The response of congenitally athymic (nude) mice to the chemical carcinogen 7, 12-dimethylbenz (a) anthracene
by Eustace Arnold Johnson

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
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Abstract:
Considerable evidence supports the theory that a thymus-dependent immunological surveillance mechanism is important in tumor prevention. To this end we expected the congenitally athymic (nude) mouse to have many spontaneous tumors. We have not observed spontaneous tumors in the nude mice in our colony. Because the nude mice have a short lifespan, we thought that maybe the mice died before spontaneous tumors had time to develop. Therefore, we attempted to induce tumors in nude mice and their phenotypically normal littermates with the carcinogen 7, 12-dimethylbenz(a) anthracene. In every experiment each normal littermate developed papillomas and none of the nudes developed a papilloma. We do not believe this result was due to the hairless condition of the nude mice as we successively induced papillomas with the same carcinogen in the hairless (hr/hr) mutant. We then considered that some thymic influence might be required for the production of some types of tumors. To test this idea, nude mice (NU), nudes implanted with thymus glands (Nu-tg), nudes injected with thymus cells (Nu-tu), and phenotypically normal littermates (NLM) were treated with 7, 12-dimethylbenz(a)anthracene and then croton oil as a promoter. All of the NLM, Nu-tg, and Nu-tc developed tumors; only one of 21 Nu mice developed a tumor.

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Date *10 October, 1973*

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MICE TO THE CHEMICAL CARCINOGEN
7, 12-DIMETHYLBENZ (A) ANTHRACENE

by

EUSTACE ARNOLD JOHNSON

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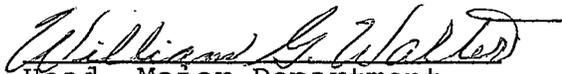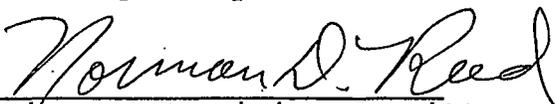fulfillment of the requirements for the degree
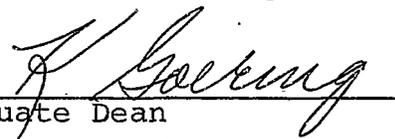
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ABSTRACT

Considerable evidence supports the theory that a
thymus-dependent immunological surveillance mechanism is
important in tumor prevention. To this end we expected the
congenitally athymic (nude) mouse to have many spontaneous
tumors. We have not observed spontaneous tumors in the
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tumor.

INTRODUCTION

Concept of Immunesurveillance. The functions of the
thymus and the effects of thymectomy have been reviewed in
an article by Miller and Osoba (1). Thymectomy results in
impairment of the immunological system bringing about an in-
creased susceptibility to infections (2), the retention of
skin grafts that would normally be rejected (3-5), a short-
ened latency period for tumor development induced by some
types of viruses (6-10) but not other viruses (11-14), accep-
tance and maintainance of tumor grafts (15-16), increased
production of production of tumors with a shortened latency
period for tumor development induced by certain carcinogens
(17-18) [but not substantiated by workers using different
chemical carcinogens (6,12,19,20)], a decrease in the number
of circulating lymphocytes (1) and a shortened lifespan with
a wasting syndrome usually present before death (2,21,22).

These observations led F. M. Burnet to suggest that a
major role of the thymus is to assist in the development of
a surveillance system designed to provide protection against
tumors or neoplasms arising in the body (22-23). According
to this theory, the thymus-dependent system of immunocytes
is almost solely responsible for surveillance; whereas, the
antibody-producing cells have an almost negligible role.

Burnet suggests that this immunesurveillance system tends to reduce the continued existence or survival of those mutant cells that occur spontaneously or those that are induced from a variety of causes. Without this system, these mutant cells might progress to malignancy. The net result is that tumors would be frequent in animals with an intact immunological surveillance system. Any activity, congenital defect, or treatment which would render damage to a functional immunological system would be expected, according to Burnet, to permit more tumors or neoplasms to develop.

Evidence in Support of Immunesurveillance. Cinader (24) summarizes evidence from many laboratories that the incidence of human tumors would be much greater were it not for this immunesurveillance system. His support is as follows: a high incidence of tumors in cases of inborn errors of the cell-mediated immune apparatus; an increased incidence of tumors in the thymectomized animals which are infected by tumor viruses or tumor cells; an increase in the minimal number of tumor cells required for tumor "take" after immunization; a high incidence of tumors in immunosuppressed patients; a difference in immunological parameters between animals infected with the same virus, but having a regressing

or progressing tumor. These observations lend support for the immunesurveillance theory which has been proposed by Burnet.

