Retinoid Feeding, Hormone Inhibition, and/or Immune Stimulation and the Genesis of Carcinogen-induced Rat Mammary Carcinomas

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ABSTRACT

Female Sprague-Dawley rats were treated at 53 days of age with a single intubation of 7,12-dimethylbenzanthracene (DMBA). Three days after carcinogen treatment, the animals were treated with retinyl acetate (RA) (at 3 dietary levels), hormone inhibition (HI) [tamoxifen (1-p-β-dimethylaminoethoxyphenyl-trans-1,2-diphenylbut-l-ene) plus 2 bromo-α-ergocryptine], and/or immune stimulation (methanol-extracted residue of Bacillus Calmette-Guérin, cell wall skeleton of Nocardia rubra, or cell particulate of DMBA-induced rat mammary carcinomas plus Freund’s complete adjuvant). RA at 0.6 or 1.0 mM concentrations per kg diet significantly reduced the incidence of mammary carcinomas; 0.2 mM concentrations of RA per kg diet did not affect tumor incidence. HI also significantly decreased mammary carcinoma incidence, an effect which was significantly enhanced by all 3 dietary levels of RA. Immune stimulation by methanol-extracted residue of Bacillus Calmette-Guérin or cell wall skeleton of Nocardia rubra did not affect mammary carcinoma incidence when administered either alone or in combination with RA and/or HI. The cell particulate of DMBA-induced rat mammary carcinomas plus Freund’s complete adjuvant significantly reduced mammary carcinoma incidence in rats fed RA but did not affect mammary carcinoma incidence in placebo-fed rats or in rats treated only with HI. However, in rats treated with the triple combination of cell particulate of DMBA-induced rat mammary carcinomas plus Freund’s complete adjuvant, RA, and HI, no mammary carcinomas were observed for the duration of treatment (20 weeks after DMBA administration). Although HI was always superior to RA feeding in the prophylaxis of this neoplastic process, a significant synergism between these two treatments was consistently observed. This distinct synergism was observed even when using the low dietary level of RA, an amount of RA which by itself was ineffective in the suppression of mammary carcinogenesis. With but one exception, immune stimulation did not significantly influence this carcinogenic process, either when administered alone or when administered to rats with a reduced mammary carcinoma burden, i.e., animals treated with RA and/or HI.

INTRODUCTION

The feeding of certain retinoids (vitamin A analogues) to carcinogen-treated female rats consistently results in a significant reduction in the incidence of mammary carcinomas (17). The retinoids are effective when fed shortly after carcinogen treatment, prior to the emergence of the palpable neoplasms. Retinoids do not appear to suppress the growth of palpable rat mammary carcinomas. A retinoid which at present appears to be efficacious in the chemoprevention of this neoplastic process is RA,2 an acetate ester of retinol (vitamin A) (11, 12).

Drug-induced HI is another effective method of suppressing the development of mammary carcinomas in carcinogen-treated rats (24). Chronic suppression of pituitary prolactin secretion with CB-154 or the utilization of tamoxifen (1-p-β-dimethylaminoethoxyphenyl-trans-1,2-diphenylbut-l-ene), a potent estrogen antagonist, have been reported to significantly inhibit the emergence of mammary carcinomas in female rats when administered shortly after carcinogen treatment (5, 21–23). When these ‘‘hormone-inhibitory’’ drugs are administered together, they are more effective than when administered individually (22). The combination of CB-154 and tamoxifen is the most effective ‘‘hormone-inhibitory’’ drug preparation that we have experienced for the chemoprevention of chemical carcinogenesis of the rat mammary gland. CB-154 and tamoxifen treatments are not solely effective in the early developmental phases of this neoplastic process inasmuch as these drugs, unlike the retinoids, are effective inhibitors in the advanced stages of this disease as well (5, 19).

Nonspecific and specific IS for the prevention of tumor development in humans and experimental animals are procedures that have had widely varying degrees of success (2, 3, 15). We are aware of only 2 reports which examined the effects of early IS on the genesis of autochthonous carcinogen-induced rat mammary gland tumors (6, 13). MER of BCG, when administered to female rats at 3 and 5 or 4 and 6 weeks after carcinogen treatment, has been reported to significantly reduce the incidence of mammary carcinomas (8). In a similar type of study, CWS of NR, when administered at biweekly intervals after carcinogen treatment, also has been reported to significantly suppress the emergence of mammary carcinomas (13).

