Synergistic Interactions of Various Doses of Diethylstilbestrol and X-Irradiation on Mammary Neoplasia in Female ACI Rats

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ABSTRACT

The intent of this study was to investigate the relationship between diethylstilbestrol (DES) dose and its synergistic interaction with X-irradiation for mammary carcinogenesis in ACI rats. In addition, the role of prolactin in this synergism was studied. Ten groups of approximately 25 female ACI rats, 84 to 91 days of age, were implanted s.c. with a compressed 20-mg pellet containing either 5, 1.67, 0.56, 0.19, or 0 mg of DES combined with cholesterol. Two days later, one group at each dose level was exposed (whole body) to 150 R of 250 kVp X-irradiation. Plasma prolactin levels were determined by radioimmunoassay on samples taken at approximately 100-day intervals. The experiment was terminated 659 days after pellet implantation. Three basic types of mammary neoplasms were found: individual mammary adenocarcinoma (MAC), multiple mammary adenocarcinoma (MMAC) consisting of four or more MAC’s within a single quadrant of breast tissue; and mammary fibroadenoma. Increasing the dose of DES (with or without irradiation) increased the number of rats with a MAC (incidence) and the number of MAC’s per rat while also decreasing the mean time of appearance of all MAC’s. MMAC’s were found only in rats treated with the two highest doses of DES (with or without irradiation), and this response appeared to be DES dose dependent. Combining irradiation with DES treatment (except at the lowest DES dose) produced a synergistic increase in the MAC and MMAC incidence and the number of MAC’s and MMAC’s per rat and decreased the time of appearance of all MAC’s. Rats receiving only X-irradiation had only a few late-appearing MAC’s. No MAC’s were seen in the cholesterol control group. Fibroadenomas were very late-occurring tumors, and their time of appearance did not appear to be DES dose dependent (with or without irradiation). Almost all the rats in the three highest-dose groups had pituitary tumors, while in all other groups only about one-half of the animals had these tumors. There appeared to be a definite relationship between the dose of DES and both the initiation and the degree of plasma prolactin elevation for all DES doses except the lowest. These data suggest that in female ACI rats, MAC responses.

INTRODUCTION

It has been shown that DES will interact synergistically with either X-irradiation (14) or neutron irradiation (18) for the production of MAC in female ACI (AxC or Irish) rats. In both of these studies, the DES treatment was the same, a single compressed pellet of 5 mg DES mixed with 15 mg cholesterol implanted s.c. into each rat. The present study was undertaken to investigate the relationship between the dose of DES and the synergistic interaction with X-irradiation for MAC production. DES-induced increases in plasma prolactin were also studied since the MAC response to DES treatment (5-mg pellet) in the female ACI rat has been associated with the induction of mammotrophic pituitary tumors (6) and very high levels of circulating prolactin (21).

MATERIALS AND METHODS

Two hundred forty-three female ACI rats were purchased from a commercial source (Microbiological Associates, Inc., Bethesda, Md.) when they were 31 to 36 days of age. All rats were maintained on commercial rat chow and water ad libitum. At 85 to 90 days of age, each rat had a 20-mg compressed pellet, containing 0, 0.19, 0.56, 1.67, or 5 mg DES combined with cholesterol, implanted s.c. in the intrascapular region, using light ether anesthesia. Two days later, one-half of the animals were irradiated with 150 R of 250 kVp X-rays as described previously (17), and the remaining animals were sham irradiated. The 10 experimental groups are shown in Table 1.

One-ml blood samples were taken from the same 10 animals in each group 133, 286, 341, 440, and 587 days after pellet implantation. These samples were collected from unanesthetized rats by an orbital sinus puncture technique. The procedure was completed within 2 min of picking up each animal to prevent stress-induced plasma prolactin elevation. Terminal blood samples were obtained by decapitation. All blood samples were collected in cold (4°C) heparinized (80 μg/ml blood) centrifuge tubes. Plasma samples were separated by centrifugation at 1000 × g for 45 min at 4°C and then stored at −25°C until assayed by radioimmunoassay for prolactin. All plasma samples were assayed at 2 or 3 dilutions in duplicate. The rat prolactin reference standard (RP-1), rat prolactin for iodination (I-1), and antiserum to rat prolactin (S-2) were obtained from the National Institute for Arthritis, Metabolism, and Digestive Diseases. The radioimmunoassay procedure used was a modification of the National Institute for Arthritis, Metabolism, and Digestive Diseases double-antibody procedure (21).

