

STUDIES WITH CARCINOGEN-INDUCED MAMMARY CARCINOMA OF THE RAT

The Trial of Auto-Immunization to Inhibit Tumor Formation after the Surgical Removal of Tumors

BY

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ABSTRACT

Auto-immunization treatment of carcinogen-fed rats using homogenates of carcinogen-induced tumors did not have any adverse effect on new tumor formation and growth as well as local recurrences. Auto-immunization treatments did not change the degree or nature of the inflammatory reaction observed in carcinogen-induced tumors.

Auto-antibodies against carcinogen-induced tumor-tissue antigen could not be demonstrated in sera to any significant extent.

However, the detection of lymphocytic infiltration of various intensity in the stroma of the tumor suggested that host defense mechanism may involve in the course of development this autochthonous tumor.

INTRODUCTION

The induction of multiple mammary carcinoma in female rats by gastric instillation of methylcholanthrene was first reported by Shay, Aegerter, Gruenstein and Komarov¹⁾ in 1949. An alternate method for the rapid production of these tumors by different carcinogenic hydrocarbons has recently been reported by Huggins, Briziarelli and Sutton²⁾ in 1959, and Huggins, Grand and Brillantes³⁾ in 1961.

These tumors have been used in experiments designed to study the endocrinological aspects of carcinogen-induced breast carcinoma²⁻⁶⁾. Because of the rapid and reliable induction procedures resulting in a high incidence of tumors, this system has also been used in chemotherapy^{7,8)} and bio-

chemical⁹⁾ studies.

This paper presents results of experiments designed to study the chemically-induced tumor-system in the light of the following considerations:

1. Following the surgical removal of all palpable tumors, whether auto-immunization treatment using homogenates of one of the tumors mixed with Freund's adjuvant has any effect on new tumor induction and growth as well as local recurrences.

2. Whether auto-antibodies against tumor tissue antigen can be demonstrated in sera.

3. Whether auto-immunization treatments change the degree of inflammatory reaction observed in carcinogen-induced tumors.

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MATERIALS AND METHODS

Tumor induction procedure:

Female Sprague-Dawley rats, 40 days old, were obtained from a commercial breeder. They were maintained in the animal room for 10 days and when 50 days old, were fed carcinogen by gastric intubation. For these experiments, a total of 40 rats were used: 7 rats that had been given 10 mg 3-methylcholanthrene (MC) three times per week for seven weeks; 7 rats that had been given a single feeding of 100 mg 3-methylcholanthrene, and 36 rats that had been given a single feeding of 15 mg 7, 12 dimethylbenz [3] anthracene (DMBA). The procedure for feeding and type and age of animals used were that recommended by Huggins and coworkers^{2,3}).

Carcinogen-fed animals were maintained in large stainless steel cages. When tumors became palpable, each animal was placed in a separate cage for individual observation. *Observation and evaluation of tumor production and growth:*

The animals were examined at weekly intervals, and body weight, number of tumors and size of tumors recorded. The average diameter of an individual tumor was obtained by measuring the maximum and minimum diameter over the skin, using a vernier caliper. In certain cases, measurements were converted, in accordance with the procedure of Huggins²), to total tumor area which was equal to the sum of the areas of all the individual tumors present in the rat at the time of observation.

Removal of tumors:

Tumor-bearing animals were anesthetized by intraperitoneal administration or Nembutal (60 mg/kg). After removal of hair with mechanical clippers, the skin was sterilized using iodinealcohol. Tumors were easily separated from the surrounding tissue by a blunt procedure. The ulcerated and

firmly attached skin and the involved muscles were removed with the tumor. Bleeding from the major vascular route, which usually entered at the base of the tumor, was controlled by ligature. The skin was closed by metal clips or continuous sutures. The operative field was inspected and described. Severe post-operative infection was not encountered.

