



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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THE RESPONSE OF CONGENITALLY ATHYMIC (NUDE)
MICE TO THE CHEMICAL CARCINOGEN
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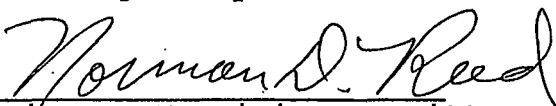
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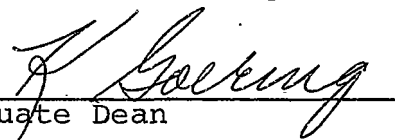
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ABSTRACT

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.

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 increased susceptibility to infections (2), the retention of skin grafts that would normally be rejected (3-5), a shortened latency period for tumor development induced by some types of viruses (6-10) but not other viruses (11-14), acceptance 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-methylcholanthren (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 investigators. 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 adenovirus 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 immunesurveillance 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-methylcholanthrene have reported no significant difference in the incidence 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 vesicant 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 nonimmune 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 development.

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 respond 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 decreased 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 Harbor, Maine. These mice are hairless, 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 Robinson 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 tweezers 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 Competence. The parameter used to test for immune competence was the ability to reject homografts or heterografts or to produce antibodies to sheep red blood cells (SRBC). This immune competence 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 dissecting board and the underlying membranous 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 overlayers with a sterile gauze pad. Above the gauze pad, a gauze 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 techniques 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 hemagglutinating 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 suspended and 0.1 ml of a 1:10 dilution of guinea pig complement 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 nude 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 succeeding 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-Dimethylbenz(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 malignant 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 scarring 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-Dimethylbenz(a)anthracene 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 Giovannella (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 thereafter 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.

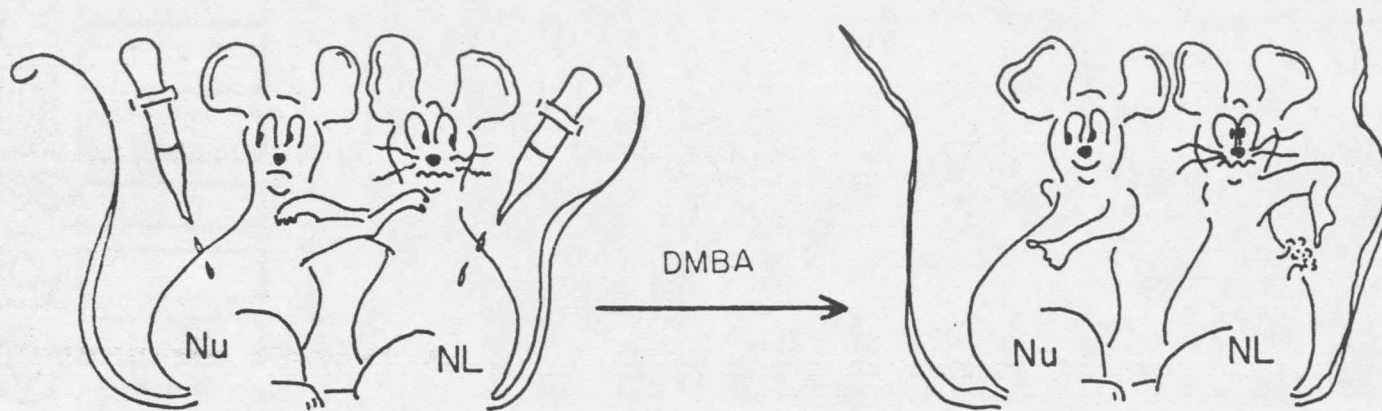


FIGURE I. Protocol showing the treatment of nudes (Nu) and normal littermates (NL) with 7, 12-dimethylbenz(a)anthracene (DMBA).