1 Research performed under Contract Y01-CP-30213, with the Biological Models Segment of the Carcinogenesis Program of the National Cancer Institute. Brookhaven National Laboratory is operated by Associated Universities, Inc., under contract to the United States Department of Energy, Contract DE-AC02-76CH00018.

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Received April 11, 1980; accepted July 30, 1980.

The abbreviations used are: DES, diethylstilbestrol; MAC, mammary adenocarcinoma; FA, fibroadenoma; MMAC, multiple mammary adenocarcinoma.

3 The abbreviations used are: DES, diethylstilbestrol; MAC, mammary adenocarcinoma; FA, fibroadenoma; MMAC, multiple mammary adenocarcinoma.
Each rat was identified by a numbered ear tag. The rats were palpated once a week, and the anatomical location of each mammary tumor was recorded using the nipples as reference points. Whenever a mammary tumor grew to about 1 cm in diameter, it was removed under ether anesthesia. If a second tumor was found at the site of a previously removed tumor, it was not recorded as a new tumor unless the site had been palpated as tumor free for 8 weeks. Mammary tumors were fixed, sectioned, stained with hematoxylin and eosin, and given a pathological classification of either MAC or FA according to the criteria of Young and Hallowes (25). If there were 4 or more MAC’s within a single quadrant of breast tissue, this was referred to as the MMAC response. When a MMAC was found, the entire quadrant of breast tissue was surgically removed, fixed, defatted, stained with hematoxylin, and cleared in methyl salicylate. The individual mammary tumors then were counted using a dissecting microscope. Even with this procedure, the total number of MAC’s per quadrant was probably underestimated because in many cases they were too crowded to be clearly distinguished. The MMAC response was usually found in all 4 quadrants within a 1- to 3-week period. However, quadrants were only removed at a maximum rate of one quadrant/week until 3 quadrants had been removed. When the fourth quadrant was removed, the animal was killed. Rats also were killed when they became moribund, when no more breast tissue remained, or 659 days after pellet implantation. At autopsy, each rat was examined for gross pathological changes, and all pellets were recovered. Abnormal pituitary glands were classified as gross tumors if they were hemorrhagic, fragile, or exceeded 30 mg in weight (4). For all time calculations, Day 0 was the day of pellet implantation.

RESULTS

Rats in the 3 highest-dose DES groups (Table 1, Groups 5 to 10) died significantly earlier than did rats in the control, X-irradiation only, or the lowest-dose DES groups. The mean days in the study (survival time, except for Groups 1 through 4) decreased in a dose-dependent manner for these high-dose groups (Table 1). The addition of X-irradiation caused significant additional dose-dependent reductions in the mean days in the study for all doses of DES. X-irradiation alone had no adverse effects on survival (Table 1). The occurrence of early pituitary tumors in DES-treated rats usually was associated with morbidity and a decreased mean time in the study.

All groups except the controls (Group 1) developed MAC’s (Table 1). X-irradiation alone (Group 2) produced a few late-appearing MAC’s. Because there was significant early mortality in the groups treated with the larger doses of DES, the data for mammary tumor incidence versus time (Charts 1, 2, and 3) were corrected for intercurrent mortality by a life table technique (13). Differences were delineated by Cox’s exact trend analysis (23). For rats treated with DES alone, there was a significant ($p < 0.01$) trend toward earlier and greater MAC incidence with increasing DES dose, except for the highest dose of DES (Chart 1). Increasing the dose of DES (without X-irradiation) also decreased the mean days to the first MAC and the mean days to appearance of all MAC’s (Table 1). An additional DES dose effect was seen as an increase in the mean number of MAC’s per rat, except for the highest DES dose (Table 1).