From the foregoing, it appears that 3 distinctly different types of biological response modifiers, i.e., retinoid feeding, HI, or IS, are capable of suppressing the genesis of mammary carcinomas in female rats treated with chemical carcinogens. It is german to point out, however, that neither of these treatments alone have been reported to completely block this tumorigenic process; tumor incidence is usually reduced no more than 25 to 75% by any one of these treatments. Although this is a noteworthy reduction, it is conceivable that a combination of these treatments may be far superior to either one alone, perhaps capable of completely blocking this neoplastic process. Thus, the purpose of this study is to determine whether or

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2 The abbreviations used are: RA, retinyl acetate; HI, hormone inhibition; CB-154, 2-bromo-α-ergocryptine; IS, immune stimulation; MER of BCG, methanol-extracted residue of Bacillus Calmette-Guérin; CWS of NR, cell wall skeleton of Nocardia rubra; i.e., intragastric; DMBA, 7,12-dimethylbenz(a)anthracene; CP of MT + FGA, cell particulate of 7,12-dimethylbenz(a)anthracene-induced rat mammary carcinomas plus Freund’s complete adjuvant; MNU, N-methyl-N-nitrosourea.

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not these treatments can enhance one another in the prophylaxis of chemical carcinogenesis of the rat mammary gland.

MATERIALS AND METHODS

Nine hundred sixty female Sprague-Dawley rats (Spartan Research Animals, Inc., Haslett, Mich.) were used in this study. All animals were housed in a temperature-controlled (24 ± 1°C S.D.) and light-controlled (14-hr/day) room and were given a diet of Wayne laboratory meal (Allied Mills, Inc., Chicago, Ill.) and water ad libitum. The vitamin A content of the Wayne laboratory meal is 15 IU of the vitamin per g of ration, as specified by the manufacturer.

Experiment 1. At 53 days of age, 320 rats were treated i.g. with 20 mg DMBA (Eastman Kodak Co., Rochester, N. Y.) dissolved in sesame oil (20 mg/ml). At 56 days of age, the rats were divided into 8 groups (40 rats/group) and treated as follows: (a) controls; (b) IS; (c) HI; (d) HI plus IS; (e) RA fed; (f) RA plus IS; (g) RA plus HI; and (h) RA plus HI plus IS. IS was accomplished by weekly (for 11 or 15 weeks) s.c. injections of CWS or NR, 150 μg/rat, suspended in sterile 0.9% NaCl solution containing 0.2% Tween 80. The preparation of CWS of NR was performed as described previously (13). HI was accomplished by daily (for 26 weeks) s.c. injections of CB-154 (400 μg/100 g body weight) and tamoxifen (ICI 46,474) (25 μg/100 g body weight). Tamoxifen was mixed with gum arabic, and this mixture was added to 0.9% NaCl solution containing CB-154. RA feeding was accomplished as in Experiments 1 and 2. The control rats were sacrificed at 12 weeks after DMBA treatment because they were inured with mammary carcinomas (157 palpable mammary tumors in 40 rats). At this time period, only 5 mammary carcinomas were observed in the HI group and one was observed in the HI plus RA group (p < 0.001). At the end of treatment (26 weeks after DMBA administration), 3 times the number of mammary carcinomas were observed in the HI group as in the HI plus RA group (p < 0.05). RA alone, IS alone, or the combination of RA plus IS did not significantly affect mammary carcinoma incidence. IS did not significantly affect mammary carcinoma incidence in HI- or HI plus RA-treated rats. IS-, RA-, and IS plus RA-treated rats were sacrificed at 11 weeks after DMBA treatment, for at this time period, like the controls, these animals were burdened with large numbers of palpable mammary carcinomas. Upon cessation of treatments (26 weeks after DMBA administration, Groups 3, 4, 7, and 8), mammary carcinoma incidence was analyzed by χ² analysis.

RESULTS

Experiment 1. In this study, rats were treated with a single high dose of DMBA (20 mg) and fed daily a low level of RA (0.2 mM in each kg of ration). The IS was CWS of NR, administered once weekly, and the HI drug regimen was a daily dose of tamoxifen and CB-154. See Chart 1 for experimental design and mammary carcinoma incidence data.

The control rats were sacrificed at 11 weeks after DMBA treatment because they were inured with mammary carcinomas (157 palpable mammary tumors in 40 rats). At this time period, only 5 mammary carcinomas were observed in the HI group and one was observed in the HI plus RA group (p < 0.001). At the end of treatment (26 weeks after DMBA administration), 3 times the number of mammary carcinomas were observed in the HI group as in the HI plus RA group (p < 0.05). RA alone, IS alone, or the combination of RA plus IS did not significantly affect mammary carcinoma incidence. IS did not significantly affect mammary carcinoma incidence in HI- or HI plus RA-treated rats. IS-, RA-, and IS plus RA-treated rats were sacrificed at 11 weeks after DMBA treatment, for at this time period, like the controls, these animals were burdened with large numbers of palpable mammary carcinomas. Upon cessation of treatments (26 weeks after DMBA administration, Groups 3, 4, 7, and 8), mammary carcinoma incidence increased; the rate of mammary carcinoma increase was less, however, than that observed in the control rats (Group 1).