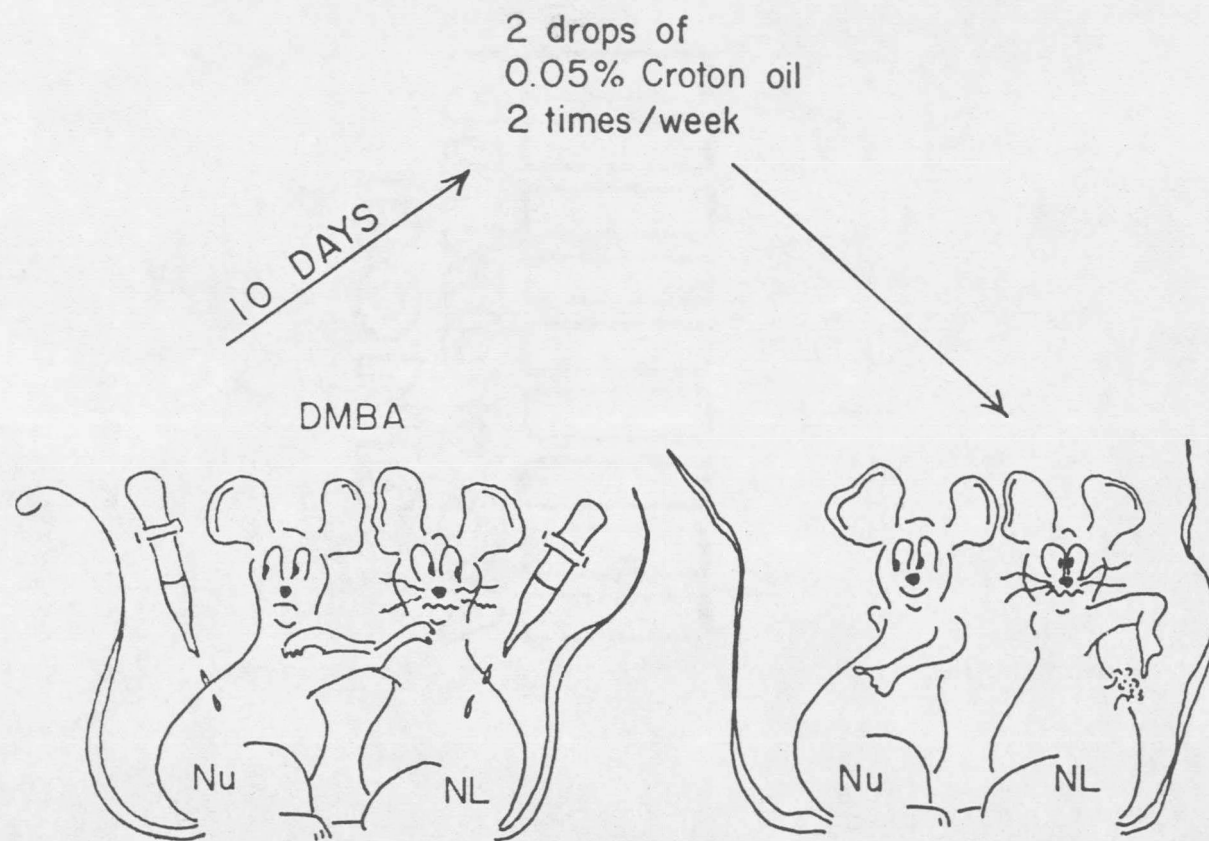


FIGURE II. Protocol showing the treatment of nudes (Nu) and normal littermates (NL) with 7, 12-dimethylbenz(a)anthracene (DMBA) and croton oil.

Response of Another Hairless Mutant (hr/hr) and Normal littermates (hr/+) to 7, 12-Dimethylbenz(a)anthracene and Croton Oil.

Because nude mice did not develop papillomas and/or tumors as we expected, we thought we would check our experimental design using DMBA and croton oil on another hairless mutant. This mutant (hr/hr) has been observed by Iversen and Iversen (51) to not develop spontaneous tumors and to be rather responsive to chemical carcinogens. These mice have intact thymuses. After treatment with 400 ug of DMBA and twice weekly treatment with two drops of 0.05 percent croton oil, the hairless mutant (hr/hr) developed papillomas. The results of treating the hairless strain and their normal littermates are shown in Table V.

It is interesting that the hairless mice developed papillomas before their littermates. This observation would coincide with the quantitative analysis studies of Tarnowski (52) demonstrating that hairless mice of the same strain we used absorbed more tritiated 7, 12-dimethylbenz(a)anthracene than their normal littermates. The observations by Tarnowski were based on autoradiographic studies and showed labeled DMBA in the epidermis, hair follicles and subcutaneous glands. Some mast cells also showed tritiated DMBA.

TABLE V

Papilloma development in hairless (hr/hr) and their normal littermates (hr/+) treated with 400 ug of DMBA and croton oil.