The addition of X-irradiation produced significant increases ($p < 0.02$) in the trend toward earlier and greater MAC incidence with increasing DES dose over that found with DES alone, except for the lowest dose of DES (Chart 1). Combining X-irradiation with DES treatment also significantly ($p < 0.01$) increased the mean number of MAC’s at all DES doses (Table 1). In addition, the mean days to the first MAC and the mean days to appearance of all MAC’s decreased significantly ($p < 0.05$) for all groups, except those given 0.19- or 0.56-mg DES pellets (Table 1, Groups 3 and 6).

When the mean time to first MAC was plotted versus the log DES dose (Chart 4), there appeared to be a linear dose-response relationship for all DES doses (with or without X-irradiation), except at the highest DES dose. Combining X-irradiation with DES treatment did not change the slope of the dose-response line but did shift significantly [$p < 0.006$ by analysis of covariance (20)] the line such that the first MAC’s

### Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Mean days in study</th>
<th>Per rat in group</th>
<th>Days to first appearance</th>
<th>Days to appearance of all</th>
<th>Pituitary tumors/rats examined</th>
<th>Per rat in group</th>
<th>Days to first appearance</th>
<th>Days to appearance of all</th>
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<td>2. Chol + X</td>
<td>25</td>
<td>630</td>
<td>0.4</td>
<td>592</td>
<td>603</td>
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<td>0</td>
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<td>634</td>
<td>0.1</td>
<td>609</td>
<td>609</td>
<td>0</td>
<td>0</td>
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<td>574(*)</td>
<td>0.7</td>
<td>593</td>
<td>613</td>
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<td>562(*)</td>
<td>0.3</td>
<td>476</td>
<td>458</td>
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<td>0</td>
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<td>510(*)</td>
<td>2.6</td>
<td>383</td>
<td>429</td>
<td>1</td>
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<td>9/15</td>
<td>14/19</td>
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<td>497</td>
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<td>7. 1.67 mg DES</td>
<td>24</td>
<td>336(*)</td>
<td>6.0</td>
<td>229</td>
<td>273</td>
<td>0.1</td>
<td>234</td>
<td>230</td>
<td>21/21</td>
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<td>0</td>
</tr>
<tr>
<td>8. 1.67 mg DES + X</td>
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<td>231(*)</td>
<td>9.0</td>
<td>160</td>
<td>207</td>
<td>0.1</td>
<td>230</td>
<td>225</td>
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<td>231(*)</td>
<td>4.0</td>
<td>165</td>
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<td>271</td>
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<td>162</td>
<td>159</td>
<td>24/24</td>
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a Study terminated on Day 659.

b Chol, cholesterol; X, 150 R X-irradiation.

c $p < 0.05$, DES plus X-irradiation versus DES ($f$ test).

d $p < 0.05$, DES plus X-irradiation versus DES ($f$ test).

e $p < 0.01$, DES plus X-irradiation versus DES ($f$ test).

f Represents one FA in one control rat.
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Chart 1. The percentage of rats (at risk) with MAC's for each treatment group plotted as a function of time in days after DES-cholesterol pellet implantation. Data were corrected at 7-day intervals for intercurrent mortality, mg, mg of DES in 20-mg compressed DES-cholesterol pellet; X, 150 R of 250 kvp X-rays given 2 days after pellet implantation; chol, control rats receiving a 20-mg cholesterol pellet.

added to the DES treatment, very significant increases (p < 0.001) were produced in the trend toward earlier and greater MMAC incidence with increasing DES over that found with DES alone (Chart 2). This trend was further confirmed by the finding that only rats receiving 5 mg DES plus X-irradiation developed 3 or 4 MMAC's per rat. The addition of X-irradiation to DES treatments also significantly (p < 0.01) increased the mean number of MMAC's per rat at both doses but decreased the days to first MMAC and the mean days to appearance of all MMAC's only at the highest DES dose (Table 1).

The FA's were very late-appearing tumors compared to the MAC'S, except for a single early FA in the control group (Table 1). Because animals in the 2 highest-dose groups (Groups 7 to 10) died early, FA's were found only in the 2 lowest-dose DES (Groups 3 to 6), the cholesterol (Group 1), and the X-ray (Group 2) groups. DES dose (without irradiation) appeared to have little effect on the mean number of FA's per rat, mean days to first FA, or the mean days of appearance of all FA's (Table 1).

