[CANCER RESEARCH 36, 1019–1022, March 1976]

Synergism between Neutron Radiation and Diethylstilbestrol in the Production of Mammary Adenocarcinomas in the Rat[1]

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SUMMARY

When young female A × C rats were given 9.6 rads of 0.43-MeV neutrons, 32 of 33 survived a 50-week follow-up period, 2 rats developed a total of 3 mammary adenocarcinomas, and 3 rats developed a total of 4 mammary fibroadenomas. For 25 rats implanted with a 20-mg pellet containing 5 mg diethylstilbestrol and 15 mg cholesterol, average survival was 284 days; 22 rats developed a total of 182 mammary adenocarcinomas, and 21 rats developed a pituitary tumor. When diethylstilbestrol was given 2 days before neutron radiation to 35 rats, the average survival was 239 days; 32 rats developed a total of 842 mammary adenocarcinomas, 1 rat developed a single mammary fibroadenoma, and 34 rats developed a pituitary tumor. All of the 31 control rats survived the 50-week study period, and none developed tumors. Twenty-one of the rats that received both diethylstilbestrol and neutron radiation and 1 rat that received only diethylstilbestrol exhibited a multiple mammary adenocarcinoma response with a range of 18 to 72 mammary adenocarcinomas per rat. These results were interpreted to mean that a synergistic interaction between diethylstilbestrol and neutron radiation on mammary adenocarcinoma formation occurs in terms of an earlier onset and a larger number of mammary adenocarcinomas. These results confirm and complement a previously reported synergistic interaction between diethylstilbestrol and X-radiation on mammary adenocarcinoma formation in A × C female rats.

INTRODUCTION

Segaloff and Maxfield (10) reported that DES[5] and X-irradiation acted in a synergistic fashion for the production of mammary adenocarcinomas in female A × C rats. This finding, when combined with the fact that in other strains of rats neutron radiation is an even more potent inducer of mammary neoplasia than are X-rays (12, 14), makes a study of the possible synergistic interaction of DES and neutron radiation of great interest. We present data indicating that there is indeed a synergistic interaction between DES and neutron radiation for the production of mammary adenocarcinomas in female A × C rats.

MATERIALS AND METHODS

The experimental plan of Segaloff and Maxfield (10) was followed in a general way. Female A × C rats were purchased from a commercial source (Microbiological Associates, Inc., Bethesda, Md.) when they were 28 to 35 days of age. Rats from 3 weekly shipments were assigned to 4 experimental groups so that each experimental group contained an approximately equal proportion of rats from each of the shipments. Thus each group was made up of rats with a range of 50 to 71 days of age and an average body weight range of 114 to 117 g when the experiment began. Twenty-five rats each received a 20-mg compressed pellet, made of 5 mg of DES and 15 mg of cholesterol (purchased from Steraloids, Pawling, N. Y.) in a pellet press (Parr Instrument Co., Moline, Ill.), implanted s.c. in the intrascapular region while under light ether anesthesia. A 2nd group of 25 rats each received 9.6 rads of 0.43-MeV neutrons 2 days after each had received a DES pellet. The neutron dose was given in about 2 hr using methods described previously (12). A 3rd group of 33 rats received only neutron radiation, and a 4th group of 31 rats were reserved as nontreated controls.

Rats were kept in an animal room equipped with a laminar flow system (1) and fed on commercial rat chow and water *ad libitum*, under conditions of 12 hr (8 a.m. to 8 p.m.) of fluorescent light per day. Each rat was identified by a numbered ear tag and each mammary tumor, as it was located by once per week palpation, was recorded as to anatomical location using the nipples as reference points.

