Synergism of Estrogens and X-Rays in Mammary Carcinogenesis in Female ACI Rats [1, 2, 3]

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ABSTRACT—A previously demonstrated synergistic interaction between diethylstilbestrol (DES) and radiation on rat mammary carcinogenesis was extended to another estrogen, 17-ethinylestradiol (EE2). These newly reported results with EE2 demonstrated that the previously reported synergistic interaction between DES and radiation is not confined to just DES. Instead, these new results implied that the synergistic interaction is a synergistic interaction between the estrogenic activity of DES and radiation on rat mammary carcinogenesis. Female inbred ACI rats were used. By the end of the experiment, no neoplasia was detected in rats bearing cholesterol pellets, with and without X-ray exposure. No significant tumor data were obtained from rats treated with 0.1 mg EE2, with and without X-rays. Approximately 50% of the rats treated with DES and approximately 90% of the rats treated with 1 mg EE2 had 1 or more mammary adenocarcinomas (MAC). X-rays synergistically increased the number of MAC per rat in the groups implanted with DES or 1 mg EE2. X-rays also increased the trend toward earlier increased incidence of rats with MAC as compared to rats treated with estrogens only. All rats treated with DES and 1 mg EE2 had pituitary tumors. The mean weight of the pituitary tumors in the groups treated with 1 mg EE2 was approximately 1.5 times that of the groups treated with DES. Mean terminal plasma prolactin levels for rats treated with 1 mg EE2 or DES were, respectively, 17.5 and 9.5 times control values.—JNCI 1981; 67:455-459.

Radiation is the only agent for which there is good statistical evidence as a cause of mammary carcinogenesis in both experimental animal models and humans. Recent epidemiologic studies suggested that exogenous estrogens are associated with an increased risk of human breast cancer in menopausal women (*1, 2*). Although we are unaware of any reports on interactive effects of radiation and estrogens in humans, DES has been demonstrated to interact synergistically with radiation in the production of MAC in female ACI (A × C or Irish) rats (*3, 4*). Combined treatment with both agents produced more MAC per rat than the numbers obtained by summation of results from DES treatment only and radiation only. Synergistic interactions between other estrogens and radiation on rat mammary carcinogenesis have not been published. Therefore, we undertook an investigation to compare the interaction of X-rays with DES to the interaction of X-rays with EE2, an estrogen that is widely used by women.

MATERIALS AND METHODS

The experimental animals were weanling females from our own colony of ACI rats. At 2-4 months of age, 170 rats were assigned to 4 groups so that each group contained rats of the same range of ages. Each rat was given a sc implant on day 0 of the experiment of a single 20-mg compressed pellet (*4*). The pellets for each group contained either cholesterol only, cholesterol and 2.3 mg DES (≈1.25 mg DES/100 g body wt), cholesterol and 1 mg EE2, or cholesterol and 0.1 mg EE2 (table 1). Two days later, approximately half the rats in each group were given total-body irradiation of 150 R of 250-kV peak X-rays, and the remaining animals were sham irradiated (table 2).

Each rat was palpated weekly for mammary tumors, and the location of each tumor was recorded by the use of the nipples as reference points. Mammary tumors were removed for histopathologic classification (*5*) when they grew to approximately 2 cm in diameter or at the termination of the experiment on day 190 after pellet implantation. The experiment was initiated in two phases with approximately equal numbers of rats for each treatment entered 1 day apart. The study was terminated on 2 sequential days so that the rats whose blood was used for prolactin determination were all killed between 0900 and 1100 hours. At termination, the rats were killed by decapitation, and trunk blood was collected by radioimmunoassay (*6*) from 5 rats in each treatment group per day for prolactin determinations. Vaginal smears were taken from the trunk of each decapitated rat that was used for the prolactin determinations. Remaining breast tissue was subjected to wholemount preparation for evaluation of mammary gland development. The preparations were scored from 1 to 5 at 0.5 intervals, with the higher scores given to more developed mammary tissues (*7*). At autopsy, other organs, especially the reproductive tract

ABBREVIATIONS USED: DES = diethylstilbestrol; EE2 = 17-ethinylestradiol; MAC = mammary adenocarcinoma(s); MMAC = multiple mammary adenocarcinoma(s).