Days after DMBA application	Number of mice with papillomas *	
	hr/hr	hr/+
38	4/8	0/12
47	6/8	0/12
49	6/8	3/12

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

TABLE VI

Papilloma development in nude mice with thymus grafts (Nu-tg) and normal littermate mice (NL) treated with 150 ug of DMBA twice per week for seven weeks.

Days after first DMBA application	Number of mice with papillomas *	
	NL	Nu-tg
58	1/3	0/4
74	1/3	1/4
110	1/3	2/4
123	3/3	3/4
127	3/3	4/4

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

It was, therefore, obvious that our experimental design was satisfactory for the induction of papillomas in at least one strain of mice that was devoid of body hair. Therefore, we began to entertain the idea of a possible requirement of an immune response as a prerequisite for tumor induction as presented by R. T. Prehn.

Response of Littermates and Thymus-implanted Nudes to 7, 12-Dimethylbenz(a)anthracene.

If an immune response is required for tumor development, then nude mice which have been immunologically reconstituted by thymus gland implantation might be expected to develop tumors.

In Table VI are listed the results of treating littermates and thymus-implanted nudes with 7, 12-dimethylbenz(a)anthracene. The protocol used is shown in Figure III. All of the littermates and all of the thymus-implanted nudes developed one or more papillomas. Two of these thymus-implanted nudes developed an apparent carcinoma from existing papillomas. Both of these suspected carcinomas became ulcerated and indurated grossly resembling a carcinoma. These impressions were not confirmed histologically. One weak point in this group was the absence of nude mice that