Because 2 distinct types of mammary tumor responses were noted, individual or multiple, each type of response was recorded separately. Individual mammary tumors were removed under ether anesthesia at a size of about 2 cm. If a 2nd individual tumor was found at the site of a previously removed tumor, it was not recorded as a 2nd individual tumor unless a 10-week period had elapsed between removal of the 1st tumor and detection of the subsequent tumor. In the other type of mammary tumor response, multiple tumors were often detected within a single quadrant of mammary tissue over a period of about 1 week. Because there were too many tumors to be counted by palpation alone, the entire quadrant was removed, fixed, defatted, stained with hematoxylin, cleared, and stored in methyl salicylate; tumors were then counted under a dissecting microscope. If additional detail was required, the quadrant

[1] Research done under Contract Y01-CP-3021 with the Biological Models Segment of the Carcinogenesis Program of the National Cancer Institute.

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[3] Supported in part by Training Grant 5 T01-CA05243 from the National Cancer Institute.

[4] Brookhaven National Laboratory is operated by Associated Universities, Inc., under contract to the U. S. Energy Research and Development Administration.

[5] The abbreviation used is: DES, diethylstilbestrol.

Received September 11, 1975; accepted November 29, 1975.

was sectioned, stained with eosin, and examined. This procedure probably underestimated the total number of multiple tumors per quadrant. The multiple tumor response within a single quadrant was usually found within all 4 quadrants within a 1 to 3-week period. However, quadrants were removed at the rate of 1/week until 3 quadrants were removed. The 4th quadrant was examined only after the rat was killed. Rats were killed when they became moribund, or when tumor removal was no longer feasible, or 50 weeks after the day of DES pellet implantation. At autopsy each rat was inspected for gross pathology, and if the rat was treated with DES the pellet was recovered. All mammary tumors were sectioned, stained with hematoxylin and eosin, and given a pathological classification of either adenocarcinoma or fibroadenoma according to criteria consistent with those published by Young and Hallowes (17). Abnormal pituitary glands were recorded as pituitary tumors if they were hemorrhagic, fragile, and exceeded 100 mg or 0.1 cu cm. Body weights and days of survival were tested for significant differences by means of the *t* test (13). Tumor incidence differences were tested by means of the χ^2 contingency test (7).

RESULTS

The DES treatment, with or without neutron radiation, depressed body weight as early as 2 days after implantation and reduced body weight gains throughout the course of the experiment. Significant mortality was found in the DES group and the DES plus neutron group (Table 1), and this mortality was associated with large pituitary tumors that produced cachexia. Pituitary tumors were found only in DES-treated rats or DES-plus-neutron-treated rats. The number of rats with mammary fibroadenomas, in any of the experimental groups, was not different from nontreated control rats (Table 1).

Almost all rats given DES or DES plus neutron radiation developed mammary adenocarcinomas (Table 1). Rats given DES plus neutron radiation tended to develop mammary adenocarcinomas somewhat sooner than those rats given only DES (Chart 1). From the 20th through the 28th weeks, the incidence of rats with 1 or more mammary adenocarcinomas was greater in the group that received both DES plus neutrons than in the group that received only DES. Many more mammary adenocarcinomas per rat were found in the DES-plus-radiation group than in the DES-only group. This was due largely to the fact that 21 of 35 rats treated with both DES and neutrons developed a multiple tumor response while only 1 of 25 rats given only DES showed the multiple mammary adenocarcinoma response.

DISCUSSION

The observation of Segaloff and Maxfield (10) of a synergistic interaction between DES and X-radiation on the pro-