Immunization:

Rats bearing tumors of a minimum size of 1.5 cm average diameter, were used. This size tumor yielded sufficient tissue for preparation of test-antigens. Rats were chosen and distributed between experimental and control groups so that latency of first tumor appearance and delay until operation were approximately equal for both groups. A group of 22 animals were used for auto-immunization treatment as described below. This group consisted of 2 rats that had been fed 10 mg MC, 3 times per week for 7 weeks; 4 rats that had been fed a single dose of 100 mg of MC; and 12 rats that had been given a single feeding of 15 mg of DMBA. The control series consisting of 18 rats designated as "non-immunized" were injected with Freund's adjuvant. This group consisted of 5 rats given multiple treatments of MC; 3 rats given a single feeding of MC; and 14 rats given a single feeding of DMBA. Three additional rats were included in the "non-immunized" group even though they did not receive Freund's adjuvant injection because their general behavior prior to, and after surgery, was similar to that of Freund's adjuvant-injected control rats. Excised tumors, which were first separated from connective tissue and necrotic portions, were minced with scalpels and divided into 5 to 7 portions of approximately 0.3 g each. Minced tissues that were not used immediately, were preserved in individual 2 ml vials at -78°C according to the method described by Hauschka¹⁰). The primary series of im-

munizations was started on the same day that all the tumors were removed. An aliquot of one of the minced tumors was rapidly frozen in a dry-ice-acetone bath and then thawed. This was done three times. The tissue was then homogenized in nine volumes of 0.86 per cent saline for five minutes in a glass homogenizer inserted in an ice bath. The suspension was mixed with an equal volume of complete Freund's adjuvant⁽¹¹⁾ and the mixture was further homogenized for 2-3 minutes. The animal from which the tumor had been removed was then given two injections of the preparation: One injection of 0.2 ml intramuscularly, and a second injection of 0.3 ml, subcutaneously. The remaining injections of the *primary* series were made on the third and fifth days following surgery. On the fourth and eighth weeks after surgery, a second and third series were given using the remaining tissue which had been stored in the frozen state. Preserved tissue, after thawing, was prepared and injected back into the rats from which it had been removed as described above. Immunized animals were maintained in individual cages and examined weekly for tumor appearance.

Control animals were treated with complete Freund's adjuvant, not containing homogenized tumor tissue.

Sampling of Blood:

Blood was obtained before or at the time of operation and at weekly intervals thereafter, by the orbital bleeding method described by Riley⁽¹²⁾. Under ether anesthesia, small samples of blood were collected in tubes for the hematocrit and when required, larger amounts, up to 2.0 cc, were obtained by collecting the overflowing blood, drop by drop in test tubes. No orbital infection was encountered.

Serum was prepared by the usual methods and was preserved at -30°C until used.

Plasma lactic dehydrogenase was evaluated by the method of Wroblewski and La Due.

Hemagglutination test:

The method used was the tanned sheep hemoagglutination test described by Boyden⁽¹³⁾ and modified by Aizawa⁽¹⁴⁾. Visible reactions were graded on the basis of Stavinsky's criteria⁽¹⁵⁾. Serum from rats was obtained and stored as described above. Tumor and liver tissue, preserved in the frozen state, were used as test antigens. Liver tissue was obtained by hepatectomy or at necropsy after the 8th post-operative week. As a positive control, sheep red blood cells (SRBC) coated with antigen were always tested against standard anti-rat tumor rabbit sera which had been prepared from 2 rabbits immunized with pooled induced-rat mammary tumor mixed with complete Freund's adjuvant. Only antigen-coated sheep blood cells which reacted with the standard serum were used in the tests. In addition, a "negative control" was used as follows: Tanned SRBC, not coated with antigen, were tested against 5 per cent rabbit serum, against standard anti-rat-rabbit serum, and against the test sera. Antigen-coated SRBC were also tested against 5 per cent rabbit sera as an extra control. Occasionally, positive reactions were observed in these "negative" controls. When this occurred, tests were repeated.

Histological Study:

All tumors obtained at operation and at autopsy were examined histologically. Sections were made on the largest cut surface of the tumor and stained by hematoxylin and eosin.

RESULTS

A: Tumor occurrence and growth:

At the fourth post-operative week, no significant differences were found between the immunized and non-immunized groups.

Tumors were found in 14 of the 18 (78 per cent) non-immunized animals and 18 of the 22 (78 per cent) immunized animals. A comparison of the average number of tumors (approx. 3.0/rat) and average tumor diameter (approx. 1.3 (0.9–1.73) cm) between the two groups showed no significant differences.

Before the 8th post-operative week, one rat of the control group and 2 from the immunized group died of recurring tumor or accident. In the 8th post-operative week tumor incidence, in the non-immunized group, was approximately 88 per cent and in the immunized group it was 90 per cent. No significant differences were observed between the two groups in the number of tumors per rat (3.8) or average tumor diameter (1.4 cm). Essentially similar results were obtained at the end of the 10th week.

