the numerical averages of latency times and survival times for each group as well as the average number of days survived.

Although there is some variation among the group averages, at a 5 per cent significance level (alpha level), as calculated using a maximum likelihood estimator in a chi-square test, there is no significant difference among any of the groups. Thus, from the results of the latency and survival times of the animals infected with virus and of those infected with virus and painted with DMBA or 3-MC, it was concluded that neither chemical had any effect upon the production of osteosarcomas by the FBJ virus.

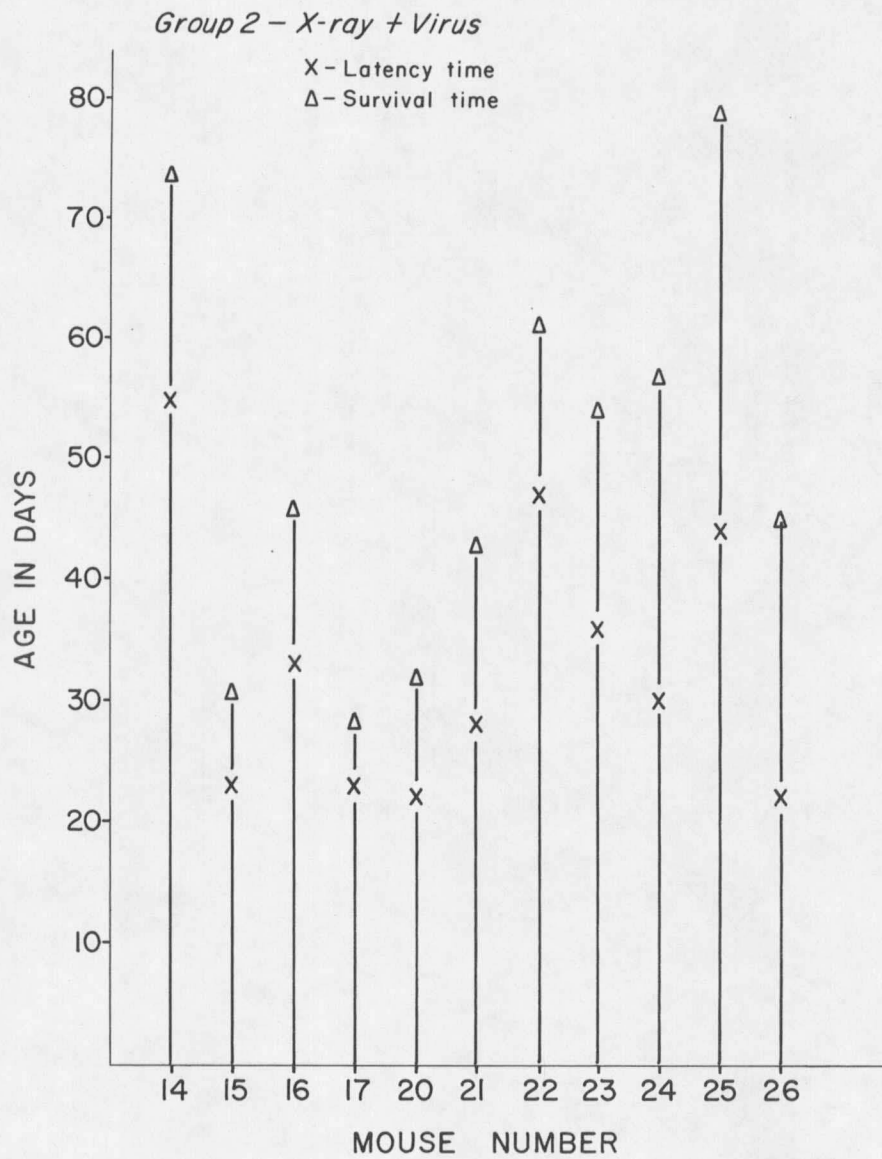


Figure 2. Distribution of latency and survival times of mice infected with virus and x-rayed (group 2)

Table 1. Latent and survival periods and location of tumors in mice infected with virus and x-rayed (group 2)

Mouse No.	Tumor Location	Day Palpated	Day Died	No. of Days Survived
14	Left hind leg; right ribs	55	74	19
15	Left hind leg	23	31	8
16	Left hind leg	33	46	13
17	Left hind leg	23	28	5
20	Left hind leg	22	32	10
21	Left hind leg*, sternum, left ribs	28	43	15
22	Left hind leg*, sternum, right ribs	47	61	14
23	Left hind leg*, entire rib cage	36	54	17
24	Left hind leg*, entire rib cage	30	57	27
25	Left ribs*(2)	44	79	35
26	Left hind leg	22	45	23

* Initial tumor

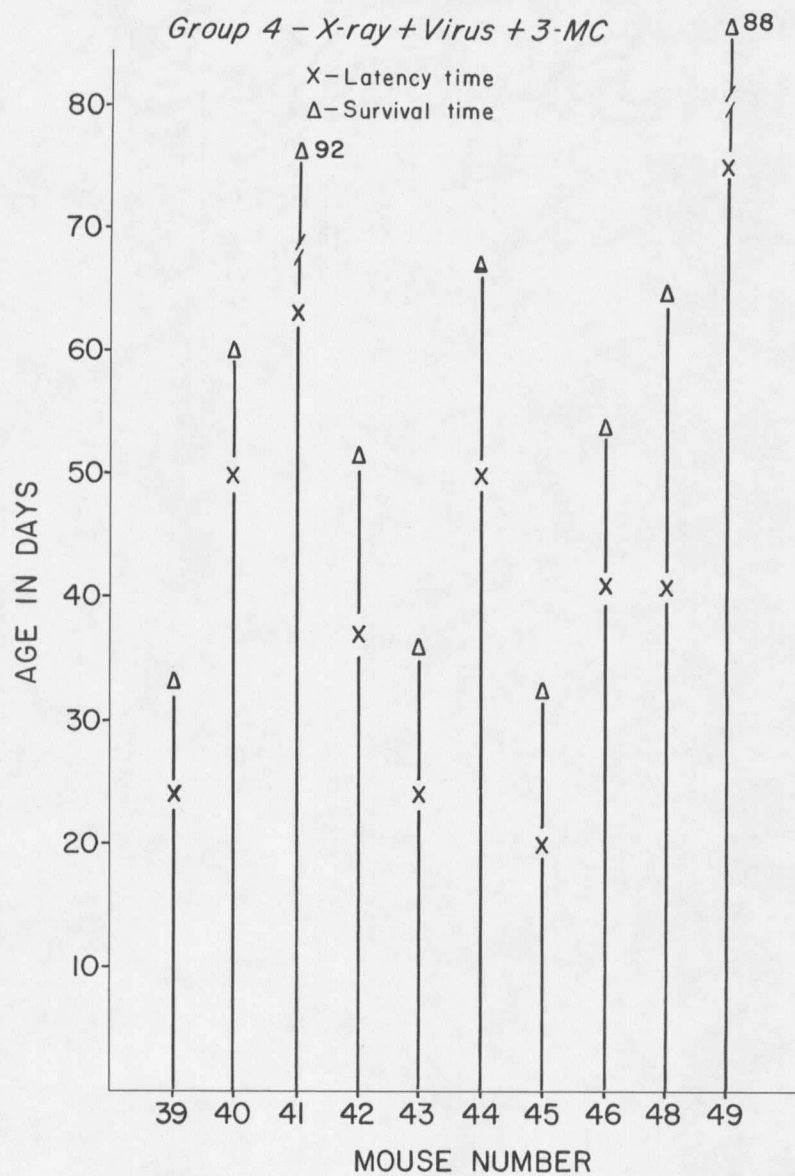


Figure 3. Distribution of latency and survival times of mice infected with virus, painted with 3-MC, and x-rayed (group 4)

Table 2. Latent and survival periods and location of tumors in mice infected with virus, painted with 3-MC, and x-rayed (group 4)