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[3] The animal care conditions fully meet or exceed the American Association for Accreditation of Laboratory Animal Care standards.

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TABLE 1.—*Responses of female ACI rats to estrogen treatment*

Group[a]	No.	Body weight gain, %[b]	Pituitary weight, mg[b]	Mammary development[c]
I Cholesterol	35	20.7±1.1	13.8±0.4[d]	1.5 (1.0–2.5)
II 2.3 mg DES	37	6.7±.08[e,f]	33.9±1.6[g]	5.0 (4.0–5.0)
III 0.1 mg EE2	48	15.1±1.1[e]	15.1±0.5[d]	3.5 (2.0–4.5)
IV 1 mg EE2	46	13.8±1.6[e]	63.2±7.4	5.0 (3.5–5.0)

[a] Each group includes animals with and without X-rays.
[b] Values are means ± SE.
[c] Scored with increasing development from 1 to 5, to the nearest 0.5. Values are means (ranges).
[d] Significantly less than group II or IV ($P<0.001$, t-test).
[e] Significantly less than group I ($P<0.001$, t-test).
[f] Significantly less than group III or IV ($P<0.01$, t-test).
[g] Significantly less than group IV ($P<0.001$, t-test).

and pituitary glands, were examined for gross abnormalities.

Each quadrant of breast tissue was scored as an MMAC response if it contained 5 or more confirmed MAC. When there were more than 5 MAC per quadrant, the tumors tended to coalesce. Therefore, each MMAC response was assigned a maximum value of 5, and the total number of MAC reported for each group that had MMAC (table 2) is an underestimate of total MAC.

The data for incidence and time of appearance of MAC and MMAC were subjected to a computerized trend and homogeneity analysis, including Cox's test and the Kruskal-Wallis test (*8*). These data were also subjected to 2 × 2 chi-square analyses. Pituitary weights and the mean times to detection of first MAC in each rat were subjected to two-way analyses of variance followed by Dunnett's and Student's t-tests (*9*).

RESULTS

Only 4 out of the 170 rats in the experiment died before the experiment was terminated (day 190). These 4 animals were randomly distributed among the following treatment groups: cholesterol, 1 mg EE2 and X-rays, 0.1 mg EE2 and X-rays, and DES. In the groups that received DES only, or 1 mg EE2 and X-rays, the individual rats that died early survived long enough (>160 days) to be included in the tumor data, and no corrections were made for intercurrent mortality.

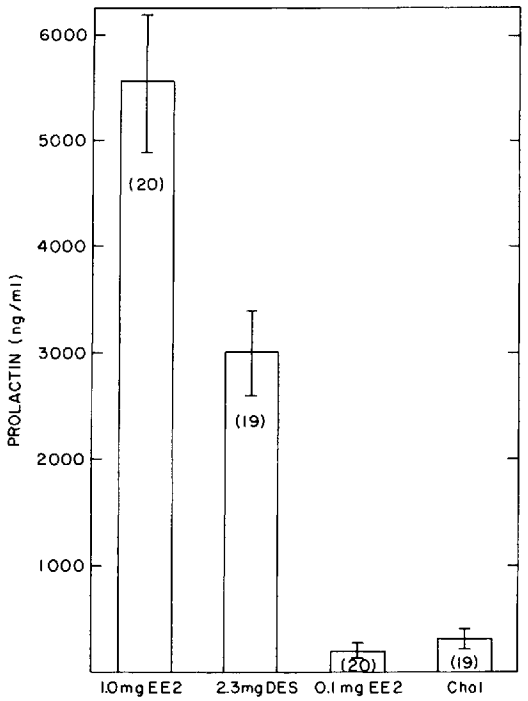
Differences in mean percentage body weight gains were related to estrogen treatment, without a significant effect of X-rays (two-way analysis of variance). Therefore, the body weight data for rats with and without X-rays were pooled (table 1). By the end of the experiment, the cholesterol controls gained significantly more than the other groups. DES-treated animals gained significantly less than the rats treated with either dose of EE2 (table 1).

