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Radiation-induced Mammary Carcinogenesis in Virgin, Pregnant, Lactating, and Postlactating Rats[1]

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ABSTRACT

Little attention has been paid to the influence of the reproductive condition of the rat at the time of irradiation upon X-ray-induced mammary carcinogenesis. At 12 weeks of age, 160 virgin female Sprague-Dawley rats were mated, and another 140 were maintained as virgins. On Days 14 to 16 of pregnancy, 36 mated females and 40 virgins were whole-body irradiated with 200 R of X-rays. On Days 2 to 5 postpartum, 40 additional mated females and 40 additional virgins were irradiated. At this time, all pups were removed from their mothers; 2 weeks later, 40 additional parous females and 40 virgins were irradiated. The remaining animals: parous females; virgins; and mated, but nonparous females were kept as unirradiated controls. Breast tissue biopsies were taken from 25% of the animals in each irradiated group on the day of irradiation, and the biopsies were examined microscopically for mammary tissue development. The experiment was terminated 10 months after irradiation. Despite marked differences in mammary tissue development among the groups at the time of irradiation, there were no significant differences among the irradiated groups for the incidence of rats with mammary adenocarcinomas or the number of mammary adenocarcinomas per rat. There were no significant differences among the irradiated groups for the incidence of rats with mammary fibroadenomas. There was an increase in the number of fibroadenomas per rat which was associated with increased age at the time of death. In contrast to mammary carcinogenesis produced by polycyclic aromatic hydrocarbons in previous studies, in the current investigation the physiological status of the host at the time of irradiation had little effect on: (*a*) the final incidence of mammary neoplasia; (*b*) the number of mammary adenocarcinomas produced; or (*c*) the type of mammary neoplasms produced.

INTRODUCTION

Mammary carcinogenesis induced by polycyclic aromatic hydrocarbons in female Sprague-Dawley rats has been shown to be dependent on the age and physiological condition of the animal at the time of p.o. or systemic carcinogen administration (1, 2, 9, 10, 17, 18, 22). Except for very young females, radiation-induced mammary carcinogenesis has not been shown to be age dependent in virgin females (8, 17). However, the effect of physiological status of the rat at the time of irradiation has received relatively little attention (18). Therefore, we undertook a study to compare the effects of irradiation during pregnancy, lactation, postlactation, and virginity upon mammary carcinogenesis in Sprague-Dawley rats.

MATERIALS AND METHODS

At approximately 12 weeks of age, 160 virgin female Sprague-Dawley rats (Taconic Farms, Germantown, N. Y.) were placed into 80 plastic tubs. Each tub contained 1 male and 2 females. Another 140 virgin females of the same age and source were kept isolated from the matings in another animal room. All rats were maintained on commercial rat chow and water *ad libitum*, under 12 hr (8 a.m. to 8 p.m.) fluorescent light per day, at 22.5 ± 1° (S.D.) and 55 ± 5% humidity.

Mating success was checked by gross examinations for vaginal plugs and microscopic examinations for sperm in vaginal smears at 9 a.m. and at 4 p.m. daily. Upon evidence of impregnation, pairs of the pregnant famales were placed in cages in another animal room.

At Days 14, 15, and 16 postfertilization, a total of 36 pregnant females (Group 1) were whole-body irradiated with 200 R of 250-kVp X-rays (21), along with a total of 40 virgin females (Group 2). Two to 5 days after parturition, another 40 lactating females (Group 3) and 40 virgins (Group 4) were irradiated. Gross external observation of milk in the stomach of the pups was used to detect lactation and milk letdown. At this time, the pups were removed from all the females that gave birth. Fourteen days later, another 40 of the parous rats (Group 5) and 40 virgins (Group 6) were irradiated. Another 28 parous rats (Group 7), 20 virgins (Group 8), and 12 mated (Group 9) but nonpregnant rats were maintained as unirradiated controls.

For each irradiated rat, time was reckoned from the day of irradiation, Day 0; for the unirradiated controls, the first day of irradiation of the pregnant females was used as Day 0. One hr after irradiation, 10 females from each of the irradiated parous and irradiated virgin control groups were subjected to light ether anesthesia. A biopsy, approximately 10 sq mm, was taken from the breast tissue medial to the middle nipple of the right lower quadrant of each anesthetized rat. The biopsies were fixed in formalin, defatted, stained, dehydrated, cleared, and mounted unsectioned for evaluation of mammary tissue development (24). The samples were scored from 1 to 5 at 0.5-unit intervals, with the larger values representing greater development (27). Because of the variation, both mean values and range are given in Table 1.