Mouse No.	Tumor Location	Day Palpated	Day Died	No. of Days Survived
39	Left hind leg	24	33	9
40	Left hind leg	50	60	10
41	Right ribs	63	92	29
42	Right ribs	37	52	15
43	Left hind leg	24	36	12
44	Right ribs*, left ribs left hind leg	50	67	17
45	Left hind leg	20	32	12
46	Left hind leg*, right ribs (3)	41	54	13
48	Left hind leg*, left ribs right ribs	41	65	24
49	Left hind leg	76	88	12

* Initial tumor

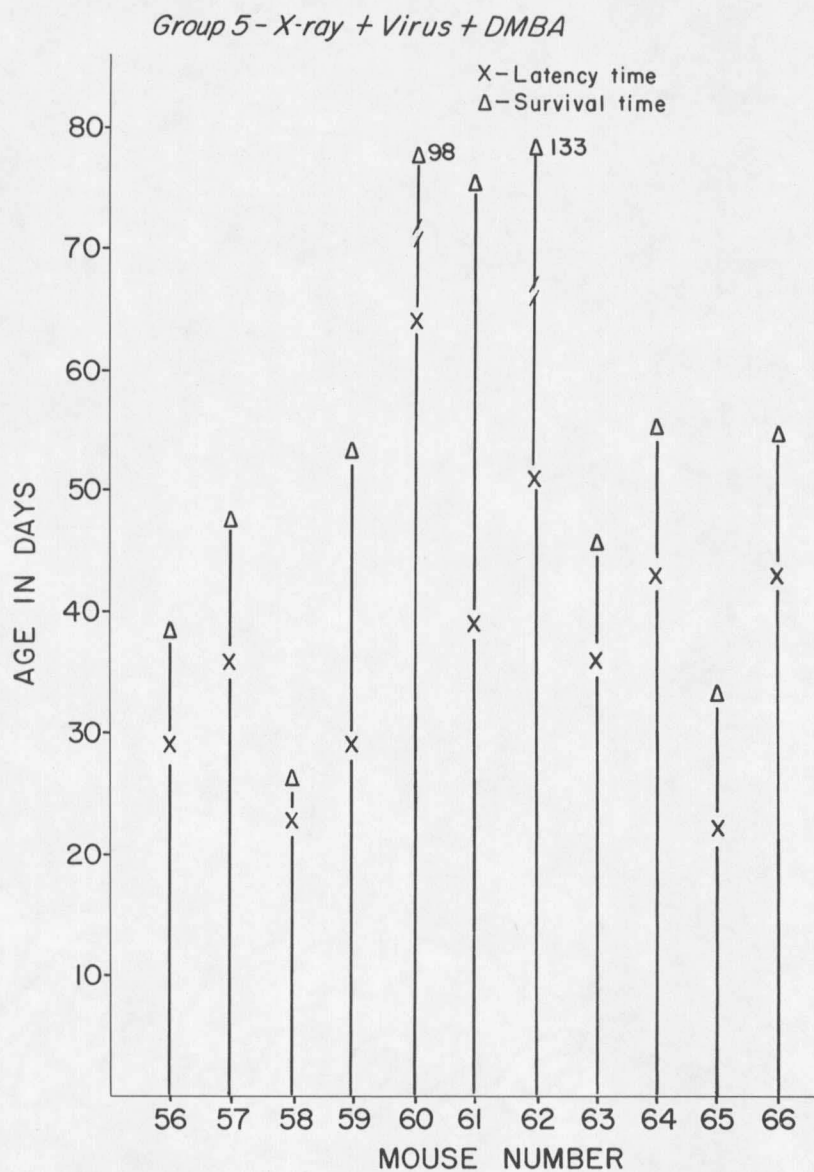


Figure 4. Distribution of latency and survival times of mice infected with virus, painted with DMBA, and x-rayed (group 5)

Table 3. Latent and survival periods and location of tumors in mice infected with virus, painted with DMBA, and x-rayed (group 5)

Mouse No.	Tumor Location	Day Palpated	Day Died	No. of Days Survived
56	Left hind leg	29	38	9
57	Left ribs	36	48	12
58	Left hind leg	23	26	3
59	Left hind leg*; left ribs (2), right ribs	29	54	25
60	Left ribs	65	98	33
61	Right ribs*, left ribs	39	76	37
62	Left hind leg	51	133	82
63	Left hind leg	36	46	10
64	Left hind leg	43	55	13
65	Left hind leg	22	33	11
66	Right ribs	43	55	13

* Initial tumor

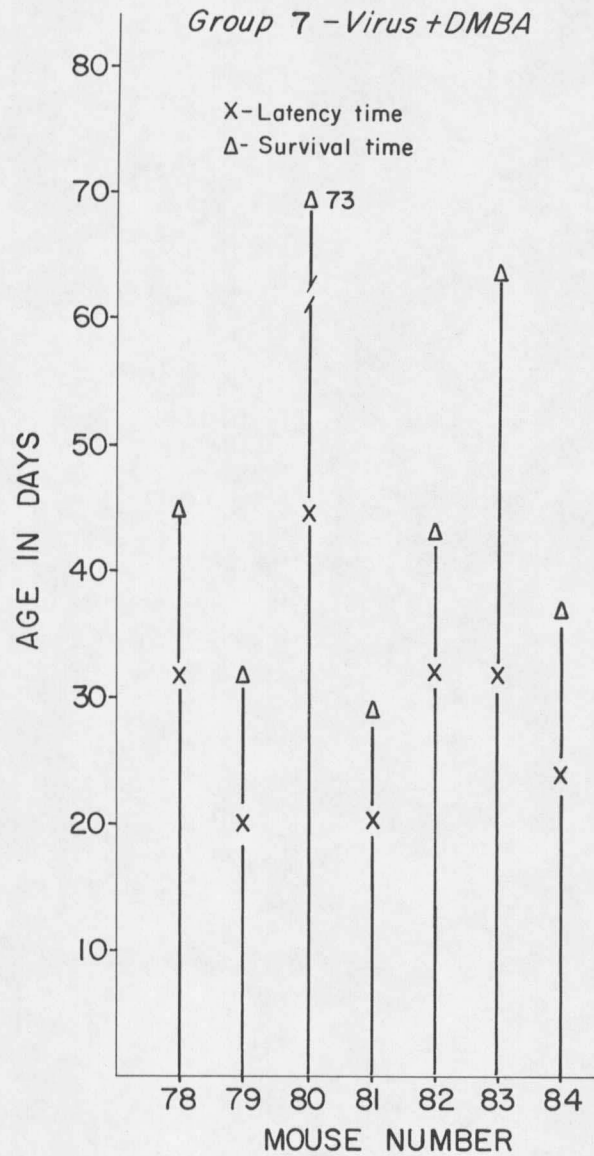


Figure 5. Distribution of latency and survival times of mice infected with virus and painted with DMBA (group 7)

Table 4. Latent and survival periods and location of tumors in mice infected with virus and painted with DMBA (group 7)

Mouse No.	Tumor Location	Day Palpated	Day Died	No. of Days Survived
78	Left hind leg	32	45	13
79	Left hind leg*, sternum	20	32	12
80	Right ribs*, left ribs (2) left femur	45	73	28
81	Left hind leg	20	29	9
82	Left hind leg*, left ribs	32	43	11
83	Left hind leg*, entire rib cage	32	64	32
84	Left hind leg	24	37	13

