

Lack of Antigenicity of Mammary Tumors Induced by Carcinogens in a Nonantigenic Preneoplastic Lesion¹

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SUMMARY

Eight mammary carcinomas were induced by either 3-methylcholanthrene or 7,12-dimethylbenz(a)anthracene in a nonviral- and noncarcinogen-induced preneoplastic (prealignant) alveolar nodule which had arisen in a hormonally stimulated BALB/c female mouse. The preneoplastic mammary nodule outgrowth was found to be nonantigenic when tested in the strain of origin. Seven of the eight carcinogen-induced carcinomas were also found to exhibit no clear-cut evidence of antigenicity. The eighth tumor, however, was shown to produce tumor-specific immunity in BALB/c mice.

The results suggest that carcinogens may not directly induce the appearance of new antigens in the tumors they produce. Furthermore, carcinogens may act in a different manner when inducing a preneoplastic change than when inducing a tumor from a preexisting preneoplastic lesion.

INTRODUCTION

During the last 2 decades, evidence collected at numerous laboratories has proven beyond a doubt that most carcinogen-induced tumors are antigenic when tested in the strain of origin. Tumors that have been shown to be antigenic include both carcinogen-induced sarcomas (3, 4, 12, 14) and carcinomas (8, 9, 13, 15). The antigens of chemically induced neoplasias generally are unique to each tumor and are not shared even with other tumors induced by the same carcinogen in the same animal (4, 10). Prehn and Slemmer (13) and Lappe (5, 6) have demonstrated that carcinogen-induced, preneoplastic, hyperplastic mammary nodules and skin papillomas already exhibit tumor-specific antigenicity.

The reverse of this relationship had not been studied. In preneoplastic lesions which are nonantigenic, would the subsequently developing neoplasias also be nonantigenic, or would such neoplasias, if induced by chemical carcinogens, exhibit tumor-specific antigenicity?

We now report that most of the tumors induced by chemical carcinogens in a hormonally induced, nonantigenic preneoplastic lesion of the mammary gland are also nonantigenic.

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MATERIALS AND METHODS

Mice. All animals used in these experiments were BALB/cCrgl mice, which are free of mammary tumor virus and have a very low incidence of spontaneous mammary tumors (7). The isoantigenic homogeneity of this strain was tested during the progress of the reported experiments by the exchange of tail or body skin between randomly selected mice. No histocompatibility differences were detected.

All of the mice were housed in temperature- and light-controlled rooms, were fed a diet of standard mouse pellets, and were given water *ad libitum*. Cages of mice to be used in the experimental and control groups were selected at random, and the animals were matched according to age and sex.

D1 Nodule Outgrowth. The hyperplastic nodule outgrowth used in these experiments and designated as HOG-D1 arose in a BALB/c female that had been hormonally hyperstimulated (7). At the beginning of the present study, HOG-D1 had been maintained for 4.5 years by serial transplantation in BALB/c mice.

The tumor-producing capability of HOG-D1 in untreated mice is 4%, the tumors appearing approximately 13 months after transplantation of the outgrowth. Treatment with carcinogen increases the tumor incidence of HOG-D1 to 60% and decreases the latent period to approximately 7 months (1).

Tumors. Four mammary carcinomas were induced in HOG-D1 by MC³ and 4 were induced by DMBA. The mammary gland rudiments were removed from the inguinal fat pads of 3-week-old mice, following the method of DeOme *et al.* (2), and a small piece of HOG-D1 was implanted in the remaining fat. DMBA tumors were induced in HOG-D1 by intragastric feeding of the carcinogen to adult mice that had previously received transplants of the outgrowth. Tumors induced by MC in a similar manner in other transplants of the same outgrowth were graciously provided by Dr. Daniel Medina. Each tumor was initially tested when it was in its 1st transplant generation.

Sensitization and Challenge with HOG-D1. The mammary gland rudiments of mice used in the HOG-D1 immunization experiments were removed at 3 weeks of age. At 12 weeks, a small incision was made in the flank of each animal, and a thin 1- x 5-mm strip of HOG-D1 was implanted *i.d.* by means of a small trocar. Three weeks later, the experimental mice, as well as the untreated control mice, received implants of small strips

³The abbreviations used are: MC, 3-methylcholanthrene; DMBA, 7,12-dimethylbenz(a)anthracene.

of HOG-D1 in each inguinal fat pad. Eight to 13 weeks later, the pads were removed from both groups of animals and were immersed in Bouin's solution. This method allowed the calculation of the percentage of occupancy by the transplant in each pad.