Differences in pituitary weights were also related to estrogen treatment without a significant effect of X-rays (two-way analysis of variance). The pituitary glands, in terms of weight and gross pathology, in the groups treated with 0.1 mg EE2 were not different from the cholesterol controls (table 1). All the rats in the other treatment groups had hemorrhagic tumors. The mean pituitary weight of the DES-treated rats was approximately threefold higher than that of controls. In the groups that received 1 mg EE2, the mean pituitary weight was more than 1.5 times that of the DES-treated rats. Plasma prolactin levels paralleled the results obtained from weighing the pituitary glands. The order of mean prolactin levels from high to low for the pellet treatments was 1 mg EE2>2.3 mg DES>0.1 mg EE2≈cholesterol (text-fig. 1). Only the rats that were pelleted with 0.1 mg EE2 or cholesterol

TABLE 2.—*Interactions of estrogens and X-rays on mammary carcinogenesis in female ACI rats*[a]

Group	Rats with MAC	Rats with MMAC[b]	Rats with 4 MMAC	Total No. of MAC[c]	MAC per rat with MAC	MAC per rat	Mean time, days, to first MAC
I Cholesterol	0/17	0	0	0	0	0	0
II Cholesterol + X-rays	0/18	0	0	0	0	0	0
III 2.3 mg DES	9/19[d]	1	0	42	4.7	2.2	148
IV 2.3 mg DES + X-rays	11/19[d]	9[e,f]	3	151	13.7	8.0	148
V 0.1 mg EE2	0/24	0	0	0	0	0	0
VI 0.1 mg EE2 + X-rays	1/24	0	0	4	4	0	144
VII 1 mg EE2	20/23[d,g]	4	0	123	6.2	5.4	165
VIII 1 mg EE2 + X-rays	21/24[d,h]	15[e,f]	10[h]	300	14.3	12.5	135

[a] Rats irradiated (150 R) 2 days after implantation of pellets containing cholesterol only or estrogen and cholesterol.
[b] Five or more MAC per individual quadrant of breast tissue (maximum count scored at 5 MAC per individual quadrant).
[c] Maximum count per rat=20 for rats with 4 MMAC.
[d] Groups III, IV, VII, and VIII are different from groups I, II, V, and VI ($P<0.01$, χ^2).
[e] Groups IV and VIII are different from groups II and VI ($P<0.01$, χ^2).
[f] Group IV vs. III and group VIII vs. VII ($P<0.01$, χ^2).
[g] Group VII are different from group III ($P<0.05$, χ^2).
[h] Group VIII vs. IV ($0.05<P<0.1$, χ^2).



TEXT-FIGURE 1.—Blood plasma prolactin levels in female ACI rats 190 days after pellet implantation. *Bars* represent SEM. *Values in parentheses* are No. of animals per treatment, with and without X-rays. 1 mg EE2 vs. all other groups, $P<0.01$, Student's *t*-test. 2.3 mg DES vs. all other groups, $P<0.01$, Student's *t*-test.

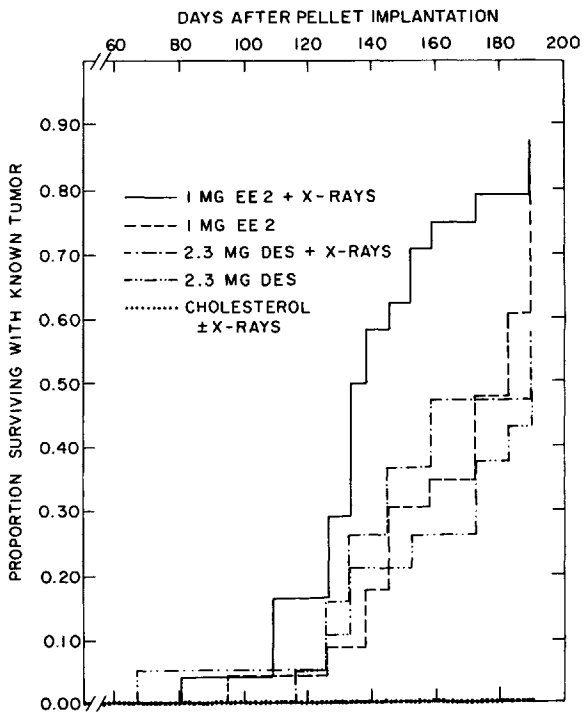
alone (with or without X-rays) showed evidence of cycling in their vaginal smears.