* Initial Tumor

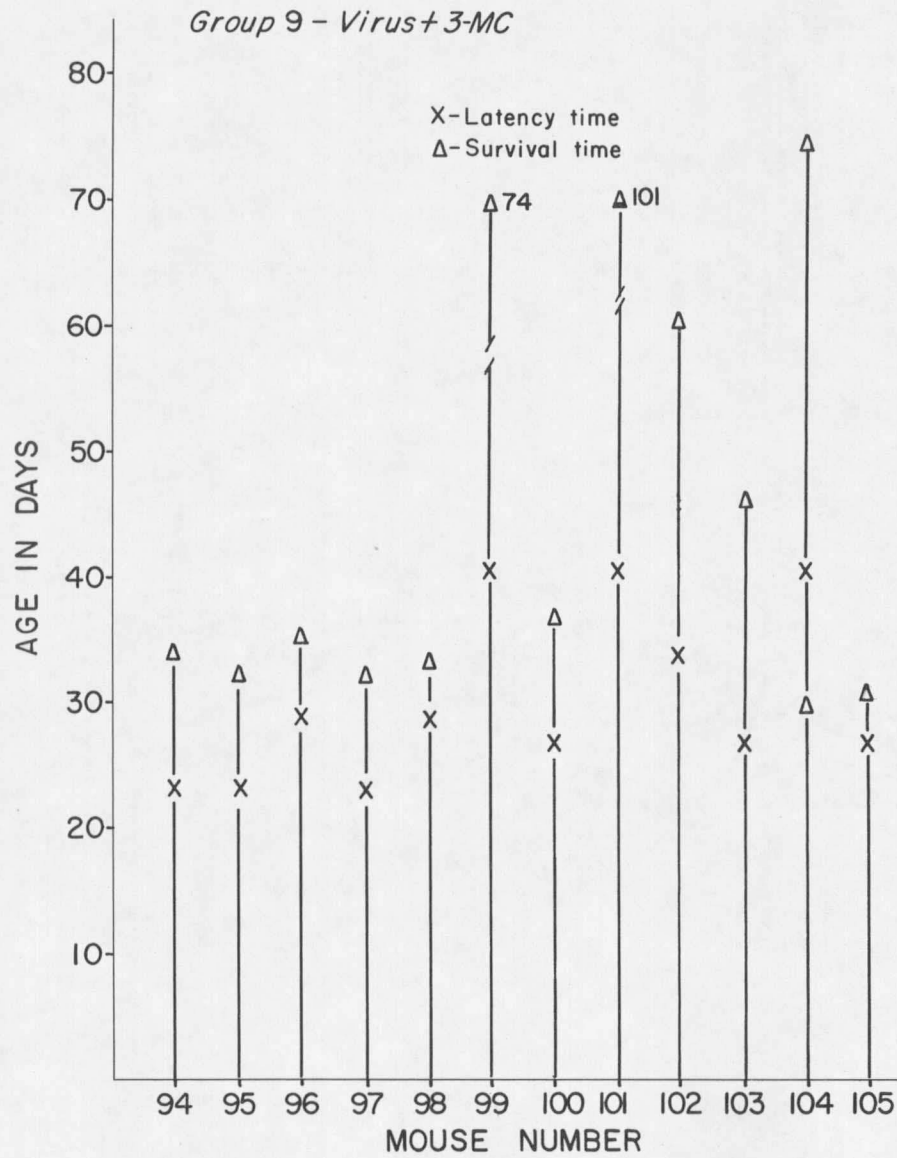


Figure 6. Distribution of latency and survival times of mice infected with virus and painted with 3-MC (group 9)

Table 5. Latent and survival periods and location of tumors in mice infected with virus and painted with 3-MC (group 9)

Mouse No.	Tumor Location	Day Palpated	Day Died	No. of Days Survived
94	Left hind leg*, left ribs left pelvis	23	34	11
95	Left hind leg	23	32	9
96	Left hind leg	29	35	6
97	Left hind leg	23	32	9
98	Left hind leg	29	33	4
99	Right ribs*, left ribs	41	74	33
100	Left hind leg*, right ribs left ribs	27	37	11
101	Right ribs	41	101	60
102	Left hind leg	34	60	26
103	Sternum*, entire rib cage	27	46	19
104	Sternum*, left ribs (2), right ribs	41	74	33
105	Left hind leg*, left ribs right ribs	27	30	3

* Initial tumor

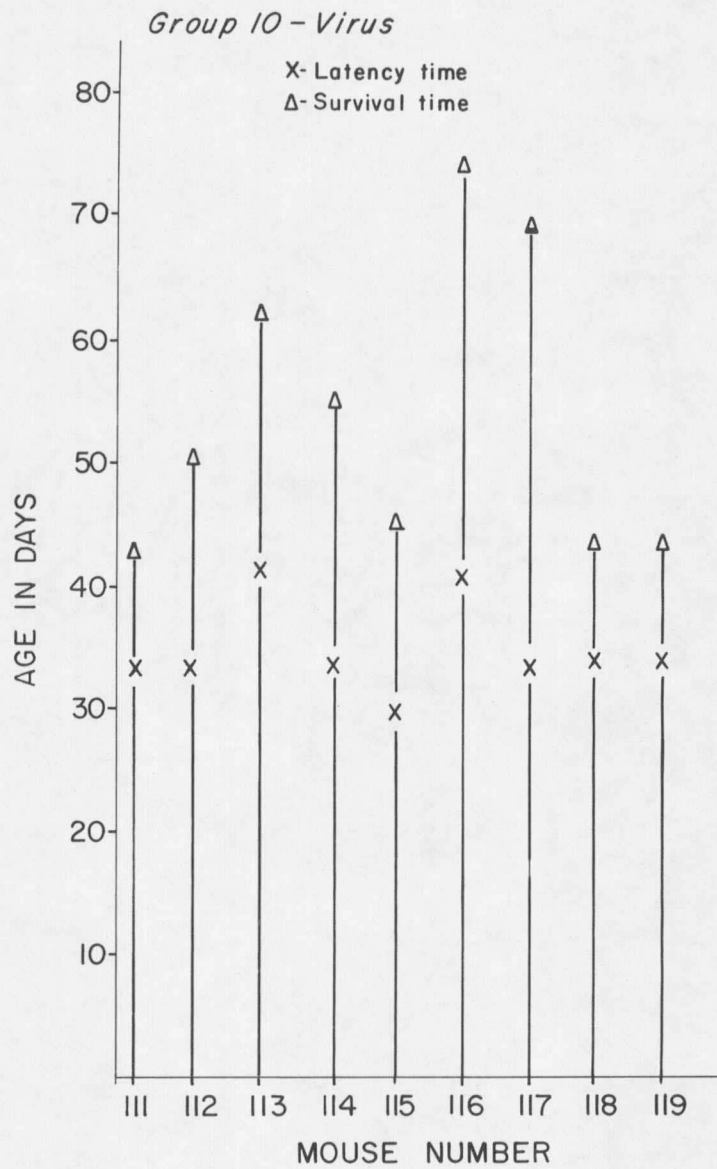


Figure 7. Distribution of latency and survival times of mice infected with virus (group 10)

Table 6. Latent and survival periods and location of tumors in mice infected with virus (group 10)

Mouse No.	Tumor Location	Day Palpated	Day Died	No. of Days Survived
111	Sternum	33	43	10
112	Right ribs*, left ribs	33	51	18
113	Right ribs* (2), right humerus	41	63	22
114	Sternum, left pelvis	3	56	23
115	Sternum	29	46	17
116	Right ribs*, left ribs	41	74	33
117	Sternum*, right ribs, left ribs	33	69	36
118	Left ribs*, right ribs	33	44	11
119	Left ribs	33	44	11

