

## Induction of Cell Proliferation, Micronuclei and Hyperdiploidy/Polyploidy in the Mammary Cells of DDT- and DMBA-Treated Pubertal Rats

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The environmental estrogen, dichlorodiphenyltrichloroethane (DDT), and its metabolites have been implicated in the development of breast cancer through mechanisms that remain to be elucidated. It has been hypothesized that exposure to DDT and its metabolites, during critical periods of development, can contribute to an elevated risk for breast cancer in adults. In the present study, we have investigated the effect of *o,p'*-DDT on mammary gland cell proliferation and chromosomal alterations, in a rat mammary cancer model (commonly used to study human cancer), to gain insights into its potential role in the development of breast cancer. Twenty-one-day-old female Sprague-Dawley (SD) rats were administered *o,p'*-DDT, 7,12-dimethylbenz[*a*]anthracene (DMBA), genistein, DDT+DMBA, or DDT+DMBA+genistein, over a 14-day period. To determine changes in chromosome number and structure, we used the micronucleus assay as well

as multicolor fluorescence *in situ* hybridization (FISH) region-specific DNA probes for rat chromosomes 4 and 19. Cell proliferation was evaluated using 5-bromo-2'-deoxyuridine (BrdU). Significant increases in BrdU-incorporated cells were seen in the rats treated with DDT+DMBA. Although micronucleus frequencies were somewhat elevated in several of the treatment groups, significant increases were not seen in any of them. Significant increases in numerical chromosomal aberrations were detected in all of the DDT- and DMBA-treated groups. Genistein significantly reduced BrdU incorporation and polyploidy in the DDT+DMBA-treated rats. These initial studies indicate that DDT and DMBA can induce cellular and chromosomal alterations in the rat mammary gland, which is consistent with the hypothesis that these agents can induce early events in mammary carcinogenesis. Environ. Mol. Mutagen. 46:43–52, 2005. © 2005 Wiley-Liss, Inc.

**Key words:** mammary gland; cancer; dichlorodiphenyltrichloroethane; 7,12-dimethylbenz[*a*]anthracene; cell proliferation; hyperdiploidy; polyploidy; fluorescence *in situ* hybridization

### INTRODUCTION

Breast cancer is the most prevalent form of cancer in females and it is the second leading cause of death in women in the United States. While breast cancer incidence has decreased in recent times, it continues to increase in women aged 50 and above. By contrast, mortality rates have declined by 2.3% per year from 1990 to 2000 in all women [ACS, 2004]. Risk factors for breast cancer, including genetic predisposition [Coughlin and Piper, 1999; Dunning et al., 1999; Sakorafas and Tsiotou, 2000] and reproductive factors [Wohlfahrt et al., 1999; Daling et al., 2002; Meeske et al., 2004], account for only 30–50% of breast cancer cases [Davis et al., 1993; Madigan et al., 1995; Coyle, 2004]. Environmental factors such as exposure to chemicals, dietary habits, and lifestyle are

believed to account for the remaining 50–70% of breast cancer cases [Desaulniers et al., 2001].

Although some evidences indicate that environmental estrogens have a role in the etiology of breast cancer as

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well as adverse reproductive outcomes, the link between dichlorodiphenyltrichloroethane (DDT) and breast cancer remains controversial [Safe, 1995; Calle et al., 2002]. DDT (1,1,1-trichloro-2,2-bis(*p*-chlorophenyl)ethane) is a pesticide that was once widely used in agriculture, to control insect pests. After 1972, the use of DDT was no longer permitted in the United States except in case of a public health emergency. DDT does not occur naturally in the environment; however, it still enters the environment because of its current use in other areas of the world. Exposure to DDT and its metabolites and derivatives occurs mainly by eating foods containing small amounts of these compounds, which in turn can expose even infants drinking mother's milk to DDT. DDT bioaccumulates in the adipose tissue [Smith, 1999; LaKind et al., 2000]. There is general agreement that human populations are continuously exposed to a wide variety of environmental estrogens such as DDT and that identifying the effects of these compounds at the cellular and molecular level is necessary to understand the health risks.

DDT is a known animal carcinogen and is a suspected human carcinogen [IARC, 1991; NTP, 2002; EPA, 2004]. DDT has also been shown to be a tumor promoter [Kimbrough, 1995; Schrenk, 1998]. Several epidemiological and animal studies indicate that DDT may be associated with cancers of the lung, liver, pancreas, and breast [Kashyap et al., 1977; Rossi et al., 1977; Garabrant et al., 1992; Aronson et al., 2000; Mathur et al., 2002; Charlier et al., 2003; Pavuk et al., 2003]. In contrast, some other epidemiological studies have been unable to establish an association between DDT exposure and risk for breast cancer [Safe, 1995; Calle et al., 2002].

In the recent years, there has been much interest in understanding the molecular and cellular mechanisms involved in the carcinogenicity of organochlorines such as DDT, polychlorinated biphenyls (PCBs), and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) [Diel et al., 2002; Jeong and Kim, 2002; Bounias, 2003; Harada et al., 2003; Lee and Edwards, 2003; Frigo et al., 2004]. Several studies suggest a modulatory role for these organochlorines in animal mammary carcinogenesis models [Scribner and Mottet, 1981; Desaulniers et al., 2001]. However, most of these studies have not focused on early events that may be associated with the development of mammary cancer.