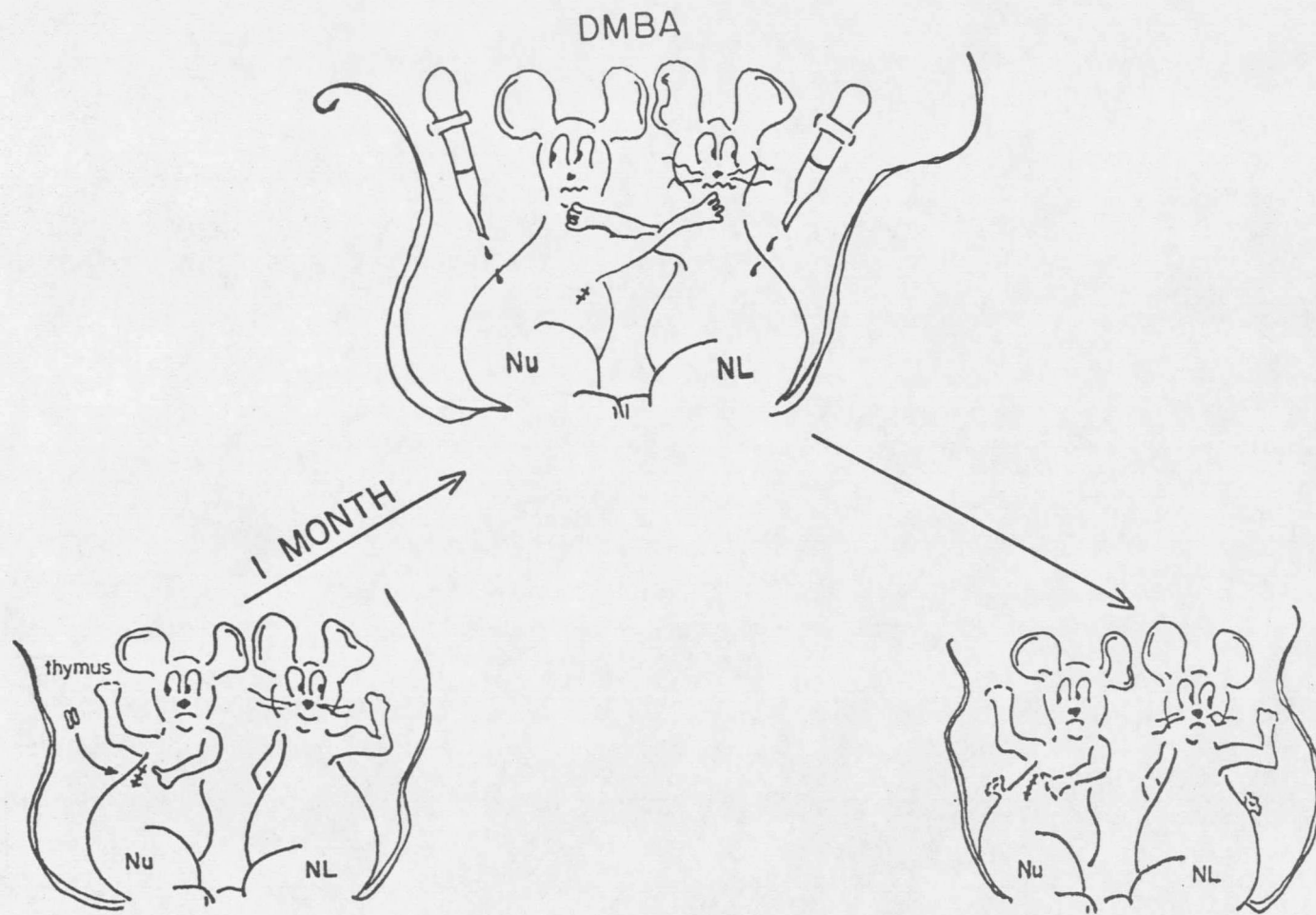


FIGURE III. Protocol showing the treatment of thymus gland-implanted nudes (Nu-tg) and normal littermates (NL) with 7,12-dimethylbenz(a)anthracene.

had not been altered by thymus-implantation or other means of providing immune competence. We, therefore, decided to embark upon an additional experiment which included nudes, littermates, thymus-implanted nudes, thymus cell-injected nudes, and thymus extract-injected nudes.

Response of Nudes, Thymus-implanted Nudes, Thymus Cell-injected Nudes, Thymus Extract-injected Nudes and Littermates to 7, 12-Dimethylbenz (a) anthracene and Croton Oil.

As mentioned previously, only one of these nude mice developed a papilloma after one treatment with 400 ug of DMBA and then twice weekly treatment with croton oil until termination of the experiment. These results are listed in Table VII and the protocols used are shown in Figures IV and V.

All of the normal littermates, all of the thymus-implanted nudes and all of the nude mice injected with thymus cells developed papillomas. The number of papillomas on the littermates were not counted, but all had at least two papillomas. The seven thymus-implanted developed papillomas in two different sets. The second set of papillomas developed after the first set of papillomas had regressed.

TABLE VII

Papilloma development in nudes (nu), normal littermates (NL), thymus-grafted nudes (Nu-tg) and thymus cell-injected nudes (Nu-tc) treated with 400 ug of DMBA and croton oil.

Days after DMBA application	NL	Nu-tg	Nu-tc	Nu *
28	1/24	0/7	0/5	0/21
31	2/24	0/7	0/5	0/21
34	4/24	0/7	0/5	0/21
35	5/24	0/7	0/5	0/21
38	8/24	4/7	0/5	0/20
40	10/24	4/7	0/5	0/20
41	11/24	4/7	0/5	0/20
44	13/24	4/7	0/5	0/20
45	15/24	5/7	0/5	0/20
47	16/24	7/7	0/5	0/20
52	16/24		0/5	1/20
55	18/24		0/5	1/19
57	20/24		0/5	1/18
66	20/24		0/5	1/15
71	22/24		0/5	1/13
75	23/24		0/5	1/9
76	23/24		3/5	1/9
83	23/24		3/5	1/8
88	23/24		3/5	0/2
102	23/24		5/5	0/2
106	23/24			0/1
107	23/24			0/0
109	24/24			

Summary of data: all NL developed papillomas
 all Nu-tg developed papillomas
 all Nu-tc developed papillomas
 one Nu developed a transient papilloma,
 this nude died on day 106.