* Initial tumor

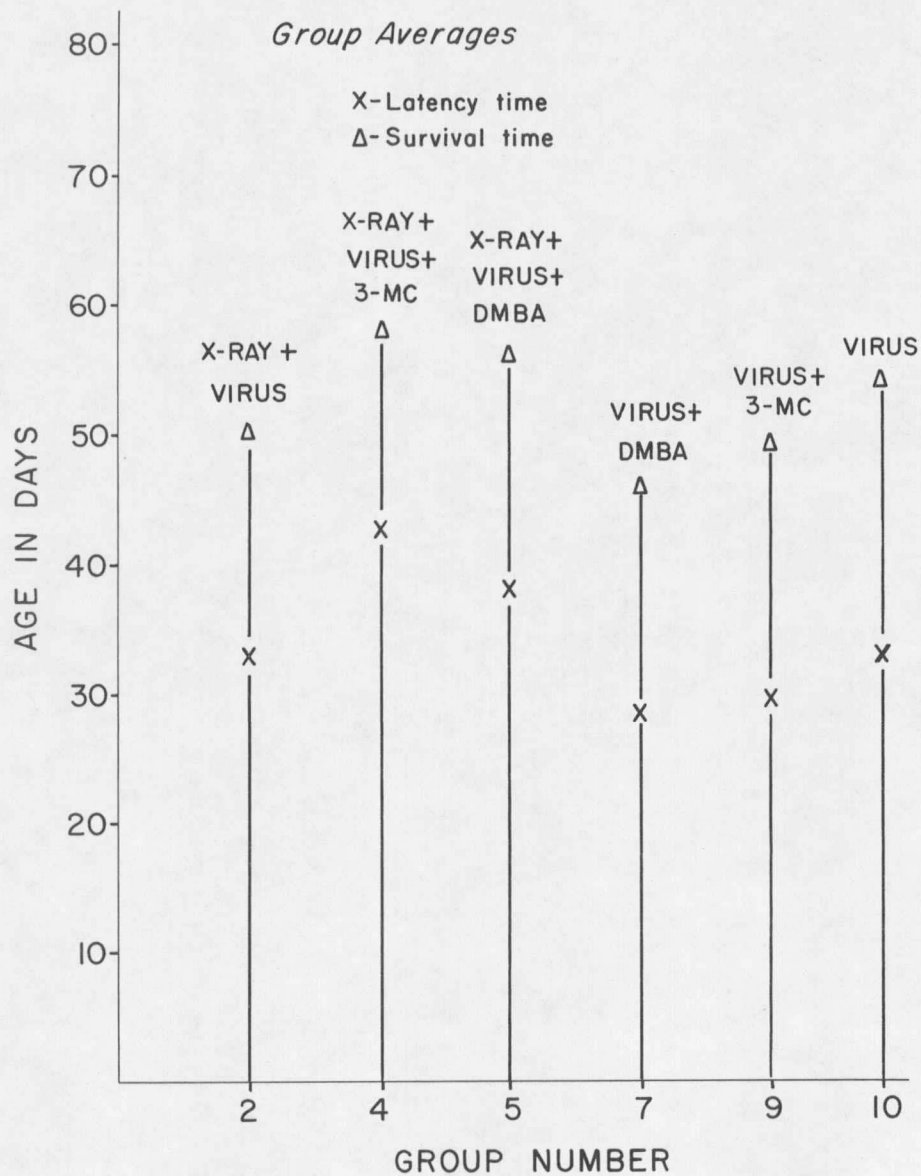


Figure 8. Distribution of the average latency and survival times for groups 2, 4, 5, 7, 9, and 10

Table 7. Average latent and survival periods for groups 2, 4, 5, 7, 9, and 10

Group No.	Test Conditions	Ave. Day Palpated	Ave. Day Died	Ave. No. of Days Survived
2	Virus + X-ray	33	50	17
4	Virus + 3-MC + X-ray	43	58	15
5	Virus + DMBA + X-ray	38	56	18
7	Virus + DMBA	29	46	17
9	Virus + 3-MC	30	49	19
10	Virus alone	34	54	20

Average weekly weights of all groups. This parameter was used to determine whether somatic growth of the mice was impaired by either DMBA or 3-MC. The weights of all the animals within each group were averaged for every week and the resulting values plotted. In addition to the averages, the maximum and minimum weights in each group were plotted. Figures 9 through 14 illustrate the plots for the first, second, third, fifth, seventh, and ninth weeks.

A trend is evident as the time lengthens. For the first three weeks (figures 9, 10, and 11) all the groups are approximately the same in average weight. By the fifth week (figure 12), however, two clusters begin to immerge which are well separated by week nine (figure 14). One cluster consists of the groups only injected with virus and those injected with virus and painted with one of the chemicals. The other cluster consists of the negative control groups and the groups receiving chemical only. The average weights of those groups infected with the virus (groups 2, 4, 5, 7, 9, and 10) are similar and, as would be expected, much lower than those of the negative control groups and the groups treated with a chemical only (1, 3, 6, and 8). The most significant aspect of this separation is that the weights of the latter groups are similar with no apparent difference between those painted with chemical and those receiving no treatment.

Thus, it was concluded that neither DMBA nor 3-MC affected the

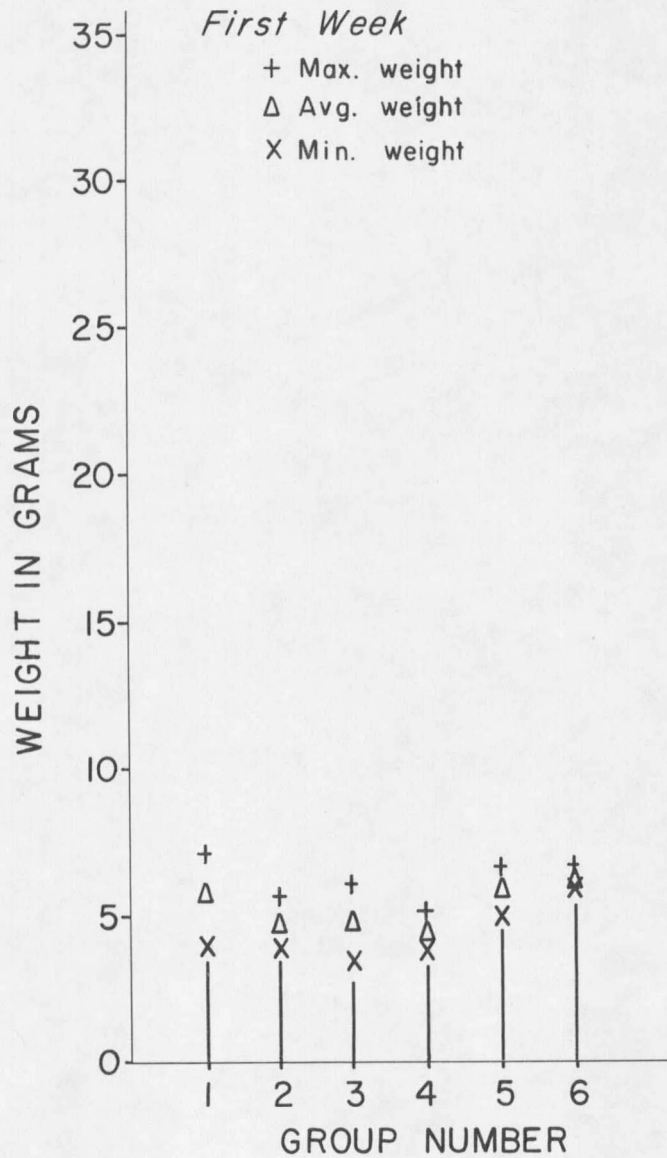


Figure 9. Distribution of the maximum, minimum, and average weights of all test and control groups at one week of age

