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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Date *August 12, 1971*

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

by

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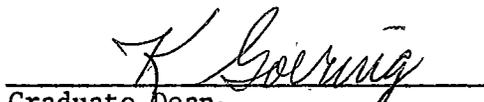fulfillment of the requirement for the degree

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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 $\underline{in vivo}$ studies the results have shown varying degrees of enhancement. However, in 1967, Fiscus $\underline{et al.}$ observed an inhibitory effect of the chemical carcinogens 7,12-dimethyl-benz(a)anthracene (DMBA) and 3-methylcholanthrene (3-MC) upon spleno-megaly 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 possiblity 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 invest-
igation 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) con-
cluded 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-Jinkins (FBJ) virus is the subject of this

paper. The project was undertaken to ellicit a possible explanation

for the results obtained by Fiscus et al. in 1967.

The FBJ virus, first isolated in 1966 by Finkel, Biskis, and

Jinkins (1966), was chosen for this investigation for two reasons.

First, it was selected because of its consistancy 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 simultan-

eously 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 micro-

scopic 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 inter-
action 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 subthreshhold 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 carcinoginesis 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 interferred 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 centri-
fugation in a refrigerated centrifuge (Reilly, 1970):

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
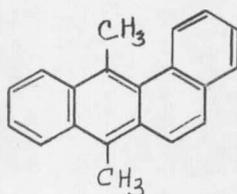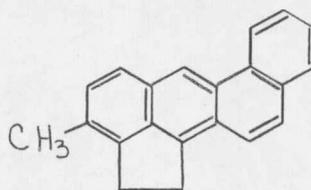
7,12-Dimethylbenz(a)anthracene   3-Methylcholanthrene
           (DMBA)   (3-MC)

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°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.

Group No.	Treatment	No. of Animals
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 alligned parallel to the x-ray tube. Redipac Kodax 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 approx-
imately 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.

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* 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 Jinkins
(1966), Finkel et al. (1966a), and by Kelloff et al. (1969). The
tumors were palapably 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 con-
siderably 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