Miller, Grant, and Roe (18) investigated the incidence of tumors in neonatally thymectomized mice and sham-operated control mice. They concluded that tumors arose earlier and more frequently in thymectomized mice in comparison to sham-operated mice. The regression rate was higher in the sham-operated control mice. Grant and Miller (17) reported tumors appearing more rapidly in thymectomized mice than in control mice following intramuscular injection of 20-methylcholan-thren (MCA). Defendi and Roosa (26) examined histologically sarcomas derived by injection of MCA and reported that a cellular infiltration of mostly lymphocytes could be seen around the necrotic tumors in the control mice but not in thymectomized mice. Several additional workers have reported similar results of increased tumor incidence induced by chemical carcinogens after prior treatment of animals with anti-lymphocyte serum (ALS) (27). For an excellent review of this material, one may read the review articles by Miller and Osoba (1) and the two review articles by Gleichmann and Gleichmann (27,28).

The effects of thymectomy on the incidence of tumors

induced by viruses have been described by several investi-
gators. Allison and Taylor (29) demonstrated an increased
number of tumors induced with polyoma, SV40, and adenovirus
type 12 in thymectomized CBA mice compared to CBA controls.
Several other investigators have also shown an increased
incidence of polyoma-induced tumors in thymectomized mice
(6-10). An increased frequency of tumors induced by adeno-
virus type 12 has been reported in neonatally thymectomized
mice and in hamsters thymectomized at three weeks of age (29)
when compared to sham-operated controls.

Evidence Against Immunesurveillance. Many experiments
can be cited with results incompatible with the immunesur-
veillance theory. For example, not all virus-induced tumors
are increased by thymectomy. Investigators have shown that
the incidence of lymphoid leukemia in mice infected with
Gross and Moloney viruses is reduced with thymectomy (11,14).
Mammary tumors have a reduced incidence in thymectomized
mice carrying the Bittner agent (29).

Several investigators using the carcinogen 20-methylcho-
lanthrene have reported no significant difference in the in-
cidence of chemically induced tumors in thymectomized and
control mice. Allison and Taylor (29) did not observe any

differences between the incidence of skin tumors in thymectomized rats over control rats after topical application of 7, 12-dimethylbenz(a)anthracene.

According to Gleichmann and Gleichmann (28) the rats of spontaneous malignancies in animals with suppressed cellular immunity does not significantly differ from that of non-immunosuppressed animals.

Gleichmann and Gleichmann (27-28) conclude their two-part review of immunosuppression and neoplasia by stating that a serious review of the literature places doubts on the general validity of the immunesurveillance theory. According to their interpretation, in light of the bulk of experimental evidence, the theory does not consider those factors in neoplasia which may be required in addition to immunosuppression, nor does it consider the tumor promoting effect of immunosuppressive drugs such as described for azathioprine in two-month old NZB mice or newborn C57Bl mice, or the tumor enhancing properties of the vessicant croton oil. Much work with croton oil has been described by Berenblum (30) and Van Duuren (31). Further, it does not adequately account for immunologic enhancement of "sneaking through," both of which demonstrate the ability of many malignant tumors to

adapt to a non-depressed immune system. They mention recent experiments by R. T. Prehn demonstrating a small degree of specific immunity having a possible stimulatory effect on tumor growth.

Prehn has suggested that only the less antigenic mutant cells can survive a lymphocyte-mediated attack (32-34). Also, according to Prehn, a few mutant cells with antigenicity may be stimulated by an immune stimulation that is relatively weak. Prehn mixed various numbers of spleen cells from specifically immunized mice with constant numbers of tumor cells. These mixtures were inoculated subcutaneously into thymectomized, X-irradiated recipients. He discovered that small numbers of immune spleen cells produced a statistically significant, and reproducible, acceleration of tumor growth in the inoculum as compared with controls or either nonimune spleen cells or spleen cells from animals immune to a different, non-cross-reacting tumor. Large numbers of specifically immune spleen cells produced inhibition of tumor growth (34). Prehn believes these data imply a dual function: 1. stimulation of tumor growth, early in the course of the disease, or whenever the immune stimulation is minimal. 2. inhibition of tumor growth at other times. Medina and Heppner (35) have demonstrated "immunostimulation"

induced by mammary tumors in virus-free Balb/c mammary
tumors. They used sensitized lymphocytes to enhance tumor
cell growth over the growth obtained with unsensitized
lymphocytes.