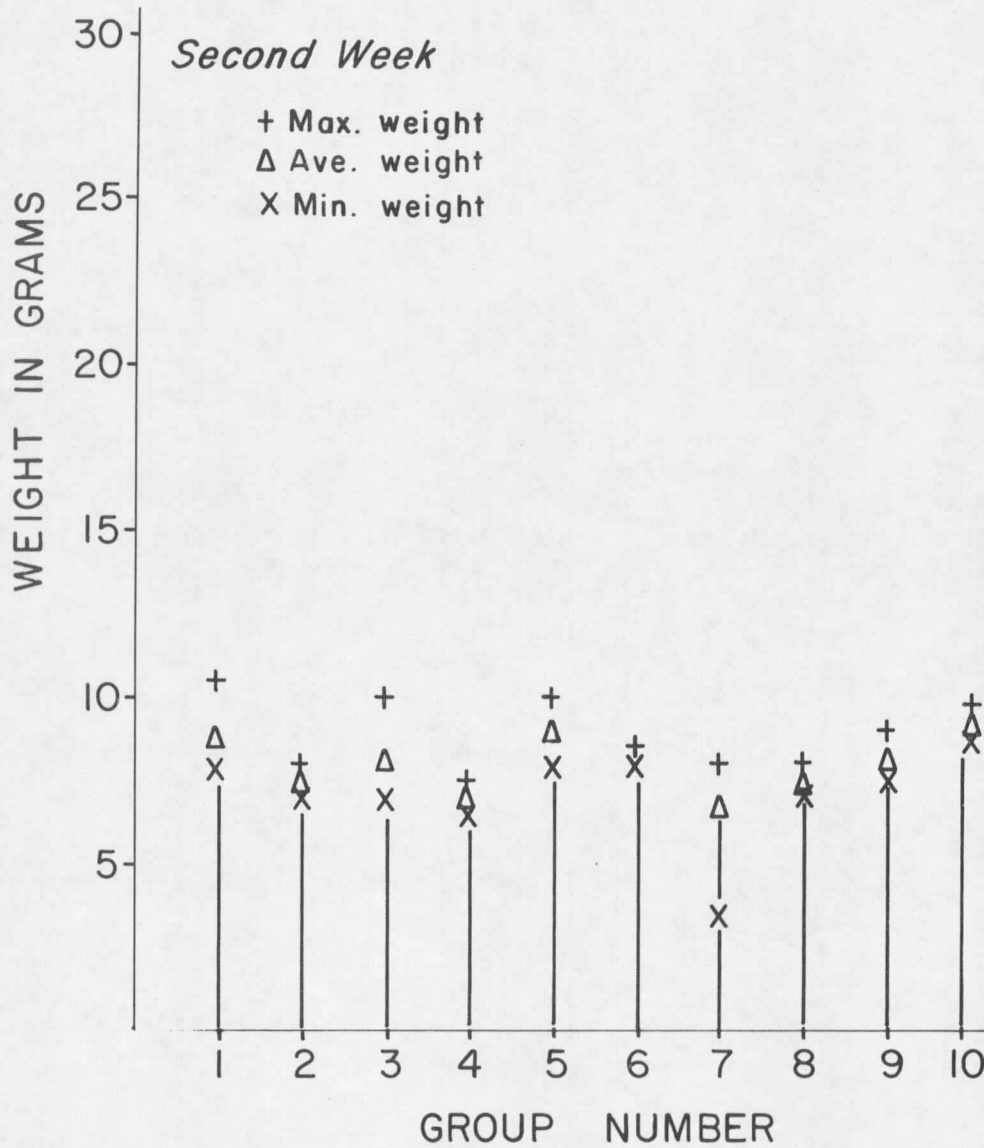


Figure 10. Distribution of the maximum, minimum, and average weights of all test and control groups at 2 weeks of age

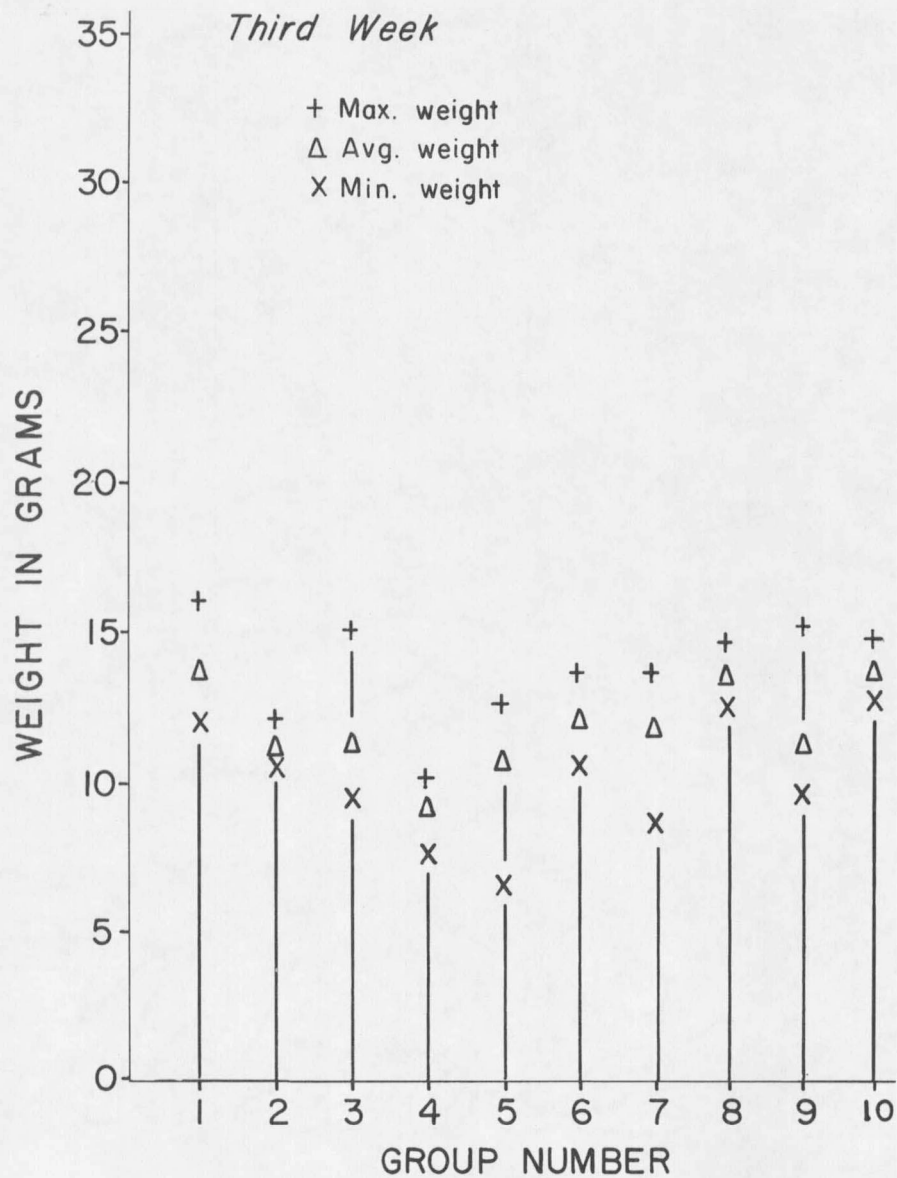


Figure 11. Distribution of the maximum, minimum, and average weights of all test and control groups at 3 weeks of age

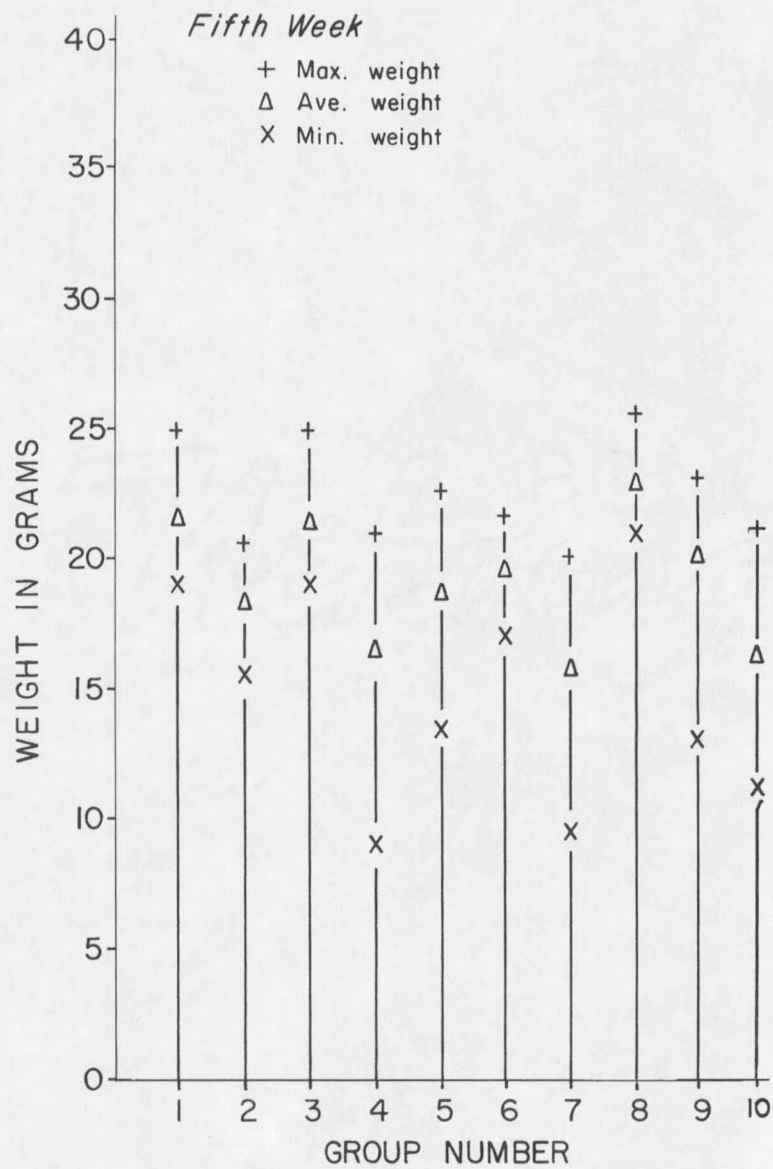


Figure 12. Distribution of the maximum, minimum, and average weights of all test and control groups at 5 weeks of age

