Occurrence of Tumors in Agent-Free Strain C3H$_f$ Male Mice Implanted with Estrogen-Cholesterol Pellets. (20230)

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A line of agent-free strain C3H mice was originated in this laboratory in 1945, from a litter of high-mammary-tumor strain C3H mice taken from their mother by cesarean section and foster-nursed upon a low-mammary-tumor strain C57BL female without the mammary tumor agent. The line is now designated as strain C3H$_f$ (formerly C3H$_b$). In contrast with the low incidence of mammary gland tumors that was originally expected in this strain, mammary tumors appeared in 38% of the breeding females although these appeared at the advanced average age of 20 months and the incidence in virgin females was only 2% (1,2). Cell-free filtrates of these tumors failed to give a positive test for the agent, and, furthermore, there was no evidence of segregation of high-tumor lines in the pedigree chart as would have been expected had the agent been present in some of the females. In addition, studies of reciprocal crosses between strain C3H$_f$ and strain C57BL have failed to give evidence of any maternal influence in the development of the tumors in the C3H$_f$ females (2).

In view of the occurrence of mammary tumors in the breeding females of this agent-free strain, it seemed desirable to ascertain whether mammary tumors would occur in males of the strain treated with estrogen, and, if so, to test these tumors for the presence of the agent. This paper reports that mammary tumors did occur in such males and that tests of cell-free filtrates of these tumors gave no evidence of the presence of the agent. The incidence of hepatomas in these estrogen-treated C3H$_f$ males is also recorded.

Procedure. Sixty-eight strain C3H$_f$ males of litters from matings distributed at random throughout the pedigree chart were set aside for this study at the time they were weaned. When they were from one to two months of age a 5 to 8 mg pellet of cholesterol containing 10% of diethylstilbestrol was implanted subcutaneously into the right axilla of each. They were then set aside to be observed regularly for the appearance of tumors. Throughout the study they were kept in plastic cages with 8 males to the cage and were given an unlimited supply of Derwood pelleted food and tap water. Each animal was autopsied following the appearance of a tumor or when it was found to be moribund or was found dead in the cage. The tumors were fixed in Fekete's modification of Tellyesniczky's fluid (70% ethyl alcohol, 20 parts; formalin, 2 parts; glacial acetic acid, 1 part), sectioned and stained with hematoxylin and eosin for histologic examination.

Results. A total of 60 males are included in the final tabulation. The seven that were deleted included 2 that died approximately one month after implantation of the pellet, one that was killed at 3 months of age and its organs used in another study, and 4 for which satisfactory records were not obtained since the animals died in the cages and were eaten by their cage mates. Tumors occurring in the 60 males are listed in Table I.

It is significant that mammary tumors occurred in 12 of these males. This incidence of 20% is not as high as the 38% incidence of mammary tumors observed in the breeding C3H$_f$ females(1), but it is significantly higher than the 2% incidence observed in C3H$_f$ females kept as virgins(2). The mammary tumors appeared in these males at from 9 to 22 months of age with an average age of 15.1 months compared with an average tumor age of 20.3 months reported in the C3H$_f$ breeding females. Histologically these 12 tumors were adenocarcinomas, 11 of which were classified as Dunn's type A, and one as Dunn's type B (3). None showed squamous metaplasia with keratinization or other unusual histologic structure such as has been observed in some of

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TABLE I. Tumors Occurring in 60 Strain C3H$_f$ Male Mice Bearing Pellets of Diethylstilbestrol in Cholesterol and Living to an Average Age of 18.9 Months.

Type of tumor	No. mice with tumor	% with tumor	Avg tumor age, mo
Mammary tumor	12	20	15.1
Hepatoma	14	23.3	22.6
Pulmonary tumor	5	8.3	19.4
Subcutaneous sarcoma	1	1.7	24
Tumor of Harderian gland	1	1.7	20

the mammary tumors that arose in C3H$_f$ females spontaneously and following x-irradiation(4), and also observed by others (5-8) in mammary tumors that arose spontaneously or following treatment with a carcinogen in females of other agent-free lines.