The rats were kept for 293 to 314 days postirradiation (Table 1). Although the mean time in the experiment was almost the same for all irradiated groups, Groups 1 and 2 were 2 to 4 weeks younger than were Groups 3 through 6 at the time of irradiation. Therefore, Groups 1 and 2 were 2 to 4 weeks younger than the other irradiated groups when the rats were killed. Each rat was identified by a numbered ear tag. Starting at 4 weeks postirradiation, the animals were palpated on a weekly basis for tumors. The anatomical location of each mammary tumor was recorded using the nipples as reference points. When approximately 2 cm in diameter, individual mammary tumors were removed from the rats under light ether anesthesia. Upon removal of a tumor, each rat was returned to the experiment. Any tumor recurring within 12 weeks at the site of removal of a previous tumor of the same type was not counted. Hematoxylin- and eosin-stained sections of the mammary tumors were classified as MACs[3] or FAs according to criteria

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[3] The abbreviations used are: MAC, mammary adenocarcinoma; FA, mammary fibroadenoma; i.g., intragastric; DMBA, 7,12-dimethylbenz(a)anthracene.

Table 1

Mammary gland neoplasia produced by whole-body X-irradiation of pregnant, lactating, postlactating, and virgin Sprague-Dawley rats

Group	No. of rats	Mammary[a] development			Mean time (days) in experiment	Rats with MACs	Total no. with MACs	Mean time (days) to		Rats with FAs	Total no. with FAs	Mean time (days) to	
		Mean	Range	No.				First MAC	All MACs			First FA	All FAs
Irradiated[b]													
1. Pregnant (14–16 days)	36	5	4–5	9	296	10	16	116[c]	150[c]	8	8	286	286
2. Virgin	40	2	1.5–3.5	9	296	13	14	116[d]	143	14	17	261[e]	268
3. Lactating (2–5 days)	40	5	5	10	293	10	15	211[c]	212[c]	14	17[f]	265	270
4. Virgin	40	2.5	2–3	9	295	10	13	175[d]	179	15	19	244	252
5. Postlactation (14–16 days)	40	3.5	3–4.5	10	298	11	18	145	182	15	29[f]	243[e]	258[e]
6. Virgin	40	2	1.5–3	9	293	13	13	138	142	15	21	246	251
Unirradiated													
7. Parous	28				314	1	1	28	28	1	1	285	285
8. Virgin	20				314	1	1	299	299	3	3	314	314
9. Mated	12				314	0	0			0	0		

[a] Graded to nearest 0.5 on biopsies taken on the day of irradiation.
[b] 200 R of 250-kVp X-rays, whole body.
[c] Group 3 greater than Group 1 ($p < 0.05$, t test).
[d] Group 2 *versus* Group 4 ($0.05 < p < 0.1$, t test).
[e] Groups 2 and 5 less than Group 1 ($p < 0.05$, t test).
[f] Tumors per rat for Groups 3 and 5 greater than for Group 1 ($p < 0.05$, t test).

of Young and Hallowes (29).

Incidence of rats with mammary neoplasia of either type, or with MACs only, or with FAs only were subjected to 2 × 2 X^2 analyses with Yates' correction for small numbers (25, 26). Mean times to detection of first MACs, or first FAs were subjected to 2-way analyses of variance and Dunnett's and Student's t tests (23). Similar analyses were applied to time to all MACs and all FAs detected in this experiment. A computerized Kaplan-Meier analysis, including Cox's test and the Kruskal-Wallis test, was used to examine treatment-related trends in time of tumor detection (28).

RESULTS

At the time of irradiation, there were clear-cut differences in the development of mammary glands among groups, and there was considerable variability within the groups that were not pregnant or lactating. In the rats of Group 1, ductal and lobuloalveolar development approached the maximum previously described for mammary glands of mid-to-late-pregnant rats (27). The mammary glands of postlactating rats (Group 5) presumably were variably regressed to give an average score that was slightly higher than the scores from the variable breast morphology of the virgins (Groups 2, 4, and 6). The number of offspring, their body weights, and suckling did not appear to be signficantly affected in the irradiated pregnant rats as compared to the other parous rats.[4]

As expected from previous studies (20), in this investigation, MACs tended to appear earlier than did FAs. The incidence of rats bearing MACs and the number of MACs per group or per rat were not significantly different among the irradiated groups. However, the time to detection of MACs was significantly shorter for pregnant rats (Group 1) than for lactating rats (Group 3).