Mammary gland development was near maximal, with extensive lobulo-alveolar and ductal growth resembling that of late pregnancy, in all the groups treated with 1 mg EE2 or DES (table 1). All the mammary tumors detected in this experiment were classified as MAC. None were found in the groups treated with cholesterol only, cholesterol and X-rays, or 0.1 mg EE2 only (table 2). Only 1 rat treated with both 0.1 mg EE2 and X-rays had 4 MAC. Approximately 50% of the DES-treated rats, with or without X-rays, and nearly 90% of the rats treated with 1 mg EE2, with or without X-rays, each bore at least 1 MAC by the end of the experiment. Most of the rats in these groups had more than 1 MAC. Analysis of variance and *t*-tests of mean times to first MAC per rat revealed an acceleration of tumor incidence for the group that received both 1 mg EE2 and X-rays as compared to the group with 1 mg EE2 only (table 2). Furthermore, trend analyses (text-fig. 2) showed that the incidence of rats with tumors was accelerated in the 1-mg EE2 groups with respect to the comparable DES groups. By day 140 after pellet implantation, the incidence of rats with tumors in the group that received 1 mg EE2 and X-rays was greater than that detected at the end of the experiment in the groups treated with DES, with or without X-rays. Similarly, by day 175 the incidence in the group that received 1 mg EE2 only was greater than that detected at the end of the experiment in the group treated with DES only. Irradiation of estrogen-treated rats also accelerated the incidence of rats with tumors as compared to rats that received estrogens only (text-fig. 2).

Irradiation of rats treated either with 1 mg EE2 or DES produced more MMAC than did either respective estrogen treatment only, and more than irradiated rats with 0.1 mg EE2 or cholesterol. Almost all the MMAC were detected at the end of the experiment. Only rats that were irradiated after implantation of 1 mg EE2 or DES exhibited "essentially total mammary carcinogenesis" (*3*), that is, a MMAC response in all four quadrants of mammary tissue per rat. However, this type of response was significant only for 1 mg EE2 (table 2). The number of MAC per tumor-bearing rat was not different when either group with 1 mg EE2, irradiated or nonirradiated, was compared to the corresponding group with DES. However, on a per-rat-in-group basis, the rats with 1 mg EE2 tended to have approximately twice as many tumors as the comparable DES-treated rats.

DISCUSSION

Our observations confirm previous reports that pelleted DES produces mammary carcinogenesis in female ACI rats, that the mammary tissue of this strain is relatively insensitive to radiation treatment alone, and that when estrogen and radiation treatment are combined there is a synergistic interaction on mammary carcinogenesis (*3*, *4*). This synergistic interaction with radiation has previously only been reported for DES.



TEXT-FIGURE 2.—Trends in incidence of rats with mammary adenocarcinomas after pellet implantation, with or without X-irradiation (150 R).

To learn if this interaction was due to the estrogenic properties of DES, we decided to extend our investigations to another estrogen. EE2 was selected because of its prolonged action and because it is in widespread use by women. We do not know what the release rate or blood levels of EE2 were in the current experiment. However, in a previous study by Blankenstein et al. (*10*), 1 mg of EE2 implanted sc in the form of a pellet produced a temporal pattern of blood levels that was compatible with our observations of an exponentially decreasing rate of estrogen release from pellets containing mixtures of DES and cholesterol (*6*). We therefore chose to use 1 mg of EE2 as our high dose, and 0.1 mg was used to estimate the lower limit for synergistic interaction of EE2 with radiation.

Synergism is defined as an interaction of two agents in a system to produce a result that exceeds the sum of the results from each agent given separately. Synergism between DES and radiation in rat mammary carcinogenesis has been reported for two parameters: the total number of mammary tumors produced (*3, 4*) and the number (incidence) of rats bearing 1 or more mammary tumors (*11*). During the course of the current experiment, both types of synergism were observed.

The first type of synergism (total number of MAC) was clearly demonstrated for the interactions of X-rays with either 1 mg of EE2 or DES by the marked increases of total MAC and MMAC above comparable individual treatments. In fact, the total MAC response was underestimated because each MMAC response was arbitrarily valued as 5 MAC.