The percentage of rats (at risk) having an FA (Chart 3) after DES treatment was not dose dependent. The addition of X-irradiation to 0.19 and 0.56 mg DES caused an increase in the incidence of FA's which was significantly above that for the DES alone (p < 0.01 and p < 0.03, respectively, by trend analysis) but was at the border line of being significantly above the incidence of irradiation alone (p < 0.06 and p < 0.08, respectively). It appears that the effects of DES and irradiation for the induction of FA probably were at the most only additive.

Pituitary tumors were found in virtually all animals examined which received the 5.0- or 1.67-mg DES pellets (Table 1, Groups 7 to 10). These tumors secreted large quantities of...
Chart 3. The percentage of rats (at risk) with a FA plotted as a function of time in days after DES-cholesterol pellet implantation. Data correction for intercurrent mortality and terms used are the same as in Chart 1.

Chart 4. The mean time to first MAC in rats treated with DES (○) and DES plus irradiation (●) plotted as a function of log DES dose (mg of DES contained in 20-mg compressed DES-cholesterol pellet). X, 150 R of 250 kVp X-rays given 2 days after pellet implantation. Data from animals treated with 5 mg DES (with or without X-irradiation) was excluded from regression calculations. The regression lines for DES (n = 27; regression coefficient = -470.2; correlation coefficient = -0.79) and DES plus irradiation (n = 48; regression coefficient = -431.4; correlation coefficient = -0.89) had slopes that were not significantly different (analysis of covariance, p > 0.5), but the lines were significantly separated (analysis of covariance, p < 0.006). Numbers in parentheses, number of rats in group with MAC’s; bars, S.E.

prolactin as evidenced by the mean plasma prolactin levels of the 5.0- and 1.67-mg DES groups increasing 26- and 13-fold, respectively, above the control value by Day 133 (Chart 5). Plasma samples were not collected at subsequent time periods from the rats selected for bleeding in these groups because most had died by the second scheduled bleeding date (Day 287) and it was decided that the surviving rats should not be subjected to the stress of bleeding. However, in an effort to obtain additional data, terminal plasma samples were obtained on Day 153 from 8 of the original 10 rats selected for bleeding in the 5.0-mg DES plus X-irradiation group. The mean prolactin level of this group was 36 times that of the Day 133 controls (Chart 5).

A very large fraction (29 of 36) of the rats which received 0.56-mg DES pellets (Groups 5 and 6) had pituitary tumors at autopsy (Table 1). The mean plasma prolactin levels in these groups had not increased significantly by Day 287, but by Day 340 the levels were 6 to 8 times the control values (Chart 5). Subsequent prolactin values were not available from this group because the Day 450 samples were lost in a freezer accident, and the animals being sampled were all dead by the next bleeding date (Day 580).

Pituitary tumors were also found at autopsy in about one-half of the 0.19-mg DES groups (Groups 3 and 4), the X-irradiation group (Group 2), and the controls (Group 1) (Table 1). The mean plasma prolactin levels of these groups (except for the X-irradiation group) were elevated on Day 580 and Day 659 compared with Day 340 (Chart 5), but this increase was largely due to 2 or 3 rats within each group developing pituitary tumors which secreted large quantities of prolactin.

DISCUSSION

In a previous study, 5-mg DES pellets implanted in female ACI rats produced hypertrophy and hyperplasia of the pituitary prolactin cells by Day 28 and prolactin cell adenomas by Day 130 (6, 21). Concurrent with this prolactin cell stimulation were plasma prolactin increases of up to 30 times the levels in the controls. The plasma prolactin levels increased rapidly with time even though the amount of DES released from the implanted pellets decreased exponentially. MAC production in these animals appeared to be related to the increased plasma prolactin levels. In the present study, there was a definite relationship between the dose of DES and both the initiation
and the degree of plasma prolactin elevation at all DES doses except the lowest (0.19 mg). This was not unexpected, since it is known that estrogens can stimulate prolactin secretion both by inhibiting hypothalamic release of prolactin-inhibiting factor and by a direct stimulatory effect on prolactin cells in the pituitary (10).

