Carcinogenic Effect of Estradiol and of Theelin in Marsh-Buffalo Mice*

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The present report is concerned with a comparison in Marsh-Buffalo mice of the action of estradiol with that of theelin when injected over a long period and in massive doses. Studies performed in this laboratory indicate that the Marsh-Buffalo strain is highly susceptible to mammary cancer and markedly resistant to the carcinogenic effect of exogenous theelin. Since the relation between estradiol and theelin in catabolism is not known, it appeared desirable to ascertain whether estradiol would be more effective, and whether the intact ovary inhibited the influence or acted synergistically to exogenous estradiol.

Suntzeff *et al.* (5) state, "In strain New Buffalo nonbreeding mice injected with estrin the percentage of tumors developing was relatively low, although it was somewhat higher than in nonbreeding control mice. There is thus some indication that the mammary gland of New Buffalo mice is relatively resistant to the stimulating action of estrin." Since these workers used only 2 to 7 mice per experiment, this work must be regarded as preliminary in nature. We were, therefore, prompted to repeat these experiments on a larger scale, since resistance to carcinogenesis caused by theelin in a high cancer strain would be a phenomenon which could not readily be ignored. The results of our experiment published to date (1), in which crystalline theelin was used, confirmed the original suggestion of Suntzeff *et al.* that the mammary gland of the Marsh-Buffalo mouse is relatively resistant to the stimulating action of estrin. The incidence of mammary cancer in our control Marsh-Buffalo mice parallels the data originally published by Marsh (3) and confirmed by Murray and Hoffman (4). It should be noted that the data of Suntzeff *et al.* indicate a considerably lower cancer incidence, and in this respect only is there a divergence in results. Since the studies reported in this paper were completed, Suntzeff *et al.* (6) have published additional data on the Marsh-Buffalo strain, and conclude that the estrogen dosage raised the incidence of tumors in nonbreeding mice to approximately that of the breeders. This deduction is obviously unwarranted as the standard deviation of the mean for the number of animals used equals the difference. In our opinion, the data again demonstrate that mice of the Marsh-Buffalo strain are highly resistant to estrogen carcinogenesis, and justify the characterization of this strain as having a relatively low incidence of cancer in Dr. Loeb's laboratory.

Experimental

At 22 days of age, litter-mate females were divided into 3 groups of 40 mice per group. One group served as a control and received injections of sesame oil. Another group received injections of estradiol.[1] The third group was ovariectomized at weaning, and received estradiol in the same amount as did the group of intact mice. The amount of estradiol administered in these experiments corresponds to the larger amounts of theelin administered in our previous experiments with theelin (1). The estradiol was dissolved in sesame oil (2 mgm. per cc.) and was administered intramuscularly or subcutaneously in doses of 0.04 cc. twice weekly for a period of 6 months. Each surviving mouse received a total dose of 3.3 to 4.2 mgm. estradiol. The controls received 0.04 cc. sesame oil twice weekly over a 6-month period. The first injections were given at the age of 1 to 2 months.

Since the purpose of these experiments was to compare the action of estradiol with that of theelin, the data of experiments with mice receiving theelin, which have been published (1), are retabulated and compared with experiments in which estradiol was used. Forty mice received theelin in each experimental group. In Tables I and II are tabulated the cumulated percentage incidences of adenocarcinoma of the breast, lymphosarcoma, spontaneous death, and combined influences removing animals from the experiment. Spontaneous death is used as a measure of toxicity. At autopsy, some of these animals were found to have lymphosarcoma. With the development of a palpable tumor, the animal was removed from the experimental group and sacrificed after the tumor had developed in diameter to 1.5 cm.

Results

Toxicity.—In the controls of both the theelin and estradiol series, the 13 and 12 per cent incidence (cumulative) of death before the 7th month is higher than is usually encountered for control mice. In a series of 120 virgin female mice housed and fed under strictly analogous conditions, but not given sesame oil, only 1 mouse died in a similar period. A slightly toxic effect of the sesame oil is indicated. In the same 7-month period, 35 per cent of the theelin-dosed mice,

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22 per cent of the estradiol-dosed mice (intact), and 20 per cent of the estradiol-dosed ovariectomized mice died. The difference for the theelin-dosed mice is statistically significant (2.4 times the standard deviation of the mean). At a later date (the 10th month) the toxic effect of the estradiol, particularly in the series of intact mice, is also clearly indicated.