The Congenitally Athymic (Nude) Mouse. In view of the
contradictory results of experiments concerning the effect
of thymectomy on tumor production and contradictory reports
relating to immunesurveillance, the "nude" mouse described
by Flanagan (36) and later found to be congenitally athymic
by Pantelouris (37), would be expected to be an excellent
model for evaluating the role of the thymus in tumor develop-
ment.

Manning, Reed, and Shaffer (41) have shown the inability
of nude mice to reject several xenografts and to retain these
grafts in a viable condition for the lifetime of the mouse.
Giovanella, Yim, Stehlin, and Williams (40), Povlsen and
Rygaard, and Rygaard and Povlsen (38-39) have reported
development of malignant human tumors in nude mice and Reed
and Manning (42) have shown nude mice to accept normal human
forskin. Reed and Jutila have shown that nudes do not re-
spond well to heterologous red blood cells (43).

Wortis, Nehlsen, and Owen (44) have reported that the grafting of thymus glands into nude mice will result in their ability to reject homografts. Manning and Reed have shown nude mice to reject xenografts if thymus glands are implanted prior to skin grafting (41). Isaak (45) also demonstrated the rejection of homografts of thymus grafted nudes but not in nudes where the thymus gland was enclosed in a millipore chamber and then grafted into a nude mouse. This would lend support to rejection phenomena being due to a cell-mediated response and not soluble factors.

The experiments described here were designed to evaluate the role of the thymus in the development of tumors induced by the chemical carcinogen 7, 12-dimethylbenz(a)anthracene.

MATERIALS AND METHODS

Mice. A breeding nucleus of mice derived from the
strain described by Flanagan (36) and carrying the nude gene
was obtained from Doctors R. C. Roberts and D. S. Falconer,
Edinburg, Scotland. These mice have since been backcrossed
onto a Balb/c strain. Because nude (nu/nu) mice show a de-
creased fertility and the females are not able to suckle
their young, our experimental stock of nude mice and their
phenotypically normal littermates (nu/+ and +/+) were derived
from heterozygous animals (46).

Mice of the (Hr/Hr) strain were obtained from the
Jackson Laboratory, Bar Arbor, Maine. These mice are hair-
less, and by our inspection have intact thymuses.

All mice were fed sterilized Purina 5010C feed and
acidified-chlorinated water (47). The mice ranged in age
from 4-5 weeks when placed into an experiment, and the sexes
were not distinguished in the experimental design.

Chemicals. The 7, 12-dimethylbenz(a)anthracene (DMBA)
was obtained from Eastman Kodak, Rochester, New York, and
dissolved in reagent grade acetone in the concentrations
indicated in the results section. The doses were applied
with a micropipet to the back of the mice. Some of the

groups of mice received in addition, two drops of 0.05
percent croton oil. The croton oil was obtained from Robin-
son Laboratories, Inc., San Francisco, California. The
croton oil was used as a promoter. The croton oil was first
applied 10 days after the last dose of DMBA was applied.
The croton oil was applied twice per week each week until
the experiment was terminated.

Thymus Grafts. Some of the nude mice received two to
four thymus glands implanted under the fascia in a pocket
prepared in the axillary region. This was done under
Nembutal anesthesia (48) when the mice were weaned at four
to five weeks of age. The thymus glands were obtained from
Balb/c mice less than three days old. Immediately preceding
transplantation, donors were sacrificed and the thymus gland
placed in phosphate buffered saline (PBS). Recipients were
anesthetized and a small incision cut through both the dermal
and inner fascia layers 1 cm caudal to each axillary region.
The thymus glands were inserted with twissors and pushed up
through a prepared pathway under the fascia to a position in
the axillary region. The incision was closed with two to
four interrupted sutures of 5-0 silk.

Thymus Cell Injections. Thymus cell suspensions were

prepared by gently screening thymuses from young adult Balb/c donors through a stainless steel mesh screen in PBS containing 1 percent fetal calf serum. Cells were washed in the cold in the PBS-fetal calf serum mixture, quantitated and 5×10^7 viable cells were injected in 0.5 ml of PBS-fetal calf serum mixture after a trypan blue exclusion test was done to determine cell viability. The injections were via a tail vein.

Thymus Cell Extracts. Thymus glands from young adult Balb/c donors were mascerated in a Serval Mini-homogenizer at 30 second bursts for five minutes with the tissue capsule suspended in ice. The extract was obtained by centrifuging the homogenate in the cold at approximately 3,000 RPM in an International Clinical Centrifuge (Model CL) for five minutes. The amount injected was 0.1 ml and was the equivalent of the supernatant material from one thymus donor.