When the present MAC and MMAC data were directly compared with the prolactin data, there was a positive relationship between increased plasma prolactin levels and increased MAC and MMAC responses. From the data, it appears that at least a 4-fold increase in plasma prolactin over that of controls was necessary to produce significant numbers of MAC’s and that even higher prolactin levels were required to produce the MMAC response. The positive relationship between increased prolactin levels and MAC or MMAC production suggests that estrogenic stimulation of pituitary prolactin secretion was the hormonal mechanism primarily responsible for the DES dose-dependent MAC and MMAC responses seen in these rats.

Plasma prolactin levels in the control, X-irradiation only, and 0.19-mg DES (with or without X-irradiation) groups increased slowly late in the experiment. This increase in the mean prolactin level was primarily due to prolactin-secreting tumors in about one-half of the animals in each of these groups. Since the pituitary tumor incidence in the treatment groups was not significantly different from the control group, these pituitary tumors probably all occurred spontaneously. Because of the late appearance of these pituitary tumors, the mammary tissue in these animals was subjected to increased levels of prolactin for only a relatively short time. Concurrently, significant numbers of MAC’s were not produced in any of these groups. These findings suggest that a prolonged stimulation of mammary tissues by sustained high levels of prolactin alone or in combination with a certain level of exogenous estrogen may be a necessary condition for significant MAC production in female ACI rats; the very important role of estrogen:prolactin ratios in mammary neoplasia has been emphasized by several investigators (2, 3, 10, 12).

In the present studies, a synergistic interaction between DES and X-irradiation was indicated by responses above that expected by additivity for (a) the number of rats with a MAC (incidence), (b) the number of MAC’s per rat, and (c) the earlier appearance of MAC’s. Similar synergistic interactions in female ACI rats have been reported by Segaloff and Maxfield (14, 15) for 5-mg DES pellets plus X-irradiation and by us (18) for 5-mg DES pellets plus neutron radiation. In a parallel study on Sprague-Dawley rats (19), no synergistic interaction on MAC production could be detected; in fact, the data suggested an inhibitory interaction. In a later study of female F344 rats, using 3.9- or 2.6-mg DES pellets and X-irradiation, we found a synergistic interaction was indicated only for MAC incidence (7). Thus, the synergistic interaction appears to be quite strain specific and variable in expression.

In the present study, the ACI rats treated with the 2 highest doses of DES produced MAC’s in such quantities ["essentially total carcinogenesis," Segaloff and Maxfield (14)] that the tumors appeared to coalesce. To more accurately quantitate this response, we used the term MMAC. The combination of the 2 highest doses of DES and X-irradiation produced a synergistic interaction in terms of significant increases in the trend toward earlier and greater MAC incidence over that seen with DES alone. The production of MMAC’s, with or without X-irradiation, was associated with the DES dose-dependent elevations of plasma prolactin levels.

The rats treated with a 0.56-mg DES pellet plus 150 R of X-

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Chart 5. The plasma prolactin levels (ng/ml) plotted as a function of time in days after DES-cholesterol pellet implantation. Terms used are the same as in Chart 1. For each point, n ≤ 10 rats; bars, S.E.
irradiation provided the most impressive example of the synergistic interaction between DES and radiation. On Day 620, the MAC incidence in this group was 4 times greater than the groups treated with either 0.56 mg DES or X-irradiation alone. This response was at least double that expected from additivity. Combining the treatments also significantly shortened the mean time to first MAC, such that MAC’s appeared about 100 days earlier with the combined treatment than with either DES or radiation alone. In addition, the mean number of MAC’s per rat was 3-fold higher for the group subjected to the combined treatment than would be expected from additivity. Since no significant differences in plasma prolactin levels were found between animals receiving 0.56 mg DES alone or DES plus X-irradiation, the greatly increased MAC response appears to be due to an interaction between the neoplastic inductive effect of radiation (1, 16) and the proliferative effect of DES via prolactin (9, 11, 24).