Lymphosarcoma.—The incidence of lymphosarcoma is definitely increased by estradiol dosage in both intact and ovariectomized mice. The increase in the theelin-dosed mice is doubtfully significant. The maximum increase in the incidence of lymphosarcoma over the controls for the theelin-dosed mice is 15 per cent, 28 per cent for the estradiol-dosed intact mice, and 47 per cent for the estradiol-dosed ovariectomized mice. However, analysis of the data does not warrant the conclusion that estradiol enhances lymphosarcoma formation over that produced by theelin. In the case of the ovariectomized mice, the low incidence of breast tumor formation gave an opportunity for more mice to develop lymphosarcoma.

Adenocarcinoma.—According to the data of Marsh, 66 per cent of 70 virgin Marsh-Buffalo mice developed tumors by the 16th month. Our own data based on 6 groups of mice, each group comprising 30 to 50 mice, are as follows: 67, 62, 63, 63, 70, and 52 per cent respectively. Within the expected normal variation our data confirm the original data of Marsh. In our present experiments 45 per cent of the controls for the theelin-dosed group and 28 per cent of the controls for the estradiol-dosed mice developed mammary tumors in a comparable period. This decrease and variation in incidence may be attributed to the injected sesame oil. The importance of controlling this factor is indicated.

A comparison of the data in Tables I and II shows that there is a doubtfully significant increase in tumor incidence in the theelin-treated mice as compared with controls and no significant difference for either the intact or ovariectomized estradiol-treated mice as compared with controls.

Since the cumulative per cent incidence of adenocarcinoma, as shown in Tables I and II, may not be a true measure of the influence of the estrogens in enhancing this form of carcinoma because of the compli-

TABLE I: CUMULATIVE INCIDENCE OF ADENOCARCINOMA OF THE BREAST, LYMPHOSARCOMA, AND SPONTANEOUS DEATH IN 40 CONTROL AND 40 THEELIN-DOSED MARSH-BUFFALO VIRGIN FEMALE MICE

	Theelin-dosed				Control			
Age in months	Per cent adeno-carcinoma	Per cent lympho-sarcoma	Per cent sponta-neous death	Per cent removed from experiment	Per cent adeno-carcinoma	Per cent lympho-sarcoma	Per cent sponta-neous death	Per cent removed from experiment
3-6	..	8	27	27	..	3	8	8
7	3	10	35	38	0	5	13	13
8	10	10	38	48	0	8	15	15
9	20	18	43	65	5	8	20	25
10	25	20	45	72	13	8	20	32
11	33	23	45	83	18	13	25	45
12	38	23	45	88	20	13	25	48
13	38	28	50	93	23	13	25	50
14	38	28	50	93	35	15	25	65
15	40	28	50	95	43	18	25	75
16	..	..	..	..	45	..	..	78

TABLE II: CUMULATIVE INCIDENCE OF ADENOCARCINOMA OF THE BREAST, LYMPHOSARCOMA, AND SPONTANEOUS DEATH IN 40 CONTROL AND 40 ESTRADIOL-DOSED MARSH-BUFFALO VIRGIN FEMALE MICE

	Intact estradiol-dosed				Ovariectomized estradiol-dosed				Control			
Age in months	Per cent adeno-carci-noma	Per cent lympho-sarcoma	Per cent spontane-ous death	Per cent removed from ex-periment	Per cent adeno-carci-noma	Per cent lympho-sarcoma	Per cent spontane-ous death	Per cent removed from ex-periment	Per cent adeno-carci-noma	Per cent lympho-sarcoma	Per cent spontane-ous death	Per cent removed from ex-periment
3-6	..	..	12	12	..	8	10	15	..	..	8	8
7	3	5	22	25	..	10	20	25	3	..	12	15
8	5	10	32	40	3	18	25	35	3	..	12	15
9	13	18	42	58	5	25	28	45	3	..	15	18
10	13	28	48	68	8	33	32	55	5	..	15	20
11	15	28	48	70	10	33	32	60	5	..	15	20
12	20	28	48	75	15	38	38	70	15	3	15	36
13	20	28	48	75	15	38	38	70	18	3	15	38
14	25	28	48	80	15	48	38	80	22	3	15	43
15	25	28	48	80	15	50	38	85	22	3	15	46
16	25	28	48	80	15	50	38	85	28	8	15	50
17	25	30	48	..	15	50	38	..	28	10	15	..

cating factors of death (toxic reaction) and development of lymphoid tumors, the incidence of carcinoma development on the basis of surviving animals should be considered. Using surviving animals as a basis of comparison, the calculation (Tables III and IV) indicates that at the 9th month there is a significant increase in tumor incidence in the theelin-dosed mice, 34 contrasted with 7 per cent, a doubtfully significant increase in the estradiol-dosed intact mice, 18 contrasted with 3 per cent, and no increase in the estradiol-dosed ovariectomized mice. At the 10th month, the increase for the theelin-dosed mice is probably significant, 48 per cent contrasted with 17 per cent, while the differences for the estradiol-dosed mice, 18 and 12 per cent contrasted with 6 per cent, are not significant. At a later date the data for estradiol-dosed intact mice indicate an increased incidence over the controls. In the case of the estradiol-dosed ovariectomized mice this difference never becomes significant.