Experiment 2. In this study, rats were treated with a single moderate dose of DMBA (10 mg) and fed daily a moderate level of RA (0.6 mM in each kg of ration). The IS was MER of BCG, administered once at 3 and 5 weeks after DMBA treatment, and the HI drug regimen was identical to that in Experiment 1. See Chart 2 for experimental design and mammary carcinoma incidence data.

The control rats were sacrificed at 12 weeks after DMBA treatment because of excessive mammary carcinoma burden. HI significantly (p < 0.001) reduced mammary carcinoma incidence when compared to controls, an effect which once again was enhanced by RA. RA alone reduced mammary...
Prophylaxis of Rat Mammary Carcinogenesis

Chart 1. Effect of RA (0.2 mM) feeding, HI (CB-154 plus tamoxifen), and IS (CWS of NR) on the genesis of DMBA (20 mg)-induced rat mammary gland carcinomas. A, 20 mg DMBA-placebo diet (Groups 1 to 4); B, 20 mg DMBA-0.2 mM RA diet (Groups 5 to 8). Percentage of rats bearing mammary carcinomas at 11 weeks after carcinogen treatment in Groups 1 to 8 were: controls, 100; IS, 87; HI, 12; HI plus IS, 29; RA, 92; RA plus IS, 95; RA plus HI, 3; RA plus HI plus IS, 14. p < 0.001, controls versus HI, HI plus IS, RA plus IS, RA plus HI, RA plus HI plus IS; p < 0.05, HI versus RA plus HI. ———, mammary carcinoma incidence after cessation of treatments.

carcinoma incidence by over 50% when compared to that of controls (p < 0.01). Twelve weeks after DMBA treatment, the total number of mammary carcinomas that were observed in the control group was 179, whereas total mammary carcinoma numbers in the RA, HI, and RA plus HI groups were 83, 5, and 2, respectively. At the end of treatments (20 weeks after DMBA administration), over twice the number of mammary carcinomas were observed in the HI group as in the HI + RA group (p < 0.05). IS alone or with RA, HI, or RA plus HI did not significantly affect the incidence of mammary carcinomas. Upon cessation of treatments (20 weeks after DMBA administration; Groups 3, 4, 7, and 8), mammary carcinoma incidence increased in all groups but at a rate less than that observed in control rats (Group 1).

Experiment 3. In this study, rats were treated with a single low dose of DMBA (5 mg) and fed daily a high level of RA (1.0 mM concentration per kg of ration). The IS was CP of MT + FCA, administered once at 1, 3, and 5 weeks after DMBA.
treatment, and the HI drug regimen was identical to that of Experiments 1 and 2. See Chart 3 for experimental design and mammary carcinoma incidence data.

HI significantly \( (p < 0.001) \) reduced mammary carcinoma incidence, an effect which, as in Experiments 1 and 2, was significantly \( (p < 0.05) \) enhanced by RA. RA alone also significantly \( (p < 0.05) \) reduced mammary tumor incidence. IS significantly \( (p < 0.05) \) reduced mammary carcinoma incidence in RA-treated animals but had no significant effect on carcinoma incidence in placebo-fed or HI-treated animals. Total numbers of mammary carcinomas in control, IS, HI, IS plus HI, RA, RA plus IS, RA plus HI, and RA plus HI plus IS groups 20 weeks after DMBA treatment were 81, 77, 7, 11, 47, 29, 2, and 0, respectively. Upon cessation of treatments (20 weeks...
Prophylaxis of Rat Mammary Carcinogenesis

Chart 3. Effect of RA (1.0 mM) feeding, HI (CB-154 plus tamoxifen), and IS (CP of MT + FCA) on the genesis of DMBA (5 mg)-induced rat mammary gland carcinomas. A, 5 mg DMBA-placebo diet (Groups 1 to 4); B, 5 mg DMBA-1.0 mM RA diet (Groups 5 to 8). Percentage of rats bearing mammary carcinomas at Week 20 after carcinogen treatment in Groups 1 to 8 were: controls, 85; IS, 74; HI, 21; HI + IS, 28; RA, 60; RA + IS, 48; RA + HI, 5; RA + HI + IS, 0. p < 0.001, controls versus HI, HI + IS, RA + HI, RA + HI + IS; p < 0.01, controls versus RA; RA versus RA + HI, RA versus RA + IS.

after DMBA treatment, Groups 1 to 8), mammary carcinoma incidence increased in all groups.