Although the incidence of rats bearing FAs appears to be small for the pregnant rats (Group 1), there were no significant differences in incidence for any of the irradiated groups. The number of FAs per group and FAs per rat tended to increase from the groups irradiated earlier (Groups 1 and 2) to those irradiated later (Groups 3, 4, 5, and 6), with significantly fewer FAs per rat in the pregnant rats than in either the lactating or postlactating rats. Among the irradiated experimental groups, there was a trend towards decreasing latency of FA detection from the groups irradiated earliest (Groups 1 and 2) to those irradiated later (Groups 3 through 6). Among the irradiated groups, the only significant differences for days to detection of first FAs were between the pregnant rats (Group 1) and their controls (Group 2) and between the pregnant rats and the postlactating rats (Group 5).

DISCUSSION

At the time of irradiation, there were clear-cut differences in mammary gland development between groups and considerable variability within some groups. The current investigation found no relationship for the status of mammary glandular tissue in female Sprague-Dawley rats (and, by extension, the physiological status of the rats) at the time of X-irradiation either to: (*a*) the resulting incidence of mammary neoplasia; (*b*) the number of mammary adenocarcinomas produced; or (*c*) the type of mammary neoplasm produced. Neither pregnant, nor lactating, nor postlactating irradiated rats were different from their respective irradiated virgin controls. Furthermore, analyses of subdivisions of the irradiated mated rat groups (*e.g.*, Group 1 irradiated on Day 14, 15, or 16) showed no differences from each other or from the totals for each group. The current study confirms and extends the results of a previous investigation by Shellabarger (18) in which inbred Sprague-Dawley rats, irradiated during lactation, did not respond differently from their irradiated-virgin controls.

In other studies, no relationships were found between the age of virgin Sprague-Dawley females at the time of irradiation and mammary carcinogenesis, except for immature rats (8, 17).

The results described above contrast markedly with those obtained with p.o. polycyclic aromatic hydrocarbons, where a

[4] Unpublished data.

critical-age treatment period has been demonstrated for maximal mammary carcinogenesis in virgin female Sprague-Dawley rats (1, 2, 9, 10). Furthermore, mammary neoplasia was reduced when pregnant or lactating rats received p.o. polycyclic aromatic hydrocarbon treatment (2, 3, 10, 18). Moon (13) found that a single pregnancy, without lactation, prior to i.g. administration of DMBA produced maximum protection against mammary carcinogenesis. Dao (4) reported that prior pregnancy inhibited mammary tumorigenesis by both p.o. DMBA or DMBA applied directly to the breast tissue. The relationship of mammary gland morphology and development in Sprague-Dawley females to chemically induced carcinogenesis has been elegantly described by Russo and Russo (16), and similar observations have been made on other rat strains (6). These investigators have concluded that maximum MAC induction occurs by p.o. DMBA administration at a stage of development that is predominated by the presence of terminal end buds, which are in turn associated with extensive cellular proliferation. However, in contrast to the observations on parous rats (4), recent publications (14, 22) reported that direct application of DMBA to mammary glands in Sprague-Dawley females did not produce a lesser incidence of MACs when the treatment was given to older rats. The youngest rats used in those studies were of the age in which p.o. or systemic DMBA administration would be expected to produce a maximal response. Those observations (14, 22) support the view that metabolism and clearance rates are important factors in mammary carcinogenesis produced by p.o. polycyclic aromatic hydrocarbon treatment (2, 5, 12, 18). It is likely that the critical period for maximal mammary carcinogenesis induction by p.o. polycyclic hydrocarbons depends both on the interactions between the development condition of the mammary tissue itself and on the metabolism and clearance rates of the polycyclic hydrocarbons. It remains to be determined as to which of these 2 factors is predominant in polycyclic hydrocarbon-induced mammary carcinogenesis.

We have insufficient information to discuss differences in the inductive action of chemicals and radiation in rat mammary carcinogenesis. In the case of radiation, it is possible that induction is occurring in a specific stem cell population which is maintained throughout the life and reproductive changes in the rat, while chemical carcinogenesis is more related to the number, rate of turnover, and types of differentiated mammary gland cells.

Specific links between chemical carcinogens and human breast cancer have not been established, although we do not exclude the existence of such relationships. Nevertheless, there is considerable evidence for radiation induction of breast cancer in humans, and the similarities to radiation-induced mammary carcinogenesis in rats have been described elsewhere (19). However, it is of some interest to note that in irradiated women the risk per rad was not very different between women who were lactating at the time of radiation and women who were not lactating at the time of radiation (11).