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

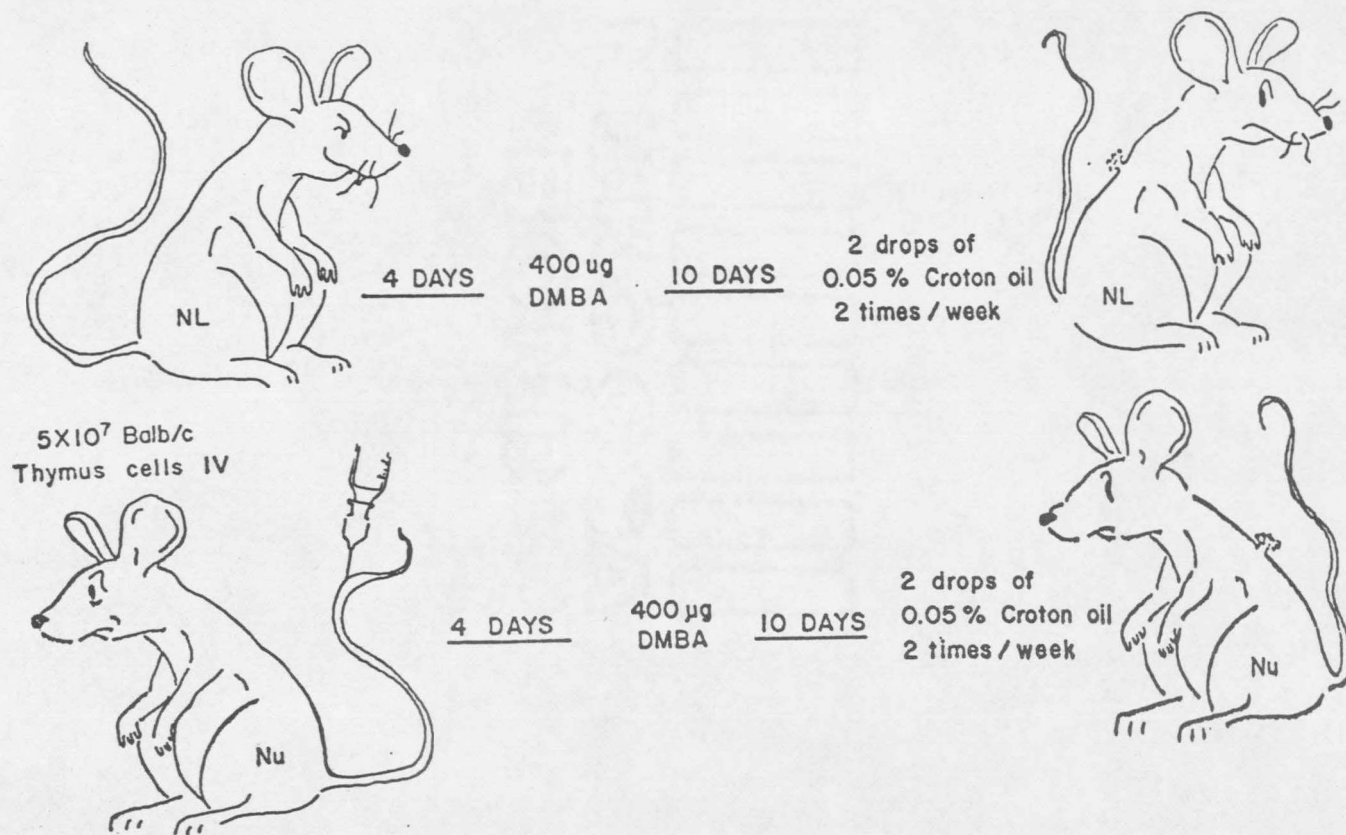
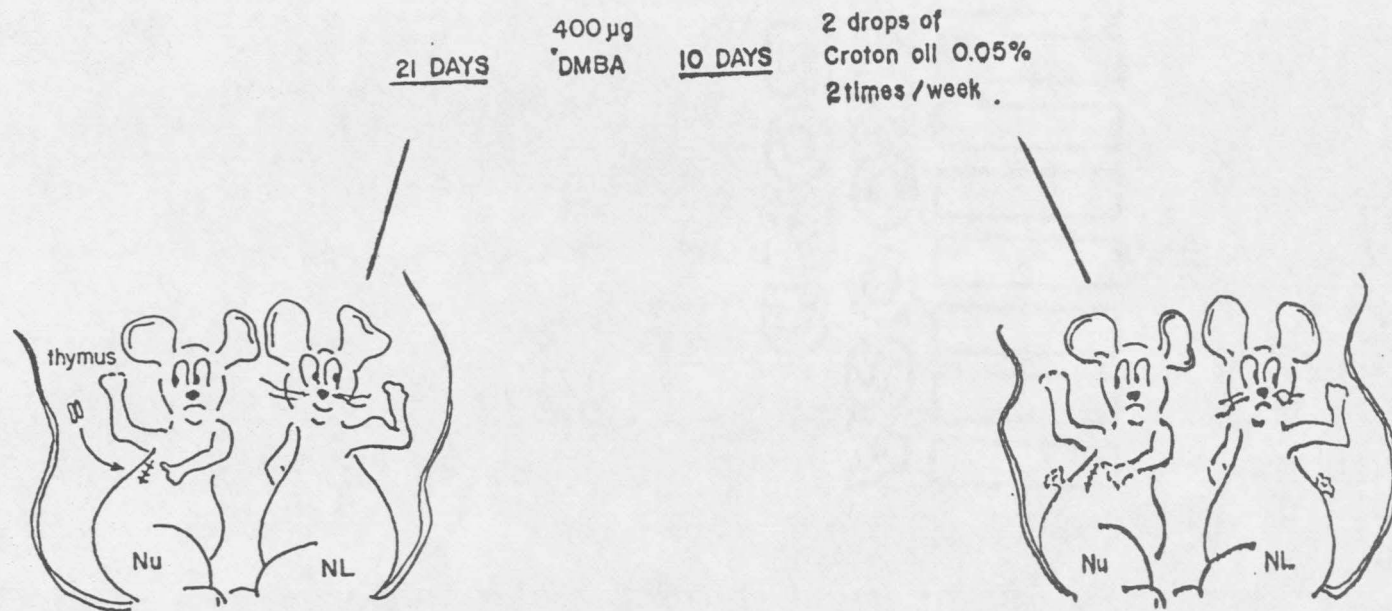