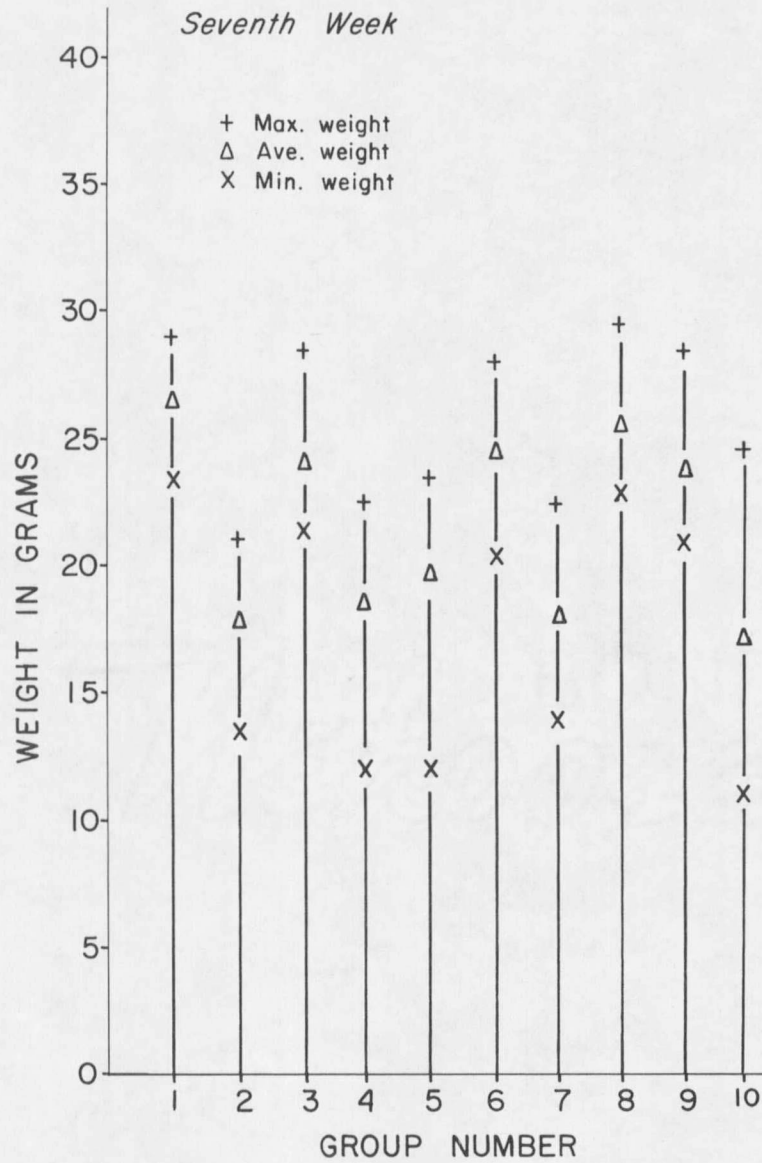


Figure 13. Distribution of the maximum, minimum, and average weights of all test and control groups at 7 weeks of age

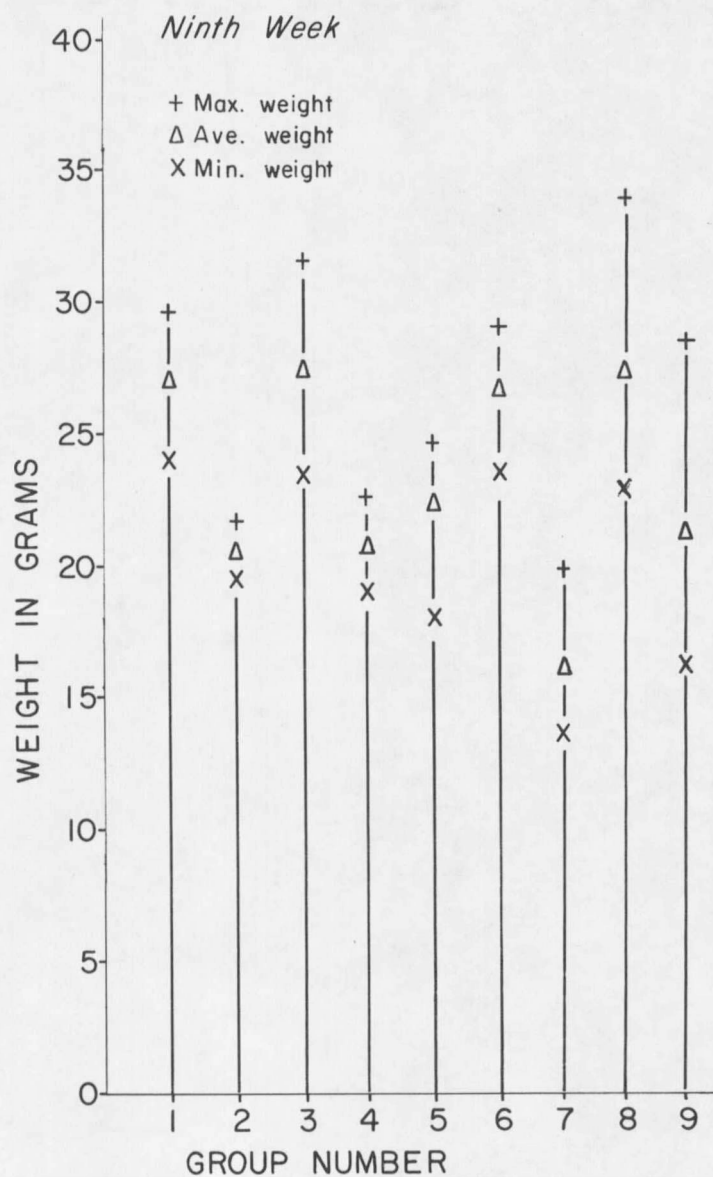


Figure 14. Distribution of the maximum, minimum, and average weights of all test and control groups at 9 weeks of age

somatic growth.

Microscopic appearance of the osteosarcomas. Tumors were histologically examined from 21 of the 60 mice which had been either infected solely with FBJ virus or painted with DMBA or 3-MC in addition to being infected with the virus (groups 2, 4, 5, 7, 9, and 10). Microscopically there was no apparent difference among the groups. Although considerable variation between sarcomas was observed, no pattern of differences could be established.

The composition of the tumors varied from simple spindle shaped cells, some producing osteoid, and no cortical involvement to more differentiated neoplastic osteoblasts with extreme cortical involvement and areas of calcification. In general, the older tumors displayed the greater differentiation just discussed. However, representatives of the spindle cell and well differentiated types, as well as of numerous gradations in between were found in tumors of all ages.