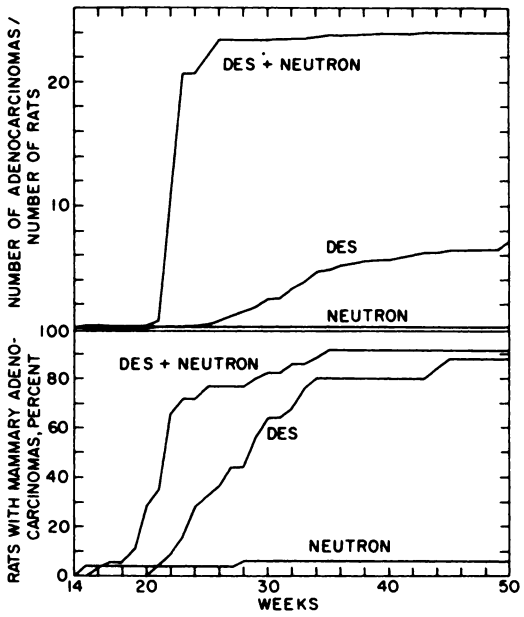

Chart 1. The percentage of rats with mammary adenocarcinomas *(bottom)* and the total number of mammary adenocarcinomas divided by the starting number of rats *(top)* for each group of treated rats plotted against weeks of DES treatment. *NEUTRON*, 9.6 rads of 0.43-MeV neutron radiation; *DES*, 5 mg DES and 15 mg cholesterol pellet. *DES + NEUTRON*, DES given 2 days before neutron radiation. No mammary adenocarcinomas were found in nontreated, control rats.

Table 1

Number of female A × C rats, number of tumors, and survival over a 50-week period following 5 mg of DES, or 5 mg of DES and 9.6 rads of 0.43-MeV neutrons 2 days later, or 9.6 rads of neutrons, or no treatment

				Rats with							
Treatment	Starting no. of rats	No. alive 50 wk later	Days until death	Pituitary tumor	Mammary adenocarcinoma	Individual (range 1–13) mammary adenocarcinomas	Multiple (range 18–72) mammary adenocarcinomas	Total no. of mammary adenocarcinomas	Rats with mammary fibroadenomas	Total no. of mammary fibroadenomas
Neutron	33	32[a]	349	0[a]	2[a]	2[a]	0	3	3	4
DES	25	3	284 ± 53[b,c]	21	22	21	1	182	0	0
Both	35	1	239 ± 44[d]	34	32	11[a]	21[a]	842	1	1
None	31	31[a]	350	0[a]	0[a]	0[a]	0	0	0	0

[a] Different from DES by χ^2 test, $p < 0.01$.
[b] Mean ± S.D.
[c] Different from neutron by *t* test, $p < 0.01$.
[d] Different from DES by *t* test, $p < 0.01$.

duction of mammary adenocarcinoma formation in female A × C rats has been confirmed by the present experiment wherein neutron radiation was substituted for X-radiation. In both experiments, DES treatment induced a small number of mammary adenocarcinomas in a large percentage of rats, while neither X-rays nor neutrons were effective in this regard. However, when DES was given with either type of radiation, a very large number of mammary adenocarcinomas were found in a large percentage of rats. The combined radiation and DES treatment evoked a multiple mammary adenocarcinoma response ["essentially total carcinogenesis" in the words of Segaloff and Maxfield (10)] in about 2/3 of the rats while either agent alone tended to produce only individual mammary adenocarcinomas. Finally, the combined radiation and DES treatment reduced the latent period of mammary adenocarcinoma appearance as compared to either agent alone. Thus all of the experimental findings in regard to mammary carcinogenesis in the female A × C rat using combined DES and X-ray treatment have been duplicated using the combined treatment of DES and neutrons.

Two additional observations, not mentioned by Segaloff and Maxfield (10) were noted. First, DES-treated rats, with or without neutron radiation, showed an immediate loss of weight and a continued depression of body weight gains. It has been known for a long time that prolonged administration of estrogen to rats depresses body weight (18). Secondly, almost all DES-treated rats, with and without neutron radiation, developed large pituitary tumors. Both of these effects, along with the large numbers of DES-induced mammary adenocarcinomas that were subjected to surgical removal, contributed to the reduced survival observed in DES-treated rats. Each DES pellet was recovered from each rat thus treated, and it seems reasonable to conclude that the amount of DES released from the pellet was a relatively large dose since it was effective in inducing pituitary and mammary tumor formation and in reducing body weight.