X-irradiation alone, at the dose used in the present study (150 R), did not have any effect on plasma prolactin levels and produced only a few late-appearing MAC’s. Our data indicate that the synergistic interaction between X-irradiation and DES treatment was produced by the stimulatory effect of elevated plasma prolactin levels, alone or in combination with exogenous estrogen, on MAC foci (or sites from which a MAC will originate) produced by irradiation. Probably only a few of the increased number of MAC foci caused by irradiation will grow to detectable size if plasma prolactin and/or estrogen levels are normal. Support for this hypothesis comes from a previous study in which a synergistic interaction for MAC production was seen in female ACI rats irradiated with neutrons followed by treatment with a DES pellet (containing 2.326 mg) as late as 200 days after irradiation. Yokoro et al. (24) also found that a graft of a prolactin-secreting pituitary tumor would stimulate MAC production in Wistar-Furth rats even when the implant was made 200 days after X-irradiation. These data indicate that the MAC foci induced by irradiation retain the potential of becoming MAC’s for a very long period of time.

From our data and those of others, it appears that DES given continuously is carcinogenic primarily because it stimulates prolactin secretion which in turn stimulates the growth of MAC foci. DES does not appear to produce MAC foci, since DES must be given continuously to produce MAC’s and, when the DES treatment is stopped, the MAC’s usually regress. The carcinogenic action of DES is not unique to this synthetic estrogen. MAC’s also have been produced in female rats by prolonged treatment with a natural estrogen, estrone (5), and with a semisynthetic estrogen, ethinyl estradiol (6). Cutts and Noble (5) found that the implantation of estrone pellets directly into mammary tissue did not increase the number of tumors in the surrounding tissue over that seen in rats having the pellet implanted s.c. in the back. Their data indicated that estrone did not act by producing MAC foci or by direct stimulation of the mammary tissue. The finding that MAC’s in our control rats did not occur until prolactin levels were at least double the level seen during the first 400 days of the experiment indicates that naturally occurring MAC foci within the breast tissue do not grow until stimulated by elevated prolactin levels.

'Days to first MAC' (the time, after Day 0, necessary for a rat's first MAC to grow to a palpable size, ~2 to 3 mm in diameter) is an indicator of the rate of MAC growth. The linear correlation found between MAC growth and the log dose of DES with or without X-irradiation (Chart 4) was indicative of a dose-effect relationship of the type common to most drug bioassays. Thus, the dose of DES appeared to be the growth rate-determining factor for MAC’s in this study. A linear correlation would be expected for groups treated with DES plus X-irradiation, if 150 R of X-irradiation produced similar numbers of MAC foci in all irradiated rats and DES dose was the MAC growth rate-determining factor. The finding that treatment with DES only produced a regression line that was parallel to the line for DES plus X-irradiation indicated that the MAC growth rate-determining factor, the DES dose, was operating similarly in both groups. Therefore, DES in this study appeared to act primarily as a ‘promoter’ stimulating the growth of spontaneously occurring MAC foci or MAC foci produced by X-irradiation.

FA’s are very late-appearing mammary tumors in female ACI rats (22). When our doses of X-irradiation and DES were similar (150 R and 5 mg, respectively) to those used by Segaloff and Pettigrew (15), our results were similar; no FA’s were produced in rats treated with DES only, and only a few late-appearing FA’s appeared in rats treated with X-irradiation only. However, in the present study, the 2 lowest doses of DES alone did produce a few late-appearing FA’s. FA’s probably did not occur with the higher doses of DES because the survival time of these rats was shorter than the latent period for the FA’s. The time to appearance and the number of FA’s per rat did not appear to be dependent on the DES dose or the plasma prolactin levels. Combining irradiation and DES did not increase the incidence of FA’s above that expected from additivity nor cause the FA’s to appear earlier than with either treatment alone. These data when compared to the MAC data indicate that for the female ACI rat the mechanisms responsible for MAC and FA induction and proliferation are basically different.

In summary, it appears that in the mammary tissue of female ACI rats, few radiation-induced MAC’s grow to a detectable size unless stimulated by a prolonged elevation of plasma prolactin alone or in combination with exogenous estrogen. In addition, MAC induction by DES alone or by the synergistic interaction of DES and X-irradiation is highly DES dose dependent. This DES dose-dependent mammary carcinogenesis appears to be directly related to the DES-induced dose-dependent elevation of plasma prolactin levels in female ACI rats.

ACKNOWLEDGMENTS

We sincerely thank Lyndora Boyd, Elizabeth Jellett, John Shanley, and Mary Sneed for their enthusiastic and capable technical support, Howard Pate and Keith Thompson for aid with statistical analysis and computer programming, and Doris Pion for her secretarial assistance.

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