Assay for Immunological Competance. The parameter used to test for immune competance was the ability to reject homografts or heterografts or to produce antibodies to sheep red blood cells (SRBC). This immune competance was provided either by thymus gland grafting, thymus cell injection, or by thymus gland extract injection.

The grafting procedures were adapted from Billingham and Silver (49). Briefly, full thickness grafts were prepared from CBA mouse skin or human foreskin. The skin was pinned and stretched on a disecting board and the underlying membraneous layers were gently scraped away with a scapel. Circular grafts were then cut with a sharpened 10 mm diameter cork borer and the grafts were rinsed twice in PBS and then suspended in a final solution of PBS. Recipient mice were anesthetized with nembutal and a circular bed cut in the skin on the right or left lateral portion of the rib cage posterior to shoulder. Following the graft bed preparation, the circular grafts were placed directly onto the graft bed. Additional trimming of the circular grafts or graft bed site was occasionally done to obtain a better "fit." The graft bed was cut a little larger than the skin graft. Dressings were then applied consisting of a sterile cloth impregnated with petroleum jelly overlayered with a sterile guaze pad. Above the guaze pad, a guaze strip was wrapped around the mouse to immobilize the skin graft. The outermost layer was a plaster-gauze cast which was also wrapped around the trunk of the mouse. The plaster casts were removed six to eight days later and the grafts examined. The grafts were then examined every two to three days there-

after and evaluated for signs of rejection. Graft rejection
was monitored by the development of an inflamed area with a
total necrosis of the graft and replacement of the skin
graft with that of host origin representing total rejection.
In order to rule out damage to the graft due to scratching
by the mouse, the grafts were kept bandaged with Band-Aids.
The grafting procedures and techinques described are those
of Manning, Reed, and Shaffer (41).

Humoral antibody titers, including hemagglutinating
(HA) titers and hemolytic (HL) titers were determined five
days after the administration of 0.25 ml of a 10 percent
suspension of SRBC intraperitoneally (I.P.). Serum hemag-
glutinating antibody levels were determined by the method
described by Isaak (45).

After bleeding from the retroorbital sinus, twofold
serial dilutions of serum samples were prepared in modified
barbitol buffer (45). Agglutinin (HA) titers were read
after centrifugation, following which the SRBC were sus-
pended and 0.1 ml of a 1:10 dilution of guinea pig comple-
ment was added to each tube. Hemolysin (HL) titers were
read after an incubation of 1 1/2 hours at 37° C.

Tabulation of Nude Mice Used in the Experiments. Only
nuce mice alive when normal littermates (NL) first began to
develop tumors were included in the experiments. All nude
mice that died before any littermates developed tumors were
excluded from the tabulation of date.

RESULTS

Illustrations showing the protocol used in the succeed-
ing experiments and the results are shown in Figures I-V.
The data derived from the experiments are listed in Tables
I-VIII.

Response of Nude and Littermate Mice to 7, 12-Dimethyl-
benz(a)anthracene (DMBA).

Nude mice have not been observed to develop spontaneous
tumors. They have been observed to have a shorter lifespan
than their normal littermates. It is possible that the
lifespan of the nude mice is not long enough for tumors to
develop. To answer this question, we attempted to induce
tumors in nude mice with a known carcinogen, DMBA (See
Figure I). We expected many more tumors in the nude mice
to arise and possibly more rapidly than in their normal
littermates.

We treated nude and littermate mice with DMBA using
different doses and dose schedules. The protocol for these
studies is portrayed in Figure I. The results are tabulated
in Tables I-III. All of the normal littermate mice developed
one or more papillomas. These growths were not examined
histologically so there is not any confirmation if any of
these growths had progressed to a maliganant carcinoma.

These papillomas developed at the site of DMBA application on the littermate mice. Approximately 36 hours after the first application of DMBA, an erythematous and indurated area developed on the littermate mice. This reddened area healed over with some scaring and hair loss. In addition to the erythema, marked thickening of the skin was noted on the littermate mice.

Surprisingly, none of the nude mice developed any papillomas or any grossly visible response to DMBA. This is not the result one would expect according to the concept of immunesurveillance.

Response of Nude Mice and Littermates to 7, 12-Dimethyl-benz(a)anthracnee and Croton Oil.