The dose of whole-body X-irradiation administered to the rats in the current experiment was selected to provide minimal interference with reproductive function, while producing a significant mammary carcinogenic response in a relatively short time (18, 20). The experiment was successful in both aims, as evidenced by the comparisons of birth, lactation, and tumor results between the irradiated parous rats (Groups 1, 3, and 5) and the unirradiated parous rats (Group 7).

We have no explanation for the apparently earlier MAC responses in the irradiated pregnant rats and their irradiated virgin controls (Groups 1 and 2), as compared to the irradiated lactating rats and their irradiated virgin controls (Groups 3 and 4). These relationships did not hold up under trend analyses.

In the current investigation with Sprague-Dawley females, which are responsive to X-irradiation in terms of mammary neoplasia, the apparently increased FA responses in Groups 3, 4, 5, and 6 in comparison to Groups 1 and 2 are readily explicable. As confirmed in the present investigation, FAs tend to appear later than MACs (20), and the increased FA responses may be related to the absolute age of the rats at the time of death, rather than to time after irradiation. This is due to the experimental design where time to detection of FAs was counted from the day of irradiation, so that groups which were irradiated later were also older when killed. Therefore, one might expect to find more FAs in those groups irradiated later. It is possible that keeping Group 1 until the same "absolute age" as groups killed later would have resulted in a similar final cumulative incidence of rats with FAs between Group 1 and the other irradiated groups. The relationship of absolute age at death to FA responses may also be an explanation for the apparently shorter latency of FA responses in those groups that were irradiated later. One way to test these suggestions is to change the order in which the experimental groups are irradiated. Nevertheless, for all the irradiated groups, the experiment confirms previous observations that radiation increases FA responses in Sprague-Dawley females as compared to unirradiated controls (19).

In a previous investigation on Wistar rats studied for 2 years after 270 R of X-irradiation at 121 days of age, Reincke *et al.* (15) detected a significantly lower incidence of "benign," *i.e.*, FA, mammary tumor-bearing females for rats irradiated while pregnant, as compared to the incidence in irradiated virgins. The incidence for benign mammary tumor-bearing Wistar females irradiated when pregnant was not different from the incidence in unirradiated controls. The study differed from the current investigation in several details. The study of Reincke *et al.* (15) was over a 2-year follow-up, while our study was completed in 10 months after irradiation. Tumors were removed as they appeared in our study but not in the study of Reincke *et al.* (15), and we used Sprague-Dawley rats while they used Wistar rats. However, the data suggest that Wistar rats are less sensitive to X-radiation than are Sprague-Dawley rats since the maximum incidence of Wistar females with "malignant," *i.e.*, MAC, tumors was only 4 of 45 irradiated virgins.

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REFERENCES

1. Dao, T. L. Mammary cancer induction by 7,12-dimethylbenz(a)-anthracene: Relation to age. Science (Wash. D. C.) *165:* 810–811, 1969.
2. Dao, T. L. Inhibition of tumor induction in chemical carcinogenesis in the mammary gland. Prog. Exp. Tumor Res., *14:* 59–88, 1971.
3. Dao, T. L., Bock, F. G., and Greiner, M. J. Mammary carcinogenesis by 3-methylcholanthrene. II. Inhibitory effect of pregnancy and lactation in tumor induction. J. Natl. Cancer Inst., *25:* 991–1003, 1960.
4. Dao, T. L., and Pazck, J. Early pregnancy protects against mammary gland