FIGURE IV. Protocol showing the treatment of normal littermates (NL) and nudes injected with thymus cells (Nu-tc) with 7, 12-dimethylbenz(a)anthracene and croton oil.



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FIGURE V. Protocol showing the treatment of normal littermates (NL) and nudes with implanted thymus glands (Nu-tg) treated with 400 ug of 7, 12-dimethylbenz(a)anthracene and croton oil.

The five nudes which received an injection of 5×10^7 thymus cells developed a total of 41 papillomas. These decreased in number after 124 days after the DMBA application. The development of papillomas in the thymus cell-injected mice did not arise in sets but as one continuing series.

The nude mice injected with thymus extracts did not tolerate this treatment very well. Out of seven nude mice injected, two were found dead in their cage four days later, two mice were found gasping for air the day after a second injection was given, and one of these mice was having convulsions and the hind legs were fully extended. A fourth nude developed hemorrhagic areas on all four feet, the abdomen and the tips of the ears. This mouse later died with necrosis of all four feet and extensive hemorrhagic involvement. The last two mice lived for awhile with continued whole body wasting that was demonstrated by all of the thymus extract-injected nude mice. The nude mice controls injected with normal saline did not show this wasting and death. Thus, the results of the experiment involving thymus extracts are inconclusive.

Assay of Thymus-implanted Nudes and Thymus Cell-injected Nudes for Immunocompetance.

The four thymus-implanted nudes that developed papillomas listed in Table IV, were grafted with CBA mouse skin grafts. Three of these mice successively rejected the CBA mouse skin grafts for average rejection time of 12 days. The fourth mouse was deemed a technical failure as the protective cast slipped, allowing the graft to slip out of the graft bed and, therefore, become necrotic. Two of the three littermates also rejected a CBA mouse skin graft by day 12.

Five of five nude mice injected with thymus cells (see Table VII) were grafted with human foreskin. One of the mice was accidentally killed with the graft intact 26 days after grafting without any signs of rejection. One of the thymus cell injected nudes showed rejection of the human foreskin graft at 20 days. It was not certain if the rejection was due to mechanical damage or immune rejection, so this mouse received a second human foreskin graft.

The skin-rejection data are tabulated in Table VIII.

Response of nude mice, nudes with thymus gland implants, nudes injected with thymus cells and normal littermate to

an injection of 0.25 ml of a 10 percent PBS suspension of sheep red blood cells (SRBC) was determined by hemagglutination (HA) titers and hemolysin (HL) titers. These results are tabulated in Table IX. The littermates and thymus gland-implanted nude mice produced a high titer of HL and HA type antibodies.

The nude mice which received thymus cells did not produce HA or HL titers significantly different than did unaltered nude mice in response to injection of SRBC.

TABLE VIII

Skin graft response of nudes (Nu), nudes with thymus implants (Nu-tg), nudes with injected thymus cells (Nu-tc) and normal littermates (NL).

GROUP	No. of ANIMALS	GRAFT	RESULT
Nu	1	Human	-Retained
Nu-tg	6	Human	-6 Rejected
Nu-tc	5	Human	-1 died with graft intact -4 Rejected
NL	2	CBA	-2 Rejected
Nu-tg	4	CBA	-3 Rejected 1 graft was a technical failure

TABLE IX

Response of mice receiving 0.25 ml of 10 percent sheep red blood cells (SRBC) intraperitoneally.