Influence of estradiol on mammary glands.—Mounts of whole mammary glands were prepared from 5 mice of each experimental group, at the age of 10 to 12 months. The alveolar ducts in 5 control mounts were not as wide as the ducts in 5 similar mounts made from mice which had received estradiol. This difference was marked; the number of ducts was not increased. In the control mice the alveoli were for the most part rudimentary, but in 2 of the 5 dosed mice the alveoli were well developed in some areas. In 5 mounts of breasts of castrated mice, which had received estradiol, the alveolar ducts were slightly less numerous than those of the control group and the ducts were slightly wider. In 2 out of 5 mounts the alveoli of the castrated mice were more developed.

Discussion

We are unable to account for the discrepancy between the tumor rate of the Marsh-Buffalo strain as observed in the laboratory of Loeb and in the Buffalo and Santa Barbara laboratories. A similar discrepancy for their strain A mice was observed by Loeb and his associates at St. Louis. They do not think it probable that the nature of the diet is the responsible factor. The relatively high incidence of lymphosarcoma in our Marsh-Buffalo mice is not described by others who maintain this strain, though Marsh noted its occurrence. Since our data indicate that the injection of sesame oil alone, which was slightly toxic, inhibited the rate of tumor appearance, the importance of the general well-being of the mice is clearly indicated. In the Marsh-Buffalo strain, this factor may assume proportions as great as any effect produced by exogenous

Table III: Per Cent Breast Tumors Developing on Basis of Surviving Mice

Age in months	Theelin-dosed				Control			
	Number tumors	Number mice surviving	Per cent tumors	Cumulative per cent	Number tumors	Number mice surviving	Per cent tumors	Cumulative per cent
3-6	..	29	..	..	..	37	..	..
7	1	25	3	3	..	35	..	..
8	3	21	12	15	..	34	..	..
9	4	14	19	34	2	30	7	7
10	2	11	14	48	3	27	10	17
11	3	7	27	75	2	22	7	24
12	2	5	28	..	1	21	5	29
13	0	3	0	..	1	20	5	34
14	0	3	0	..	5	14	25	59
15	1	2	33	..	3	10	21	80
16	..	..	..	..	1	9	..	..

Table IV: Per Cent Breast Tumors Developing on Basis of Surviving Mice

Age in months	Intact estradiol-dosed				Ovariectomized estradiol-dosed				Control			
	Number tumors	Number mice surviving	Per cent tumors	Cumulative per cent	Number tumors	Number mice surviving	Per cent tumors	Cumulative per cent	Number tumors	Number mice surviving	Per cent tumors	Cumulative per cent
3-6	..	35	..	..	..	34	..	..	..	37	..	..
7	1	30	3	3	..	30	..	..	1	34	3	3
8	1	24	3	6	1	26	3	3	..	34	..	3
9	3	17	12	18	1	22	4	7	..	33	..	3
10	0	13	..	18	1	18	5	12	1	32	3	6
11	1	12	8	26	1	16	6	18	..	32	..	6
12	2	10	17	43	2	12	13	31	4	26	13	19
13	0	10	..	43	..	8	..	..	1	25	4	23
14	2	8	20	63	..	6	..	..	2	23	8	31
15	..	..	..	..	..	..	..	..	0	22	0	31
16	..	..	..	..	..	..	..	..	2	..	9	40

estrogens. It is obvious that control of this factor is of the greatest significance.

Earlier studies (1) showed that dosage of 60, 180, or 1,000 rat units of theelin per mouse during a 6- to 15-day period at the age of 50 to 90 days produced no discernible changes in the mammary glands of Marsh-Buffalo mice. Two thousand rat units of theelin brought about slight changes. It is apparent from the present studies that long-continued injections of massive doses of an estrogen produce the changes which others have succeeded in producing in other strains of mice in a relatively short period and with less material. It is also apparent that the intact ovary acts synergistically, and that in the absence of the ovary, estradiol alone is able to maintain both ductal and alveolar development.