In Experiments 1, 2, and 3, the treatments did not significantly alter body weight gains, when compared with placebo-fed animals. The only exception to this was in the RA plus HI-treated animals of Experiment 3 (Groups 7 and 8) in which a 25% reduction (p < 0.05) in body weight gains was observed.

Mammary fibroadenomas were excluded from data computation in all experiments; the numbers of these benign tumors in each group were too small for statistical analyses.

DISCUSSION

The salient observations in this study are as follows. (a) HI
significantly reduced mammary carcinoma incidence, an effect which was enhanced consistently by concurrent feeding of RA. Even the low dietary level of RA (0.2 mM concentration per kg of diet) was effective in further reducing mammary carcinoma incidence in HI-treated animals; this level of RA alone did not significantly affect mammary carcinoma incidence. (b) Non-specific IS with MER of BCG or CWS of NR, alone or in conjunction with RA feeding and/or HI treatment, did not significantly influence mammary carcinoma incidence. (c) IS with CP of MT + FCA significantly reduced mammary carcinoma incidence in RA-fed animals, but when administered alone this immune stimulant did not significantly influence the incidence of these tumors. The combination of RA feeding, HI treatment, and IS with CP of MT + FCA, however, completely blocked mammary carcinoma development; no palpable mammary carcinomas were observed in these animals for the entire 20-week treatment period.

Tamoxifen, a potent estrogen antagonist (6, 7), and CB-154, an efficacious inhibitor of prolactin secretion (20, 21), are effective suppressors of the genesis of carcinogen-induced rat mammary gland tumors when administered shortly after carcinogen treatment (5, 22, 23). In combination, these 2 drugs are more effective than when administered individually in the prophylaxis of this neoplastic process (22). RA, when fed shortly after carcinogen treatment, is also an effective inhibitor of rat mammary gland carcinogenesis (11). It is clear from the results of this study that, when these 2 very different types of treatments are administered together, a distinct synergism is observed; i.e., significantly fewer mammary carcinomas are observed than when these treatments are administered individually. A synergism between RA feeding and drug-mediated HI treatment in polycyclic hydrocarbon-induced mammary tumorigenesis has not heretofore been reported. Recently, our laboratory reported a synergism between RA feeding and CB-154 treatment in the prophylaxis of MNU-induced rat mammary gland tumors (20). More recent reports from 2 independent laboratories (9, 18) have shown a significant synergism between retinoid feeding and ovariectomy in the inhibition of the genesis of DMBA- or MNU-induced rat mammary gland tumors. Thus, it appears that mammary tumors induced in rats by 2 distinctly different types of carcinogens (DMBA and MNU) are significantly responsive to a synergistic action of RA feeding and hormone inhibition.

The lack of any significant effect of nonspecific IS, either alone or in combination with RA feeding and/or HI treatment, on the genesis of rat mammary gland carcinomas was disappointing. Kollmorgen et al. (8) reported that MER of BCG reduced mammary tumor incidence by approximately 50% in female Sprague-Dawley rats treated with DMBA and fed a low (2%)-fat diet. We administered MER of BCG as described by Kollmorgen et al. (8) and reduced mammary tumor incidence by only 15%. The only apparent reason for this discrepancy is that our diets contained approximately twice the amount of fat (~5%) in the diet used by Kollmorgen et al. (8). They reported that, upon raising the level of dietary fat, the therapeutic benefits of MER of BCG were nullified. Nagasawa et al. (13) reported that CWS of NR reduced mammary tumor incidence by approximately 33% in DMBA-treated female Sprague-Dawley rats. Using the methods of harvesting and administering CWS of NR, as described by Nagasawa et al. (13), we were able to achieve only a 13% reduction in mammary tumor incidence. The papers by Kollmorgen et al. (8) and Nagasawa et al. (13) are the only reports of which we are aware which attempted to influence, via immune stimulation, the early development of autochthonous DMBA-induced rat mammary gland tumors. Our failure to confirm these studies was not due to the inability of our bacteria preparations to elicit immune reactivity, because these preparations, when administered as described in this study, cause a delayed hypersensitivity type of reaction as determined by the method of footpad enlargement.