carcinogenesis. Proc. Am. Assoc. Cancer Res., *22:* 96, 1981.
5. Feuer, G., and Kellen, J. A. Link between carcinogenicity and hepatic metabolism of 7,12-dimethylbenz(a)anthracene. Oncology (Basel), *30:* 499–508, 1974.
6. Haslam, S. Z. The effect of age on the histopathogenesis of 7,12-dimethylbenz(a)anthracene-induced mammary tumors in the Lewis rat. Int. J. Cancer, *26:* 349–356, 1980.
7. Holtzman, S., Stone, J. P., and Shellabarger, C. J. Effects of reproductive state upon radiation induction of rat mammary carcinogenesis. Proc. Am. Assoc. Cancer Res., *22:* 105, 1981.
8. Huggins, C., and Fukinishi, R. Cancer in the rat after single exposure to irradiation or hydrocarbons. Radiat. Res., *20:* 493–503, 1963.
9. Huggins, C., Grand, L. C., and Brillantes, F. P. Mammary cancer induced by a single feeding of polynuclear hydrocarbons, and its suppression. Nature (Lond.), *189:* 204–207, 1961.
10. Kellen, J. A. Suppression of DMBA-tumor induction in rat mammary glands by pregnancy or chlorotrianisene. Z. Krebsforsch., *79:* 75–77, 1973.
11. Land, C. E., Boice, J. D., Jr., Shore, R. E., Norman, J. E., and Tokunaga, M. Breast cancer risk from low-dose exposures to ionizing radiation: results of parallel analysis of three exposed populations of women. J. Natl. Cancer Inst., *65:* 353–376, 1980.
12. McCay, P. B., King, M., Rikans, L. E., and Pitha, J. V. Interactions between dietary fats and antioxidants on DMBA-induced mammary carcinogenesis and on AAAF-induced hyperplastic nodules and hepatomas. J. Environ. Pathol. Toxicol., *3:* 451–456, 1980.
13. Moon, R. C. Relationship between previous reproductive history and chemically induced mammary cancer in rats. Int. J. Cancer, *4:* 312–317, 1969.
14. Morii, S., Tsubura, A., Takaoka, S., and Shikata, N. Dehydrogenases in local carcinogenesis in rat mammary gland by dusting of DMBA-powder. Acta Histochem. Cytochem., *13:* 287–297, 1980.
15. Reincke, U., Stutz, E., and Wegner, G. Tumoren nach einmaliger Rontgenbestrahlung weiser Ratten in verschiedenem Lebensalter. Z. Krebsforsch., *66:* 165–186, 1964.
16. Russo, J., and Russo, I. H. Influence of differentiation and cell kinetics on the susceptibility of the rat mammary gland to carcinogenesis. Cancer Res., *40:* 2677–2687, 1980.
17. Shellabarger, C. J. A comparison of rat mammary carcinogenesis following total-body irradiation at different ages. Radiat. Res., *59:* 103, 1974.
18. Shellabarger, C. J. Mammary carinogenesis in irradiated, lactating Sprague-Dawley female rats. *In:* P. Bucalossi, V. Veronesi, and N. Cascinelli (eds.), Proceedings of the Eleventh International Cancer Congress, Florence, Italy, October 20 to 26, 1974. p. 634. Milan: Casa Editrice Ambrosiana, 1974.
19. Shellabarger, C. J. Pituitary and steroid hormones in radiation induced mammary tumors. *In:* M. Pike, P. K. Siiteri, and C. W. Welsch (eds.), Banbury Report 8, Hormones and Breast Cancer, pp. 339–351. Cold Spring Harbor, N. Y., Cold Spring Harbor Laboratory, 1981.
20. Shellabarger, C. J., Bond, V. P., Aponte, G. E., and Cronkite, E. P. Results of fractionation and protection of total body radiation on rat mammary neoplasia. Cancer Res., *26:* 509–513, 1966.
21. Shellabarger, C. J., Bond, V. P., and Cronkite, E. P. Studies on radiation-induced mammary gland neoplasia in the rat. VII. The effects of fractionation and protraction of sublethal total-body irradiation. Radiat. Res., *17:* 101–109, 1962.
22. Sinha, D. K., and Dao, T. L. Induction of mammary tumors in aging rats by 7,12-dimethylbenz(a)anthracene: role of DNA synthesis during carcinogenesis. J. Natl. Cancer Inst., *64:* 519–521, 1980.
23. Steel, R. G., and Torrie, J. H. Principles and Procedures of Statistics. New York: McGraw-Hill Book Co., Inc., 1960.
24. Stone, J. P., Holtzman, S., and Shellabarger, C. J. Neoplastic responses and correlated plasma prolactin levels in diethylstilbestrol-treated ACI and Sprague-Dawley rats. Cancer Res., *39:* 773–778, 1979.
25. Swinscow, T. D. Statistics at square one. XIV—The X^2 tests. Br. Med. J., *2:* 462–463, 1976.
26. Swinscow, T. D. Statistics at square one. XV—The X^2 tests (continued). Br. Med. J., *2:* 513–514, 1976.
27. Talwalker, P. K., and Meites, J. Mammary lobular-alveolar growth induced by anterior pituitary hormones in adrenoovariectomized and adrenoovariectomized-hypophysectomized rats. Proc. Soc. Exp. Biol. Med., *107:* 880–883, 1961.
28. Thomas, D. G., Breslow, N., and Gartt, J. J. Trend and homogeneity analyses of proportions and life table data. Comput. Biomed. Res., *10:* 373–381, 1977.
29. Young, S., and Hallowes, R. C. Tumors of the mammary gland. *In:* V. S. Turosov (ed.), Pathology of Tumors in Laboratory Animals, Vol. 1, pp. 31–74. Lyon, France: International Agency for Research on Cancer, 1973.