Because the nude mice did not develop papillomas or respond to the DMBA in any grossly visible fashion, we thought it might be due to some abnormal character of their hairless skin. Indeed, it has been reported by Giovanella (50) that hairless mice are generally refractory to chemical carcinogenesis. Others such as Iversen and Iversen (51) do not agree. It has been reported that croton oil has an enhancing effect on tumor production when applied topically (21,28,30).

Table IV shown the results of treatment of nude mice and littermates with one dose of 400 ug of DMBA and croton oil applied 10 days later with applications continued twice per week until the experiment was terminated. Figure III shows the protocol used.

All of the normal littermates developed one or more papillomas and only one nude developed a papilloma that regressed after about one month.

TABLE I

Papilloma development in nude (Nu) and normal littermate (NL) mice treated with 200 ug of DMBA three times per week for four weeks.

Days after first DMBA application	Number of mice with papillomas *	
	NL	Nu
31	1/12	0/2
37	8/12	0/2
41	10/12	0/2
60	11/12	0/1
72	12/12	0/0

*   The numerator represents the number of mice with at least one papilloma. The denominator represents the number of living treated mice.

TABLE II

Papilloma development in nude (Nu) and normal littermate (NL) mice treated with 200 ug of DMBA twice per week for two weeks.

Days after first DMBA application	Number of mice with papillomas *	
	NL	Nu
28	2/3	0/3
32	3/3	0/3
43	3/3	0/2
47	3/3	0/1
56	3/3	0/0

*   The numerator represents the number of mice with at least one papilloma. The denominator represents the number of living treated mice.

TABLE III

Papilloma development in nude (Nu) and normal littermate (NL) mice treated with 200 ug of DMBA once per week for eight weeks.

Days after first DMBA application	Number of mice with papillomas *	
	NL	Nu
47	1/4	0/1
48	2/4	0/1
83	4/4	0/0

* The numerator represents the number of mice with at least one papilloma. The denominator represents the number of living treated mice.

TABLE IV

Papilloma development in nude (Nu) and normal littermate (NL) mice treated with 400 ug of DMBA and then 10 days later with an initial dose of two drops of 0.05 percent croton oil which was repeated twice per week therafter until the experiment was terminated.

Days after the DMBA application	Number of mice with papillomas *	
	NL	Nu
28	1/24	0/21
40	10/24	0/20
52	16/24	1/20
88	23/24	0/ 2
106	23/24	0/ 1
109	24/24	0/ 0

* The numerator represents the number of mice with at least one papilloma. The denominator represents the number of living treated mice.
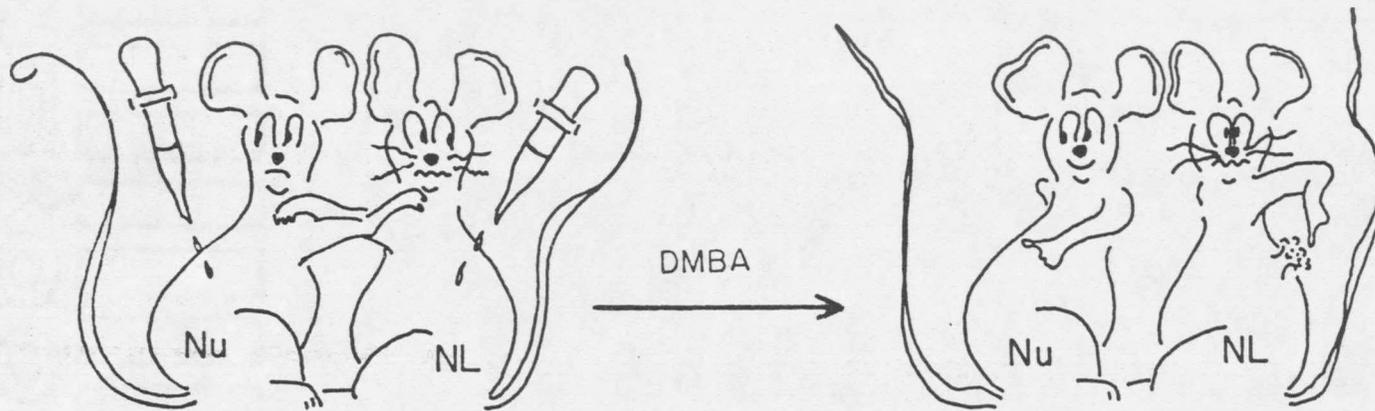
FIGURE I.   Protocol showing the treatment of nudes (Nu) and normal littermates (NL) with
7, 12-dimethylbenz(a)anthracene (DMBA).