GROUP	SERUM AB	
	HA	HL
4 Normal Littermates (NL)	480	560
5 Nudes + Thymus Glands (Nu-tg)	384	384
5 Nudes + Thymus Cells (Nu-tc)	52	130
4 Nudes (nu)	50	140

DISCUSSION

There have been numerous reports in the literature that have given evidence that an immunesurveillance system provides protection against neoplasms arising in the body. This theory supplies a simple explanation for the reported high incidence of neoplasms in animals and humans with an impaired immunological system (1-10,21,22,27-28).

It would appear that the congenitally athymic (nude) mouse would support the concept of immunesurveillance by exhibiting many spontaneous tumors arising in its body because it would be totally lacking in protection against neoplastic cells. By inspection, these nude mice have not been observed to elaborate neoplastic tumors in our colony. This was a surprise that required an explanation and possibly a reevaluation of the immunesurveillance theory or at least some refinement.

One explanation for the absence of spontaneous tumors in nude mice is that they do not live long enough for spontaneous growths to develop. In an effort to determine if this was the reason nude mice do not exhibit tumors, we tried to induce papillomas and/or carcinomas by topical application of the carcinogen 7, 12-dimethylbenz(a)anthracene (DMBA). To our surprise, again, the nude mice so treated did

develop papillomas (Figure I and Tables I-III). This result led us to suspect that the hairless condition of the skin might be contributing an abnormal character involving possibly abnormal hair follicles or keratinization which prevented a response to the DMBA.

To test the idea that the hairless condition prevented the nude mice from responding to the treatment of DMBA, we secured another hairless mutant (hr/hr) mouse and commenced treatment of this mutant with DMBA. These mutants produced papillomas even before their normal littermates (hr/+). Therefore, it was evident that our protocol should produce tumors in nude mice unless something else was lacking to prevent a response to the DMBA.

Because we expected the nude mice to develop spontaneous tumors and certainly to develop many tumors in response to topical applications of a known chemical carcinogen, which they did not do, we then considered the possible influence of some thymic property.

Nude mice that received implantations of thymus glands or injections of thymus cells produced papillomas after treatment with DMBA (Figure III and Table IV). These mice

did not develop tumors as rapidly as the normal littermates. The reason may be that the implantation of a thymus gland reconstitutes a nude for some factors or functions but not all. The nudes which received injections of thymus cells produced tumors after the thymus gland-implanted nudes. These observations would appear to be substantiated by the longer rejection time for nudes that received thymus cells to reject skin grafts and also the lower antibody levels in response to sheep red blood cells as compared to normal littermates. However, the skin rejection time was approximately the same for thymus grafted nudes and normal littermates.

The thymus cell-injected nude mice produced approximately the same titer of HA and HL antibodies as nude mice. The thymus cell-injected nude mice were able to successively reject skin grafts. This would suggest that a selected immune reconstitution results from the injection of thymus cells whereas, a thymus graft rendered almost normal competence to nude mice using HA and HL antibody production as a measure of immune competence when compared to normal littermates.

We are not prepared to say that the immune competence demonstrated by the reconstituted nudes is responsible for the induction of papillomas. We demonstrated that reconstituted nude mice rejected skin and produced antibodies against sheep red blood cells injected intraperitoneally; these same nude mice developed papillomas induced by DMBA. It would appear that a thymus is a requirement for the induction or development of some types of tumors. This thymic influence has yet to be identified. The one unaltered nude that developed one transient papilloma would not be considered significant enough to alter our contention.

SUMMARY

The treatment of congenitally athymic nude (Nu) mice with a known chemical carcinogen, 7, 12-dimethylbenz(a)anthracene (DMBA), did not result in the production of papillomas as one would expect in accordance with the immunosurveillance theory.

Immunosurveillance in reconstituted nude mice was shown by their ability to reject CBA mouse skin grafts or human foreskin grafts and by the production of anti-sheep erythrocyte hemagglutinating or hemolysin type antibodies. Nude mice were able to produce papillomas in response to topical application of DMBA only if a thymus gland was implanted or thymus cells were injected.

It was concluded that the implantation of a thymus gland or the injection of thymus cells provided the nude mouse with immunocompetence. The presence of a thymus appears to be required for the production of certain types of tumors. This does not necessarily imply immunocompetence, but may possibly be some product elicited by a thymus.

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