Almost all of the rats with mammary adenocarcinomas were from rats treated with DES and almost all of these rats exhibited a pituitary tumor. Since it is known that DES treatment often induces pituitary tumors that secrete large amounts of prolactin (4), it seems logical to suggest that the observed pituitary tumors were prolactin-secreting tumors and that prolactin plays a role in the genesis of the mammary adenocarcinomas in response to DES and possibly in the synergistic mammary adenocarcinoma response to DES plus neutron radiation. Serum prolactin determinations and immunohistochemical studies of pituitary glands from similarly treated A × C rats are in progress in order to learn more about the role of prolactin with regard to the mammary adenocarcinoma responses to DES and to DES plus neutron radiation.

The synergistic interaction between radiation and DES on mammary adenocarcinoma formation as presently observed in female A × C rats may not hold for other rat strains. When 31 female Sprague-Dawley rats were given 400 R of X-rays on the 40th day of age, 11 mammary adenocarcinomas and 29 mammary fibroadenomas were found over a 1-year follow-up period (11). When a fused cholesterol pellet containing 1.5 mg of DES was implanted immediately after 400 R to Sprague-Dawley rats, only 2 mammary adenocarcinomas and 9 fibroadenomas were found in 31 rats given both agents. DES by itself was without effect in this experiment. It would appear that DES alone has the capacity to induce mammary adenocarcinoma formation in the A × C strain but not in the Sprague-Dawley strain, and that DES acts in a synergistic fashion with radiation on mammary adenocarcinoma formation in A × C rats, but that in Sprague-Dawley rats DES and radiation interact in an inhibitory fashion on both mammary adenocarcinoma and fibroadenoma formation. Perhaps, a clue to these strain differences may be found at the level of the pituitary gland response to DES. Although the experiments with female Sprague-Dawley rats and female A × C rats were not identical in all respects, the fact remains that the DES-treated A × C rats exhibited pituitary tumors while the DES-treated Sprague-Dawley rats did not exhibit pituitary tumors. However, the finding of a synergism between DES and radiation, along with pituitary tumors that presumably secrete prolactin, in A × C female rats is in general agreement with the finding of Yokoro and Furth (16) who reported a synergism between radiation and transplanted, prolactin-secreting pituitary tumors on mammary carcinogenesis in female W/Fu rats.

The fact that both neutron radiation, as used in the present experiment, and X-radiation, as used by Segaloff and Maxfield (10), acted synergistically with DES on mammary adenocarcinoma formation is of some radiobiological interest. Neutrons interact with the atomic nuclei of biological material to produce dense ionization while X-rays interact with orbital electrons to produce diffuse ionization (2). Since both types of radiation showed a synergistic interaction with DES on mammary adenocarcinoma formation, it can be concluded that both dense ionization from neutron radiation and diffuse ionization from X-rays produced a result that was qualitatively similar. However, in other experiments with Sprague-Dawley rats (12, 14), neutron radiation produced more mammary neoplasia than did X-radiation when the 2 types of radiation were compared on an energy absorbed per g basis. This would indicate that neutron radiation is more efficient than X-radiation for the induction of mammary neoplasia, and thus the 2 types of radiation produce results that are quantitatively different yet qualitatively similar. X-ray exposure has been associated with an increased risk of developing breast cancer in the human female (5, 6, 8, 15), and some authors (9) have suggested that the risk of developing leukemia in man may be greater for neutron exposure than for γ-ray exposure. Because it is likely that an increasing number of women will be exposed to neutrons as *in vivo* neutron activation analysis techniques come into increasing use and because many women now receive exogenous estrogens, it would seem prudent to develop model systems to study the possible interactions of estrogens and neutron radiation on mammary gland carcinogenesis (3).

REFERENCES

1. Beall, J. R., Torning, F. E., and Runkle, R. S. A Laminar Flow System for Animal Maintenance. Lab. Animal Sci., *21:* 206–212, 1971.