B: Serum antibody:

This study was made with 10 of the 22 auto-immunized rats. A total of approximately 100 sera were tested against soluble tumor antigen prepared from tumor tissue that was used for immunization and from soluble liver antigen prepared from pieces of liver obtained by hepatectomy from the same animal. The anti-rat tissue rabbit sera, prepared as positive controls, showed titers against both tumor and liver-tissue-antigen coated sheep red blood cells ranging from 1:600 to 1:2,000,000.

In one rat from the 3-MC, and one from the DMBA group, titers of 1:10 were detected on the 4th to 5th weeks after immunization while no reactions were found with liver tissue antigens at this level of serum dilution. One of the rats in the 3-MC group (fed a single dose of carcinogen) remained free of tumors for 8 weeks after total tumor removal and auto-immunization, but circulating auto-antibodies were not detected. A second rat of this group which did not have

palpable tumors on the 4th week and failed to show any circulating iso-antibodies, had palpable tumors on the eighth week after total removal of all tumors.

C: Observation of inflammatory changes:

The surgically removed tumors on histologic examination showed a high incidence of diffuse round-cell infiltration. Of 42 tumors examined in the auto-immunized group, 6 had moderate to severe inflammatory changes; of 46 tumors examined from the non-immunized group, 11 tumors had moderate to severe inflammatory changes. Therefore, no essential differences were noted between the two groups.

DISCUSSION AND CONCLUSION

Attempts to control cancer cells by injection of excised tumor specimens and the search for auto-antibodies against tumor antigens have been made by Graham and Graham^{16,17}, Witebsky and coworkers¹⁸, and Finney and coworkers¹⁹.

In the present experiment, the effects of auto-immunization were studied in rats bearing carcinogen-induced mammary tumors. Because tumors appeared as localized growths at the body surface, gross tumor development could be determined by external measurement.

The mammary carcinoma produced by carcinogen feeding showed a variety of morphological patterns. The majority of the tumors, however, could be classified as papillary carcinomas, similar to those of the human breast. This type of carcinoma is seldom observed clinically. One particular characteristic is its low grade of malignancy compared with the more common, strongly invasive type of human breast carcinoma. We had evidence of metastases to the lung and regional lymphnodes, but these occurred infrequently and only in the terminal stages of the disease.

Auto-immunization was not effective against either newly-formed tumors, or tumors which recurred at surgical sites. In this study, a rat was immunized with only one of its tumors. Significant elevations in serum antibody levels, as determined by the tanned sheep red blood cell hemagglutination method, were not found in the majority of animals examined. Although two of the immunized animals did produce circulating auto-antibodies which reacted more intensively with tumor antigen than liver tissue antigen, titers were not significantly high, and tumor formation and growth was not significantly prevented in these animals. The failure to detect circulating antibodies in the majority of the rats tested may be because of a reduced immunological capacity of carcinogen-treated animals²⁰⁻²². An alternate explanation may be that circulating antibodies are absorbed by recurring tumors. If so, it may be necessary to repeatedly remove newly-produced or recurrent tumors or to use other routes of immunization as suggested by Heymann and coworkers²³, and Hunter and coworkers²⁴.

The appearance of certain inflammatory changes as seen in encephalitis^{25,26}, neuritis²⁷, adrenalitis²⁸, nephritis²⁹, and thyroiditis³⁰⁻³¹, have been ascribed to auto-immune processes. However, the pathogenesis of inflammatory reactions in neoplastic tissue is less well-defined. A number of investigators³²⁻³⁶, have suggested that inflammatory reactions in and about tumor tissue are manifestation of host defense against cancer. Other investigators have not considered inflammatory changes reliable indicators of host defense, but rather secondary phenomena associated with necrotic change³⁷ or lymphatic blockage by tumor cells³⁸. In the present study, tumors with necrotic changes were not considered in the analysis of inflammatory change. Because

these induced tumors showed no tendency to invade lymphatic routes, except in a few terminal cases and the round-cell infiltration was not significantly affected by auto-immunization procedures, other factors such as repair processes^{39,40} or bacterial infections⁴¹ may be involved in the inflammatory change. If bacterial infection is set aside for the moment, the presence of the round-cell infiltration, therefore, might be explained in terms of the processes involved in the repair of connective tissue destroyed by rapidly growing tumors³⁹. This explanation is plausible for the fast growing tumors because severe inflammatory changes were most often associated with them and mast cells, which contribute to the development of fibrosis^{42,43}, were also found to be associated with the inflammatory change.

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