Sensitization and Challenge with Tumors. Mice between the ages of 3 and 6 months were used in these experiments. Tumor growth in the experimental animals was initiated by the s.c. implantation of small pieces of live tumors. After each tumor had grown to 1.5 to 2 cm in diameter, it was removed by blunt dissection. Two days to 2 weeks after removal of the sensitizing implants, experimental and untreated control animals were challenged with a suspension of live cells from the same tumor used for sensitization. The cell suspension was prepared by the method of Vaage (16). The animals were challenged by the s.c. injection (into the left flank of each mouse) of 0.05 ml of the live tumor cell suspension. The incidence and size of the tumors were checked at weekly intervals. The experiments were terminated when mice in both the experimental and control groups had begun to die from progressive tumor growth, or at 4 months after the challenge inoculum, if only a portion or none of the mice had developed tumors.

Analysis of Results. Results are discussed in terms of tumor incidence, latent periods, and differences in the size of the tumors at various intervals following challenge. The χ^2 and Fisher tests were used to evaluate the significance of differences in the incidence of tumors between sensitized and control groups of mice. Differences between groups were considered significant when the *p* value was 0.05 or smaller.

RESULTS

Tests for Antigenicity of HOG-D1. Two experiments were performed to test the immunogenicity of the HOG-D1 nodule outgrowth. In both experiments, a small strip of HOG-D1 was implanted intradermally into the right flank of syngeneic BALB/c hosts. Three weeks later, a small strip of the outgrowth was implanted in both cleared, inguinal fat pads of the pretreated mice, as well as into those of untreated controls. In the 1st experiment, the pads of both groups were examined after 13 weeks while, in the 2nd experiment, growth of the challenge implant was examined after only 8 weeks. The results of these 2 experiments are summarized in Table 1.

In the 1st experiment, in the pretreated animals, 17 of 22 fat pads (11 animals) were completely filled with nodule outgrowth. Ten of 11 mice showed growth in both pads. The ratio for the controls was comparable. In the 2nd experiment, the results were similar. In the pretreated animals, only 4 of 12 pads (6 animals) were completely filled with the outgrowth, while 8 of 10 pads (5 animals) in the controls showed 75 to 100% growth. However, the number of animals in both experimental and control groups that showed complete or partial growth was proportionately the same, and all animals in both groups showed at least 60% growth in both pads. Careful histological examination of the HOG-D1 outgrowth from pretreated animals in both experiments revealed a complete lack of lymphocytic infiltration.

Weiss *et al.* (17) were likewise unable to show any evidence

of immunogenicity when they challenged with HOG-D1 after pretreatment with the same outgrowth, although pretreatment with a different outgrowth did appear to confer some protection against HOG-D1.

Tests for Antigenicity of Tumors Induced in HOG-D1 by Treatment with MC. Four mammary carcinomas induced in HOG-D1 by MC were tested for immunogenicity. These tumors, designated as D1-MC1, D1-MC2, D1-MC3, and D1-MC4, were always tested during their 1st and subsequent transplant generations. Small pieces of the sensitizing tumor were implanted s.c. in the right flank of BALB/c hosts. When the implanted tumors had grown, they were removed, and 2 days to 2 weeks later, both pretreated and control mice were challenged with a cell suspension prepared from the same tumor used for sensitization. Each tumor was tested several times in order to determine the response of the animals to different doses of tumor cells. A minimal dose necessary for tumor growth in untreated controls was established for 3 of the 4 tumors. Challenge doses ranged from 1×10^3 to 1×10^5 cells.

None of the tumors tested were capable of inducing resistance in BALB/c mice. The incidence, latent periods, and growth curves of the 4 tumors were similar in the sensitized and control groups. The results of these tests are summarized in Table 2.

In the tests with Tumor D1-MC3, it is possible that the smallest challenge dose used (3×10^4 cells) was too large to demonstrate very weak immunity. However, the growth curves and latent periods for this tumor were similar in both the sensitized and untreated animals. Furthermore, histological examination of this tumor revealed no evidence of lymphocytic infiltration.

Tests for Antigenicity of Tumors Induced in HOG-D1 by Treatment with DMBA. Four mammary carcinomas induced in HOG-D1 by DMBA were tested for antigenicity in BALB/c hosts. These tumors, designated as D1-DMBA1, D1-DMBA2, D1-DMBA3, and D1-DMBA4 were tested during their 1st and subsequent transplant generations. The methods used for sensitization and challenge were identical to those described for the MC-induced tumors. Challenge doses ranged from 1×10^3 to 4×10^4 cells. The minimal dose necessary for tumor growth was determined for all tumors tested. Results of the tests with these tumors are summarized in Table 2.

Table 1
Growth of a transplant of HOG-D1 in the inguinal fat pads of BALB/c mice

Sensitized animals had been pretreated with an intradermal implant of the same outgrowth, while controls had received no previous treatment. The data presented in this table represent the combined results of 2 experiments.

Treatment	No. of fat pads that were 75–100% filled/no. tested	No. of fat pads at least 50% filled/no. tested	No. of mice with growth in both pads/no. tested
Sensitized	21/34 ^a	31/34	16/17
Controls	18/25	22/25	12/13

^a By χ^2 test, the difference between sensitized and control animals is not significant; *p* > 0.50.