Figures 15 through 18 illustrate various types of growth observed; all are sections of tumors occurring in the bones of the hind legs. Figure 15 is a spindle cell neoplasm which has invaded the skeletal muscle, while figure 16 is a representative of a well differentiated tumor displaying large neoplastic osteoblasts. The feathered cortex of a differentiated neoplasm is shown in figure 17, and a representative of tumors with areas of calcification is shown in figure 18.

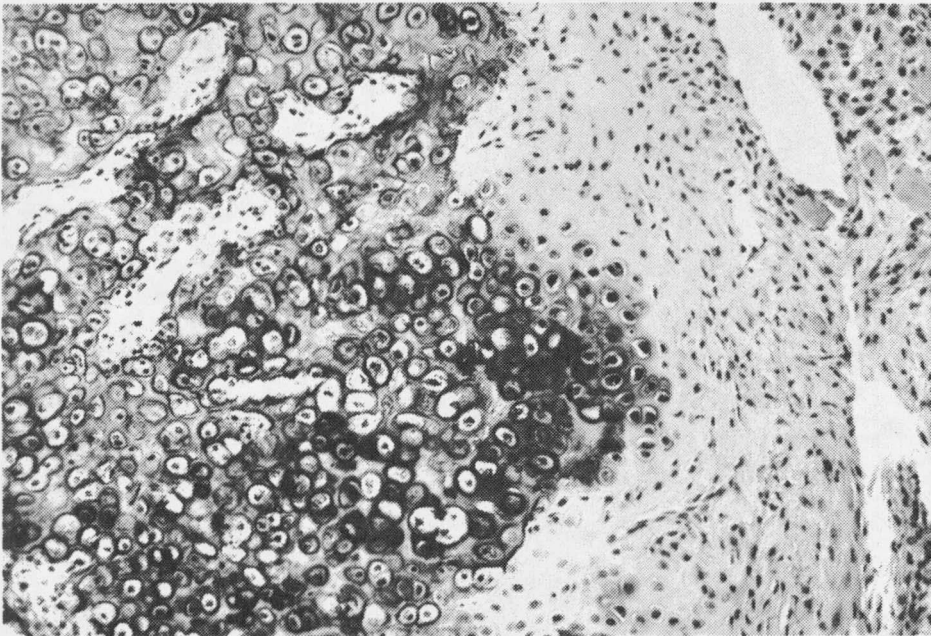
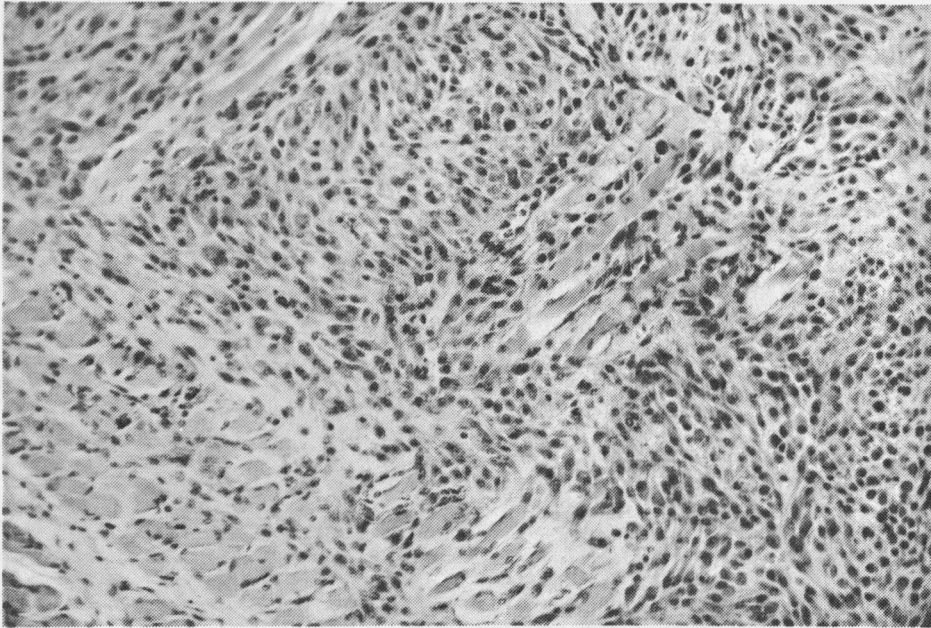


Figure 15. Invasion of the skeletal muscle by spindle cells of an osteosarcoma (200x)

Figure 16. Large neoplastic osteoblasts of a well differentiated osteosarcoma (200x)

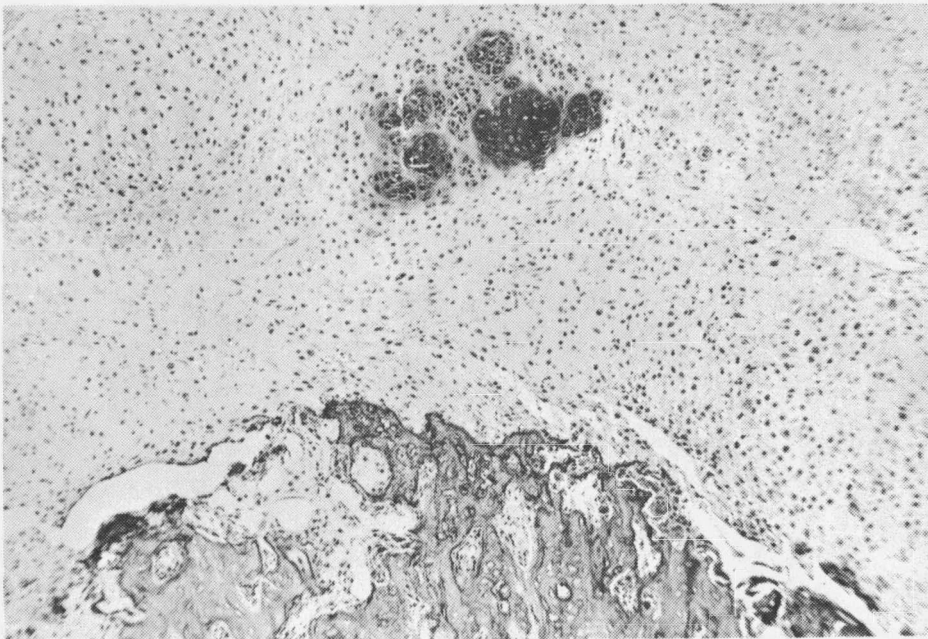
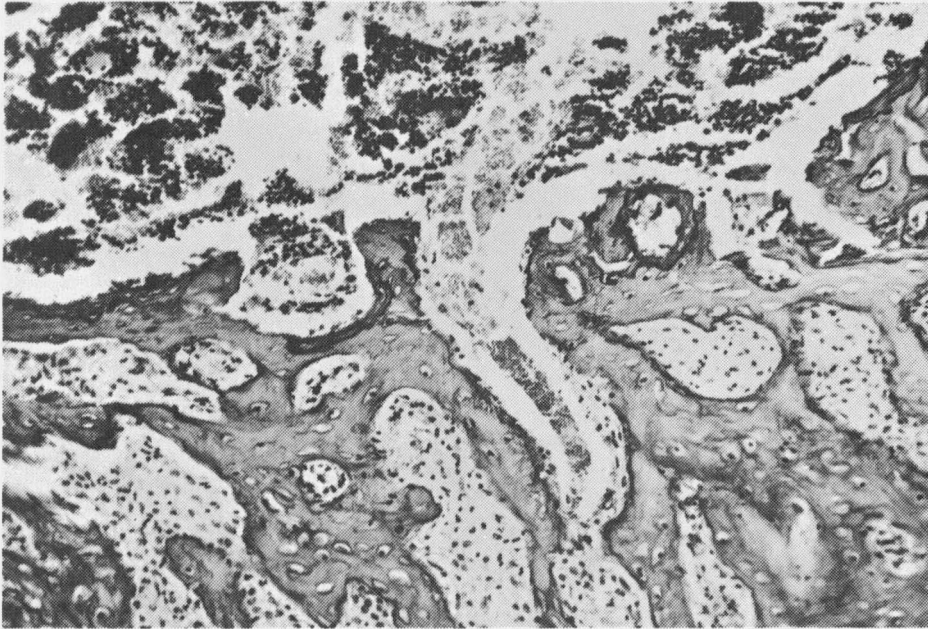


Figure 17. The irregular lace-like appearance of the cortex often seen in the osteosarcomas (200x)

Figure 18. A small area of calcification located at a distance from the thickened irregular cortex (80x)

From the microscopic examination of the neoplasms induced by the FBJ virus, the author has concluded that neither DMBA nor 3-MC produced an effect upon the virus or the osteosarcomas that it produced.