2. Casarett, A. P. Radiation Units. *In:* Radiation Biology, pp. 31–33. Englewood Cliffs, N. J.: Prentice-Hall, Inc., 1968.
3. Cohn, S. H., Ellis, K. J., and Wallach, S. *In Vivo* Neutron Activation Analysis, Clinical Potential in Body Composition Studies. Am. J. Med., *57:* 683–686, 1974.
4. Furth, J., and Clifton, K. H. Experimental Pituitary Tumors. *In:* G. W. Harris and B. T. Donovan (eds.), The Pituitary Gland, Vol. 2, pp. 460–497. London: Butterworths, 1966.
5. Grundy, G. W., and Uzman, B. G. Breast Cancer Associated with Repeated Fluoroscopy. J. Natl. Cancer Inst., *51:* 1339–1340, 1973.
6. MacKenzie, I. Breast Cancer following Multiple Fluoroscopies. Brit. J. Cancer, *19:* 1–8, 1965.
7. Mainland, D., and Murray, I. M. Tables for Use in Fourfold Contingency Tests. Science, *116:* 591–594, 1952.
8. Myrden, J. A., and Hiltz, J. E. Breast Cancer following Multiple Fluoroscopies during Artificial Pneumothorax Treatment of Pulmonary Tuberculosis. Can. Med. Assoc. J., *100:* 1032–1034, 1969.
9. Rossi, H. H., and Kellerer, A. M. The Validity of Risk Estimates of Leukemia Incidence Based on Japanese Data. Radiation Res., *58:* 131–140, 1974.
10. Segaloff, A., and Maxfield, W. S. The Synergism between Radiation and Estrogen in the Production of Mammary Cancer in the Rat. Cancer Res., *31:* 166–168, 1971.
11. Shellabarger, C. J., Aponte, G. E., Cronkite, E. P., and Bond, V. P. Studies on Radiation-Induced Mammary Gland Neoplasia in the Rat. VI. The Effect of Changes in Thyroid Function, Ovarian Function, and Pregnancy. Radiation Res., *17:* 492–507, 1962.
12. Shellabarger, C. J., Brown, R. D., Rao, A. R., Shanley, J. P., Bond, V. P., Kellerer, A. M., Rossi, H. H., Goodman, L. J., and Mills, R. E. Rat Mammary Carcinogenesis following Neutron or X-Radiation. *In:* Biological Effects of Neutron Radiation, pp. 391–400. Vienna: International Atomic Energy Agency, 1974.
13. Snedecor, G. W. The Comparison of Two Groups. *In:* Statistical Methods, Ed. 4, Chapt. 4, pp. 75–88, Ames, Iowa: The Iowa State College Press, 1946.
14. Vogel, H. H., Jr., and Zaldivar, R. Neutron-induced Mammary Neoplasms in the Rat. Cancer Res., *32:* 933–938, 1972.
15. Wanebo, C. K., Johnson, K. G., Sato, K., and Thorslund, T. W. Breast Cancer after Exposure to the Atomic Bombings of Hiroshima and Nagasaki. New Engl. J. Med., *279:* 667–671, 1968.
16. Yokoro, K., and Furth, J. Relation of Mammotropes to Mammary Tumors. V. Role of Mammotropes in Radiation Carcinogenesis. Proc. Soc. Exptl. Biol. Med., *107:* 921–924, 1961.
17. Young, S., and Hallowes, R. C. Tumors of the Mammary Gland. *In:* V. S. Turusov (ed.), Pathology of Tumours in Laboratory Animals, Vol. I, Tumors of the Rat, part 1, pp. 31–73. IACR Scientific Publication No. 5. Lyon, France: International Agency for Cancer Research, 1973.
18. Zondek, B. Tumor of the Pituitary Induced with Follicular Hormone. Lancet, *1:* 776–778, 1936.

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