Table 2
Growth in BALB/c mice of challenge inocula of carcinomas which had been induced in HOG-D1 by treatment with MC or DMBA

Unless otherwise indicated, sensitized animals had been pretreated with an implant of the same tumor used for challenge, while controls had received no previous treatment.

Nonantigenic tumors ^a	Incidence of tumors with			
	Doses above threshold		Doses near threshold	
	Sensitized	Controls	Sensitized	Controls
D1-MC1 (9) ^b	35/40	33/39	2/29	2/32
D1-MC2 (3)	12/14	13/15	7/8	3/5
D1-MC3 (2)	14/14	16/16	N.T. ^c	N.T.
D1-MC4 (4)	7/12	10/12	2/13	3/16
D1-DMBA1 (3)	7/7	8/8	6/12	5/16
D1-DMBA2 (3)	12/12	13/13	6/7	6/8
D1-DMBA4 (4)	22/24	22/24	6/8	5/8

Antigenic tumor	Incidence of tumors	
	Sensitized	Controls
D1-DMBA3 (2)	1/16	15/16 ^d
Sensitization with D1-DMBA3 and challenge with D1-DMBA4 (1)	7/7	8/8

^a By the χ^2 test and Fisher tests, results of comparisons between sensitized and controls for all tumors in this group were not significant.

^b Numbers in parentheses, number of times tested.

^c N.T., not tested.

^d χ^2 test; $p < 0.001$. The difference in tumor incidence between the sensitized and control group is highly significant.

Of the 4 tumors tested, 3 appeared incapable of inducing resistance in BALB/c mice. Sensitized and control groups were similar in terms of incidence, growth rates, or latent periods.

The 4th tumor, D1-DMBA3, was tested twice and in both cases was found to be immunogenic. In the 1st test, with a dose of 2.4×10^4 cells, 7 of 8 animals in the experimental group showed total resistance to tumor growth, while all of the controls developed tumors. When a dose of 1.8×10^4 cells was used, all 8 sensitized animals in the 2nd test were resistant to tumor growth, while 7 of 8 untreated control mice developed tumors. To ensure that the resistance induced by D1-DMBA3 was tumor specific, another test was performed. BALB/c mice were sensitized with D1-DMBA3 and challenged with D1-DMBA4. The results (Table 2) clearly show that pretreatment with the antigenic D1-DMBA3 tumor is unable to protect against challenge with a different nonantigenic tumor.

DISCUSSION

The evidence presented in this paper demonstrates that the majority of tumors induced in a nonantigenic preneoplastic mammary nodule outgrowth do not possess new antigens. The results obtained with the 1 antigenic carcinoma show that BALB/cCrgl mice are immunologically sensitive to carcinogen-induced, tumor-specific antigens, and that the negative results obtained with the other 5 tumors and with HOG-D1 were not due to a lack of immunological reactivity in these mice.

Most carcinogen-induced tumors are antigenic. However, the occurrence of some nonantigenic, carcinogen-induced tumors has been reported. Prehn and Main (12) and Klein *et al.* (4) found that 14 to 25% of the carcinogen-induced tumors they tested were not antigenic. Old *et al.* (8) could not demonstrate new antigens in 19% of tumors induced in females or in 45% of those induced in males, after treatment with MC. Furthermore, carcinogen-induced lung adenomas are notable for their lack of tumor-specific antigens (10, 11). This phenomenon has been explained as being due to an unusual characteristic of lung tissue. However, Prehn and Slemmer's (13) demonstration of tumor-specific antigens in hyperplastic alveolar nodules and mammary carcinomas, the report by Smith (15) of an antigenic MC-induced mammary carcinoma, and the present demonstration of an antigenic DMBA-induced carcinoma indicate that mammary carcinomas are similar to sarcomas with respect to carcinogen-induced antigenicity.

Prehn and Slemmer (13) and Lappé (5, 6) have demonstrated that tumor-specific antigens are already present in carcinogen-induced preneoplastic lesions. Thus, it appears that, in inducing the preneoplastic transformation, chemical carcinogens are able to bring about the appearance of new antigens that are later expressed in the tumors that arise in these preneoplastic lesions. The present study, however, demonstrates that carcinogen treatment of nonantigenic, preneoplastic nodules does not usually produce antigenic tumors. While the significance of these findings is not yet clear, they suggest that carcinogens may act in a different

manner when inducing a preneoplastic change in normal tissue than when inducing a tumor from a preexisting, noncarcinogen-induced, preneoplastic lesion.

Lappé (6) has suggested that the antigenic properties of preneoplastic cell populations are carried over into the resulting tumors after the neoplastic transformation has occurred and that antigenicity, or lack of it, is a property that is already established in the preneoplastic stages of tumor development. Prehn and Slemmer (13) have offered evidence that this is true in the case of antigenic tumors. The present findings constitute some evidence that the same is true in the case of nonantigenic tumors. Thus, a nonantigenic nodule outgrowth may usually give rise to nonantigenic tumors, regardless of carcinogen treatment.

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