DISCUSSION

This investigation concurs with the conclusion of Elliot et al (1970) that the inhibitory effect of DMBA on Friend virus leukemia observed by Fiscus et al (1967) was due to a direct effect of the carcinogen upon the leukemic process itself and not due to indirect non-specific effects. It also indicates the same possible explanation for the inhibitory effects of DMBA on Rauscher virus leukemia and of 3-MC on both Rauscher and Friend virus leukemias.

Four hypotheses were proposed by Fiscus and his co-workers to explain the inhibition of the two murine leukemias by the carcinogens. The first was that the carcinogens acted directly on the viruses. The second hypothesis was that the neoplastic aspect itself was inhibited. Inhibition of somatic growth and thus tumor growth was the third, and production of hormone-mimetic effects by the chemicals, the fourth.

The first hypothesis, that of specific virus-chemical interaction, was eliminated by Fiscus et al. (1967) due to a lack of supporting evidence. However, several investigations into the inhibition of viral replication by chemical carcinogens have been done. DeMaeyer and DeMaeyer-Guignard (1964) studied the in vitro effect of two chemical carcinogens upon viral replication. Although the results showed that DMBA and benzo(a)pyrene inhibited replication of herpes and vaccinia, two DNA viruses; the RNA virus tested, sindbis, was not affected. The production of another DNA virus, Shope fibroma virus, has been shown to be inhibited by the carcinogen N-hydroxyurethan both in vitro

and in vivo (DeSausa et al., 1965). In view of these findings it is believed that the hypothesis of direct action on the viruses by DMBA and 3-MC is plausible. However, it should be noted that the available information concerning virus inhibition by chemical carcinogens pertains to DNA viruses. Because there was no effect by DMBA or 3-MC upon the incidence of osteosarcomas produced by the FBJ virus, an RNA virus; it is hypothesized that the chemicals did not affect viral reproduction.

The fourth hypothesis, that of the chemicals producing hormone-mimetic effects, was eliminated by Fiscus and his co-workers for reasons discussed previously. This possible explanation was not studied further and will therefore not be discussed.

The results obtained from the study of the average weekly weights of all the groups (virus infected, treated and untreated with chemicals; chemical controls; and negative controls) indicated no difference in weight between those groups only painted with chemical and the negative controls. Since somatic growth is a normal occurrence and a function entirely removed from both neoplasms, it would be expected that, if the chemical carcinogens inhibited this process and thus, indirectly, the growth of the neoplasm, they would also inhibit the somatic growth when no neoplasm was present. However, such was not the case; no inhibition of somatic growth occurred in the animals only painted with DMBA or 3-MC. Therefore, the hypothesis

that the inhibition of the leukemias which occurred (Fiscus et al., 1967) was due to the inhibition of the somatic growth should be eliminated.

The results obtained from the study of the FBJ virus in combination with both DMBA and 3-MC indicate that neither chemical produced any effect upon the latency and survival times of mice infected with the virus. These are in contrast to the extended latency and survival periods observed by Fiscus et al. (1967) in the leukemias produced by the Friend and Rauscher viruses in combination with the two chemical carcinogens. Therefore these results support the hypothesis that the antagonistic effect was caused by inhibition of the neoplastic aspect itself and concur with the direct evidence produced by Elliot et al. (1970). The target of the FBJ virus is bone tissue. If the latency and survival times for mice infected with FBJ virus had been extended by DMBA or 3-MC, this hypothesis would have required further investigation to determine if the chemicals were coincidentally specific for bone cells also or if some other factors were involved.

The histologic examination of the osteosarcomas induced by the FBJ virus both in animals painted with a chemical carcinogen and in animals not painted supports the hypothesis that the inhibition was in the leukemic process itself. The sections were examined to determine the occurrence of unusual cells or cell conditions in the tumors from mice infected with virus and painted with a chemical

which were not found in tumors from mice infected with virus only. No such differences were seen.

In addition to the qualitative aspect of the microscopic examination, the sections were evaluated on a semi-quantitative basis with respect to time. Sections representing various time intervals (9, 13, 25, 33 days) were examined from each of the test and control groups. Although considerable variation was observed, no distinction among groups could be made on the basis of tumor differentiation in relation to time. The variation found among the tumors has been noted by earlier investigators (Kelloff et al., 1969; Finkel and Biskis, 1969; Finkel et al., 1966) and, therefore, was not unexpected.

These findings support the hypothesis that specific inhibition of the leukemic process occurred since, if the inhibition was instead due to non-specific cellular action by the chemicals it seems reasonable to assume that the bone cells would also be involved. These results also support the observations made by Elliott et al. (1970). Considerable difference was evident between the spleens of the animals infected with Friend virus and painted with DMBA and those only infected with Friend virus. It was this observation which led to the conclusion that the inhibition by DMBA on the murine virus-induced leukemia was due to an initial delay in the neoplastic transformation of the reticulum cells and a much slower infiltration of the spleen thereafter (Elliott et al., 1970).

It should be noted at this point that the disease process produced by the Friend and Rauscher viruses has two aspects - the neoplastic reticulum cell aspect just discussed and the erythroblastotic aspect which causes the severe splenomegaly characteristic of the two viral diseases. Fiscus et al., (1967) hypothesized originally that it was the prevention of the splenic erythroblastosis aspect of the disease which was responsible for the inhibition. Elliot et al., (1970), however, proposed instead that the inhibition was due to the inhibition of the neoplastic reticulum cell aspect because of the discovery that the erythroblasts which engorge the spleen are descendants from the virus-transformed reticulum cell and not the erythroblasts of normal splenic erythropoiesis as originally thought.

The results of this investigation have been discussed with the assumption that the painted chemicals reached the site of viral action - the periosteum and cortex of the bone. Since painting of the mice began when the animals were 36 hours old or younger (while the bone tissue was still relatively soft and rapidly metabolizing) it would seem logical that the chemicals were able to reach the target cells,

The basic problem of this investigation was to determine whether the results obtained by Fiscus et al. (1967) were specific or non-specific for the reticulum cell sarcomas produced by the Friend and Rauscher viruses. By studying the effect of the same chemical carcino-

gens, DMBA and 3-MC, upon a virus (FBJ virus) which affects a tissue or cell type completely different from the reticulum cells affected by the leukemia viruses, it was felt the question could be answered. If the effect observed was non-specific, it seems reasonable to assume that an inhibition of the osteosarcoma would have occurred. However, since the bone tumors were not affected by either DMBA or 3-MC, it is postulated that the antagonistic effect of the two polycyclic aromatic hydrocarbons on the murine leukemias produced by Friend and Rauscher viruses (Fiscus et al., 1967) was a specific interaction between the chemicals and the neoplastic cells.

SUMMARY

The most probable explanation of the inhibitory effect of DMBA and 3-MC upon leukemias induced in mice by the Friend and Rauscher viruses (Fiscus et al., 1967) has been determined by studying the effect of the same carcinogens upon a different tumor tissue, an osteosarcoma, induced by the FBJ virus.

Weekly skin paintings of the mice infected with the virus failed to produce any effect upon the incidence of tumors, latency and survival times, or the microscopic appearance of the tumors. Weekly skin paintings of mice not infected with virus failed to inhibit their general growth. It was therefore concluded that the antagonistic effect of the polycyclic aromatic hydrocarbons upon the two murine leukemias was a specific effect upon the leukemic process itself.

Reasoning leading to this conclusion and supportive evidence for it are discussed.

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