While it is remarkable that 20% of these diethylstilbestrol-treated agent-free C3H$_f$ males did develop mammary tumors, this incidence is not as high as Shimkin and Wyman(9) observed in similarly treated C3H males of Andervont's subline with the mammary tumor agent. They reported an incidence as high as 86% in those implanted with pellets containing .6 mg diethylstilbestrol, a dosage comparable with that used in the present studies. Comparison between these incidences is probably justified since our subline was derived from Andervont's subline in 1941.

To compare with these treated C3H$_f$ males, 20% of which developed mammary tumors, 241 untreated C3H$_f$ males of the breeding colony have died or been killed and examined at an average age of 12.5 months and none has had a mammary tumor. Furthermore, mammary tumors have never been observed in males of the strain C3H breeding colony, and have been reported as occurring only very rarely in untreated male mice in other laboratories.

From a review of the literature it appears that the present observation is the first observation of the development of mammary tumors in estrogen-treated males without the agent. In Shimkin's review(10) a number of groups of male mice including strain 17 n.c., 19 x R_3 hybrids, strain C57BL, strain Ax, C57BL x CBA hybrids, strain C, and strain CBA, all of which did not carry the milk agent, were listed as having been treated with estrogen, and in no group did mammary tumors appear. It should be emphasized, however, that undoubtedly the C3H$_f$ mice have a more potent genetic influence for the development of mammary gland tumors than any of these other strains or hybrids.

Hepatomas occurred in 14 of the 60 C3H$_f$ males bearing estrogen pellets. The average tumor age was 22.6 months. In a group of 39 untreated C3H$_f$ males that lived to an average age comparable with that of these treated males, and that were controls in a concurrent study, hepatomas occurred in 18 at an average tumor age of 20.2 months(14). Comparison of these two groups indicates that the treatment with diethylstilbestrol reduced the occurrence of hepatomas for there is a significant difference between the two incidences; $X^2 = 5.63$; P is between .01 and .02. Andervont (12) observed incidences of hepatomas in groups of untreated C3H males ranging from 12 to 55%. When his groups are combined the incidence in the total of 409 males is 32.76% which is greater than that of the diethylstilbestrol-treated C3H$_f$ males reported here. He also noted an incidence in untreated C3H males without the agent that was higher than that of these treated C3H$_f$ males. A decrease in incidence of hepatomas was noted in castrated C3H males with the agent and in those without the agent.

The treatment with diethylstilbestrol did not, however, lower the incidence of hepatomas in the C3H$_f$ males to that of untreated C3H$_f$ females. In 188 C3H$_f$ breeding females that lived to an average age of 19.1 months, hepatomas occurred in only 9, and these occurred at an average age of 21.3 months(1). Furthermore, in a group of 100 strain C3H$_f$ virgin females that lived to an average age of 19.9 months, hepatomas occurred in 17 and at an average age of 23.8 months(2). Agnew and Gardner(13) noted a more striking reduction in incidence of hepatomas than that reported here when they injected estrogens into C3H male mice. The only hepatomas they observed in the treated males occurred in those that received very small doses of estrogen (1 μg or 3.3 μg per week). No hepatomas were observed in males that had received 8.3

TABLE II. Tests for Presence of Mammary Tumor Agent in Tumors that Arose in Treated C3H$_f$ Male Mice and in Breeding C3H Female Mice.

Tumor filtrate No.	Age tumor arose, mo	Test C3H$_f$ females: Number	Avg age, mo	No. with tumor	Avg tumor age, mo
		From treated C3H$_f$ ♂			
149	10	5	18.4	0	—
154	12	5	15.4	0	—
155	12	5	19.6	0	—
157	14	4	11.8	0	—
164	22	4	22	0	—
		From breeding C3H ♀			
150	7	5	17.6	2	16.5
151	*	4	14.8	3	14.7
152	*	4	10	1	9
153	7	5	14.8	2	19
156	10	4	17.3	1	14
158	7	5	22.8	4	21
161	9	4	16.3	3	17
162	13	4	16	4	16
163	9	4	21	1	11

* Unknown.

μg or more per week. Previous observations on the effect of estrogens on the incidence of hepatomas in male mice are reviewed by Agnew and Gardner(13).

Other tumors observed in the diethylstilbestrol-treated C3H$_f$ males included pulmonary tumors in 5 males, a subcutaneous sarcoma in one and a tumor of the Harderian gland in one. This incidence of pulmonary tumors is comparable with incidences recorded for untreated C3H$_f$ males and females(1,2,11).