Gardner (2) has shown that in male mice of the C3H strain mammary growth and tumor development are inhibited by large doses of estrogens, and increased by smaller doses. In this connection our theelin experiments previously recorded are of interest. The injection of 2.2 mgm. theelin per mouse in 8 months did not increase the incidence of cancer over controls or mice which received 1.1 mgm. theelin over the same period. The 2.2 mgm. dose was toxic, 43 per cent of the mice dying. In another experiment, the one retabulated in this paper, the 3.8 mgm. theelin per mouse was administered in the minimum amount of sesame oil. The toxicity was reduced and the rate of tumor appearance increased over that of controls which had received the same amount of oil.

The present status of research regarding carcinogenesis in the Marsh-Buffalo mice may be summarized as follows: 1. The mouse belongs to a high cancer strain as indicated by data (1, 3, 4) obtained in three laboratories, but to an intermediate cancer strain as indicated by data obtained in a fourth laboratory (5, 6). 2. The development of mammary tumors is influenced by the general well-being. 3. Increasing the incidence of breast tumor formation significantly by exogenous estrogens in nontoxic doses has not been accomplished (1, 5, 6). 4. Toxic doses of theelin or estradiol enhance development both of lymphosarcoma and of breast tumors (not clearly established in the case of breast tumors). 5. Formation of breast tumors may be produced in the ovariectomized mouse by estrogen dosage (toxic doses). 6. Success has not attended the production of mammary gland development by estrogen dosage which would compare with that produced by gonadotropin dosage in a short interval of time (1). 7. Gonadotropin dosage at intervals over a long period either has no influence (prolan) or significantly decreases the incidence of breast tumor formation (equine and sheep pituitary gonadotropin) (1).

While we have succeeded in enhancing breast tumor formation in the Marsh-Buffalo mouse this has not been accomplished without enhancing the development of lymphosarcoma. There is, therefore, nothing specific about the estrogenic carcinogenesis, certainly nothing to indicate that the breast is more responsive. It is not known whether tumor formation could be produced in ovariectomized mice by nontoxic doses of estrogens. In view of the negative response to such doses in both intact male and female mice in enhancing carcinogenesis, this information would be desirable.

Summary and Conclusions

Mice of the Marsh-Buffalo strain receiving 0.08 cc. weekly injections of sesame oil showed a lower tumor rate than did mice that were not so treated. A 12 to 13 per cent incidence of early death indicated a toxic effect.

In groups of 40 mice, 35 per cent of theelin-dosed mice and 20 per cent of estradiol-dosed mice died within a 7-month period. Approximately 4 mgm. of estrogen were administered per mouse in a 6-month period.

Estradiol was not more toxic in ovariectomized mice than in intact mice and not more toxic than theelin, on a weight basis.

The incidence of lymphosarcoma was definitely increased by estradiol dosage in both intact and ovariectomized mice. The increase in the theelin-dosed mice was doubtfully significant.

On the basis of cumulative percentage incidence, theelin produced a doubtfully significant increase and estradiol no significant increase in breast tumor formation as compared with controls. In ovariectomized mice estradiol maintained the rate at approximately that of the controls. When the incidence was calculated on the basis of surviving mice, a significant increase in tumor formation was produced in the intact treated mice but not in the series of ovariectomized mice treated with estradiol.

REFERENCES

1. Bischoff, F., M. L. Long, J. J. Rupp, and G. J. Clarke. Endocrine Factors Influencing Tumor Development. The Effect of the Gonadotropins and Theelin Upon the Marsh-Buffalo Adenocarcinoma and Lymphosarcoma. Endocrinology, **28**:769-779. 1941.
2. Gardner, W. U. Inhibition of Mammary Growth by Large Amounts of Estrogen. Endocrinology, **28**:53-61. 1941.
3. Marsh, M. C. Spontaneous Mammary Cancer in Mice. J. Cancer Research, **13**:313-339. 1929.
4. Murray, W. S., and J. C. Hoffman. Physiological Age as a Basis for the Comparison of Strains of Mice Subject to Spontaneous Mammary Carcinoma. Cancer Research, **1**: 298-307. 1941.
5. Suntzeff, V., E. L. Burns, M. Moskop, and L. Loeb. The Effect of Injections of Estrin on the Incidence of Mammary Cancer in Various Strains of Mice. Am. J. Cancer, **27**:229-245. 1936.
6. Suntzeff, V., M. Moskop Kirtz, H. T. Blumenthal, and L. Loeb. The Incidence of Mammary Gland Carcinoma and Cancer Age in Mice Injected with Estrogen and in Noninjected Mice of Different Strains. Cancer Research, **1**:446-456. 1941.