The second type of synergism (incidence of rats with MAC) was most clearly exemplified in the groups that were treated with X-rays and 1 mg EE2, or X-rays and DES, by the incidences of rats with MMAC, and the incidence of rats with total involvement of their mammary tissues (4 MMAC/rat). In the current study, the final incidence of rats with MAC was not different for treatment with EE2 and X-rays versus EE2 only, or for DES and X-rays versus DES only. However, during the course of the experiment, differences in the rate of increase of incidence of rats bearing at least 1 MAC were readily apparent (text-fig. 2). In a previous study on F344 rats, we implied that this type of synergism represents an acceleration of carcinogenesis (*11*).

The present experiment also indicates that the interaction of EE2 and DES with X-rays is primarily due to their estrogenic properties. Under the conditions of this experiment, 1 mg of the steroidal estrogen EE2, when administered in cholesterol pellets, was a more effective synergist than 2.3 mg of DES. We believe that the greater mammary tumorigenic effect of EE2 is due to its more potent estrogenic activity (per milligram or per mole), as evidenced by the greater pituitary growth and prolactin secretory responses to EE2 than to DES. The overall poorer carcinogenic responses to DES may be related to higher toxicity, as reflected in the poorer gain in body weight by DES-treated rats than for those that received EE2. In a dose-response study of DES (up to 5 mg, with and without X-rays) in which the data were corrected for intercurrent mortality, mammary carcinogenesis increased with increasing doses of DES (*12*). This increase would not be expected if DES toxicity severely limited mammary adenocarcinoma induction. Furthermore, in the current investigation mortality was not an important factor.

We have previously suggested that the mammary carcinogenic response to DES is largely mediated by stimulation of prolactin secretion from the pituitary gland or by the formation of prolactin-secreting pituitary tumors (*6, 12, 13*). In the current study, the pituitary gland responses could explain why the mammary neoplastic responses to EE2 were more marked and more accelerated than to DES. Yokoro and his coworkers (*14, 15*) have reported that implantation of prolactin-secreting pituitary tumor tissue in Wistar/Furth rats, as much as 12 months after irradiation, resulted in increased mammary carcinogenic responses over those produced by radiation only. Even at low doses of radiation which alone yielded no MAC, subsequent tumor implantation produced mammary carcinogenesis. Under the conditions of the current experiment, 0.1 mg EE2, with or without X-rays, yielded neither significant mammary tumor responses, pituitary tumors, nor circulating prolactin levels above those of controls. However, it is possible that we would have detected such responses had we continued the experiment for a longer duration. The only rat that had an MAC response, out of 40 rats that received 0.1 mg EE2, also had an enlarged pituitary gland (26.5 mg). Unfortunately, a blood sample was not taken from this rat for prolactin measurement. In a previous experiment, the two types of synergism described above were obtained with as little as 0.56 mg of pelleted DES (*12*).

Radiation is the only carcinogenic agent for which there is widely accepted epidemiologic evidence for a role in human breast cancer, but the role of estrogens is still controversial (*16*). However, the ACI rat model suggests that segments of the human population may be at increased risk for tumors from the interactions of therapeutically administered estrogen treatments and irradiation. The current experiment suggests that part of this increased risk may be due to an acceleration of the mammary neoplastic response by the interaction of estrogens and radiation. Although there is no evidence that radiation alone reduces the latency of human breast cancer development (*17*), the contribution of a positive interaction with other factors, such as exogenous estrogens, has not been analyzed. The current study with continuous estrogen administration is especially relevant, because intradermal steroid implantation is being investigated in women (*18, 19*). The series of investigations, which include the current study, are also relevant because the group of women associated with an increased risk of breast cancer from postmenopausal estrogen-replacement therapy (*1, 2*) is at the age that is also exposed to radiation from mammographic examinations. If prolactin is involved in human mammary carcinogenesis, a dose of EE2 as low as 25 μg/day can elevate circulating prolactin levels in postmenopausal women, but it takes a tenfold higher dose for this response in premenopausal women (*20–22*). Even

though the risk from each factor may be decreased by reduction of the exposure to each factor, exposure of individual women to both estrogens and radiation may still increase the risk for breast cancer because of the synergistic interactions of these factors.

In conclusion, this investigation demonstrates that previously described synergistic interactions between DES and radiation on rat mammary carcinogenesis were due to the estrogenic properties of DES rather than intrinsic carcinogenic properties of this non-steroidal estrogen or its metabolites.

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