Tests for mammary tumor agent. Five of the mammary tumors that arose in the treated C3H$_f$ males and that were selected at random were tested for the presence of the mammary tumor agent. Five percent extracts of each tumor were prepared in distilled water with a Waring blendor; cleared in a centrifuge at 1800 to 2000 RPM for 10 minutes; and filtered through a Selas micro-porous porcelain filter (porosity 03, maximum pore size .6 μ). To test for the agent .2 cc of the filtrate was injected intraperitoneally into 4 or 5 C3H$_f$ females 21 to 28 days of age. The test females were individually identified and observed throughout their life span for the development of mammary tumors.

Results of the tests of these C3H$_f$ tumors are given in Table II along with results of identical tests concurrently made of mammary tumors from breeding C3H females that have the agent. No mammary tumors appeared in any of the 23 females used to test the tumors from the treated C3H$_f$ males. In contrast, mammary tumors occurred in 21 of the 39 females used to test the 9 tumors from the C3H females, and none of the 9 filtrates failed to produce at least one tumor in the 4 or 5 test females. These tests, thus failed to give any evidence of the mammary tumor agent in the tumors of the C3H$_f$ males just as similar tests previously failed to give evidence of the agent in the mammary tumors that arose in the breeding females of this strain. Therefore, although mammary tumors arose in 20% of these diethylstilbestrol-treated C3H$_f$ males, they apparently arose in the absence of the agent.

Summary. 1. Mammary tumors occurred in 12 or 20% of 60 agent-free strain C3H$_f$ male mice implanted with pellets of cholesterol containing 10% of diethylstilbestrol. The average age at which the tumor arose was 15.1 months. Tests of cell-free filtrates of 5 of these tumors selected at random failed to give evidence of the presence of the mammary tumor agent. 2. Hepatomas occurred in 14 or 23% of the diethylstilbestrol-treated males at an average age of 22.6 months. This incidence is significantly less than that recorded for untreated strain C3H$_f$ males of a comparable age, but greater than that recorded

for untreated C3H$_f$ females of comparable age.

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An Aldoheptose Phosphate in a Polysaccharide Isolated from *Shigella flexneri*. (20231)

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The polysaccharide of the somatic antigen of *Shigella flexneri*, type 3, has been shown to consist mainly of D-glucose, L-rhamnose and D-glucosamine(1-3). Approximately 1% of the dry weight of the polysaccharide preparation was found to consist of non-dialyzable, acid-stable, organically bound phosphorus. After acid hydrolysis of the polysaccharide and isolation of the water soluble barium salts of the phosphate esters, the latter were found to contain some hexose-6-phosphate and a heptose phosphate(1,3).

Materials. The degraded polysaccharide corresponding to fraction 3 or 4 was used as a source of the phosphate esters(3). Dialyzed human seminal plasma was used as a source of acid phosphatase. Amberlite IR-120 cation exchange resin was used after charging with HCl and IR-4B anion exchange resin was charged with Na_2CO_3. We are indebted to Dr. N. K. Richtmyer at the National Institutes of Health for generous gifts of several heptoses.

General methods. Phosphorus was determined by the method of Fiske and Subbarow (4). Heptose determinations were made by the sulfuric acid-cysteine procedure (CyR) described by Dische for other sugars(5).* We have found it possible to characterize all 7-carbon sugars which have been made available to us by comparing spectra of each sugar after CyR4 and CyR10 reactions (*i.e.*, after heating for 4 and 10 minutes, respectively). The ketoheptoses produce spectra which differ from each other only slightly, but have a much greater absorption at 510 mμ than do the aldoheptoses. The latter produce spectra which are similar in the visible region, but vary in their ultraviolet portions. However, aldoheptoses having mirror-image configurations about the first five carbon atoms produce very similar CyR spectra. Results with the CyR10 reaction are shown in Fig. 1. B and C which have enantiomorphic configurations about the first 5 carbons have similar spectra. In like manner, the pair F and G show a marked resemblance in the ultraviolet

* Dr. Dische suggested in April, 1952 that the pink color having a maximum absorption at about 510 mμ which we obtained with our phosphate ester fraction in his CyR10 reaction might be due to the presence of a 7-carbon sugar. A paper by Dr. Dische on color reactions of heptoses is in press for the *J. Biol. Chem.*