Stimulation of the immune system by the use of CP of MT + FCA significantly reduced the incidence of mammary carcinomas in RA-fed rats. This therapeutic regimen did not reduce mammary carcinoma incidence in placebo-fed or in HI-treated rats. Carcinogen-induced rodent tumors are known to have a wide variety of tumor-associated antigens, each antigen distinctly different from the others (14). Consequently, we pooled 25 discrete mammary carcinomas from DMBA-treated rats, a procedure which may have markedly contributed to the success of this therapy. In placebo-fed rats, such therapy reduced mammary carcinoma incidence by only 9%. However, in RA-fed rats, mammary carcinoma incidence was reduced by -41% by CP of MT + FCA treatment. In HI-treated rats, CP of MT + FCA treatment did not reduce mammary carcinoma incidence; but in HI rats that were fed RA and treated with CP of MT + FCA, no palpable mammary carcinomas developed during the 20-week treatment period. Retinoids have been reported to enhance immune reactivity. For example, the administration of retinoids to laboratory animals has been reported to cause thymus and lymph node enlargement (10), to enhance humoral antibody response to a variety of antigens (4), to enhance certain cell-mediated responses (1) and to enhance tumoricidal macrophage activity (16). It is conceivable that an immune system which is modified by high levels of retinoids is a system which can more efficaciously respond to a specific immune stimulus, i.e., the mammary carcinoma cell particulates mixed with Freund's complete adjuvant (killed Mycobacterium butyricum in an emulsifying oil), than to a nonspecific stimulus (i.e., CWS of NR and MER of BCG). Presently, we are examining the contribution of the Mycobacterium in this process. Notwithstanding, these results provide evidence, heretofore not reported, of a significant synergism between retinoid feeding and IS in the prophylaxis of rat mammary gland carcinogenesis.

Upon cessation of treatments (26 and 20 weeks after DMBA treatment in Experiment 1 and in Experiments 2 and 3, respectively), a moderate resurgence of mammary carcinoma incidence was observed in all treatment groups. This was disappointing, because we had hoped that by keeping mammary tumor burden low via RA feeding and/or HI treatments, the therapeutic adjuvant of IS would be more efficacious and therefore sharply reduce the emergence of mammary carcinomas. This clearly did not occur, because the rate of mammary carcinoma occurrence upon cessation of treatments was comparable in all IS groups when compared with that of the matched controls. Thus, to what extent, if any, CP of MT + FCA induced significant cellular and/or humoral tumor cytolytic effects cannot be assuredly determined. The fact that IS with CP of MT + FCA in RA-treated rats significantly reduced mammary carcinoma incidence during the treatment period, suggests, however that "killing" of incipient neoplasms did indeed occur. The emergence of mammary carcinomas upon
cessation of RA feeding and/or HI treatment is consistent with previous reports (20). HI interferes with the growth-promoting action of estrogen and prolactin on the tumor cells; elimination of this inhibitory effect results in prompt phenotypic expression of tumor growth. The mechanism by which RA feeding impedes the growth of incipient mammary tumors is unknown but may involve the recruitment of tumor cells into a more differentiated or differentiation-responsive state. Removal of the inhibitory effects of RA results in a prompt emergence of the incipient neoplasms to palpable tumors. These therapeutic modalities, acting epigenetically, are effective only at the time period for which they are administered; little or no enduring effect of these treatments is noted.

During the 20- to 25-week treatment periods, only 2 groups of rats showed a significant reduction in body weight gains when compared with the placebo-fed controls. The rats in Experiment 3 which received the combination treatment of HI and high dietary level of RA (1.0 mM concentration per kg ration) (Groups 7 and 8) had body weights approximately 25% less than the controls at the termination of treatment. HI alone or RA alone, while significantly reducing mammary tumor incidence, did not significantly affect body weight gains. Only when these 2 treatments were combined were body weight gains significantly reduced. To what extent the reduction in body weight gains in these groups of animals contributed to the suppression of tumorigenesis remains to be determined. Although these animals weighed less than controls, they were in good health; no apparent signs of illness were observed. It should be noted that in Experiments 1 and 2 (reduced dietary levels of RA) a synergism among RA and HI treatments was observed without a concomitant reduction in body weight gains. When these 2 treatments were combined were body weight gains significantly reduced. To what extent the reduction in body weight gains in these groups of animals contributed to the suppression of tumorigenesis remains to be determined. Although these animals weighed less than controls, they were all in good health; no apparent signs of illness were observed. It should be noted that in Experiments 1 and 2 (reduced dietary levels of RA) a synergism among RA and HI treatments was observed without a concomitant reduction in body weight gains. Thus, a significant synergism between HI treatment and RA feeding was observed in these studies without the complicating factor of reduced body weight gains. IS treatment, alone or in combination, did not affect body weight gains.

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