Previous studies have shown numerous ways by which DDT exposure can enhance carcinogenesis. DDT can induce cell proliferation [Snedeker and Diaugustine, 1996], inhibit gap junctional intercellular communications [Kang et al., 1996], enhance breast cell growth, and increase unscheduled DNA synthesis [Busser and Lutz, 1987]. DDT and its metabolites also activate the activator protein-1 (AP-1) transcription factor [Frigo et al., 2004], which has been associated with cell proliferation, differentiation, cellular stress, and cell death [Gupta et al.,

1999; Wisdom, 1999; Shaulian and Karin, 2001]. In addition, another mechanism by which DDT may cause breast cancer has been hypothesized to be through an alteration of estradiol metabolism. DDT increases levels of 16 $\alpha$ -hydroxyestrone (16 $\alpha$ -OH E<sub>1</sub>), a genotoxic metabolite of estradiol, which can also enhance breast cell growth [Bradlow et al., 1995]. DDT has been reported to bind to and activate the estrogen receptor (ER) [Steinmetz et al., 1996]. *o,p'*-DDT is an established environmental estrogen and both *o,p'*-DDT and its analog *p,p'*-DDT have been shown to bind to the human estrogen receptor (hER) [Mason and Schulte, 1981; Chen et al., 1997]. Furthermore, *o,p'*-DDT activates a number of cancer-related genes including Neu protein tyrosine kinase (C-erbB-encoded proto-oncogene product) [Hatakeyama and Matsumura, 1999].

7,12-Dimethylbenz[*a*]anthracene (DMBA) is a synthetic, polycyclic aromatic hydrocarbon, which is used as a prototype chemical carcinogen. A single feeding of DMBA to rats results in a high yield of mammary tumors in them [Huggins et al., 1961; Gruenstein et al., 1966]. The DMBA-induced rat mammary tumor model has found a broad application as a tool for assessing chemopreventive agents in the preclinical evaluation of drugs [Steele et al., 1994]. This rat mammary carcinoma model, developed a quarter of a century ago by Dr. Huggins, is the standard laboratory model for studying human breast cancer [Welsch, 1985].

In our study, we investigated the effects of *o,p'*-DDT on mammary gland cell proliferation and chromosomal alterations, in the rat mammary cancer model, to gain insights into the potential role of *o,p'*-DDT in the development of breast cancer. Since DMBA is widely used and validated in this *in vivo* carcinogenesis system, we have chosen this compound as an initiator to study the effects of DDT on the early chromosomal and cellular events in the carcinogenesis process.

Genistein is a natural isoflavonoid phytoestrogen found in soy products that is thought to possess breast cancer preventive properties. Genistein has been shown to give protection against DMBA-induced mammary cancer, in a rodent model [Lamartiniere et al., 1998]. Genistein is a planar molecule and is structurally similar to steroidal estrogens. Several potential mechanisms through which soy isoflavones may prevent cancer have been proposed. These include binding to ER- $\alpha$  [So et al., 1997], inhibition of tyrosine kinases [Akiyama et al., 1987], and suppression of mitogenic signaling. Genistein inhibits the growth of both ER-negative and ER-positive human breast cancer cells [Peterson and Barnes, 1991] and may have a protective effect against breast cancer [Hilakivi-Clarke et al., 1999; Day et al., 2001]. In our studies, we used genistein as a chemopreventive agent, to examine its protective effects in the DDT-DMBA-induced mammary tumor model.

**TABLE I. Experimental Design and Treatment Regimen in Animals Randomized into Groups I–VI**

Group	Treatment	Dose	Day administered
I (Control)	Sesame oil	200 $\mu$ l/rat	21, 23, 25, 27, 29, 31, 32, 34
II	DDT	50 mg/kg body wt	21, 23, 25, 27, 29, 31, 32, 34
III	DDT	50 mg/kg body wt	21, 23, 25, 27, 29, 31, 32, 34
	+ DMBA	40 mg/kg body wt	28
IV	DMBA	40 mg/kg body wt	28
V	DDT	50 mg/kg body wt	21, 23, 25, 27, 29, 31, 32, 34
	+ DMBA	40 mg/kg body wt	28
	+ Genistein	250 ppm	21–35 (mixed in the diet)
VI	Genistein	250 ppm	21–35 (mixed in the diet)

## MATERIALS AND METHODS

### In Vivo Treatment

Fourteen-day-old female Sprague-Dawley CD (SD) rats were purchased from Harlan Sprague-Dawley Laboratories (Indianapolis, IN). Upon receipt, the animals were placed on AIN-76A diet (Harlan Teklad, Madison, Wisconsin). The rats were maintained in a climate-controlled room at 22°C on a 12-hr light/dark cycle. Diet and tap water were available *ad libitum*. The rats were randomly divided into six groups (I–VI) and treatments were started at 3 weeks of age. Details of the experimental design are provided in Table I.

DDT and its metabolites, after absorption, are readily distributed through the lymph and blood to all the body tissues and ultimately stored in the lipid content of the tissues regardless of the route of exposure [Morgan and Roan, 1971]. Hence, we injected DDT subcutaneously in the rats. Although DMBA exposure can occur by various routes, the purpose of our study was to test the susceptibility of the mammary gland cells to DDT pesticides and hence we used the intragastric route of exposure. DDT was purchased from Sigma-Aldrich (St. Louis, MO). Rats in group I were injected subcutaneously with the sesame oil (Sigma-Aldrich) vehicle (200  $\mu$ l/rat). Animals from groups II, III, and V were injected subcutaneously with 50 mg/kg body wt *o,p'*-DDT on days 21, 23, 25, 27, 29, 31, 32, and 34 postpartum. Rats in groups III, IV, and V were gavaged on day 28 with 40 mg/kg body wt DMBA (Sigma-Aldrich). Rats in groups V and VI were also fed a diet containing 250 ppm genistein (LKT Laboratories, St. Paul, MN). In an earlier study, no toxicity to the female reproductive tract was observed at this dose [Fritz et al., 1998]. Two hours before killing, 5-bromo-2'-deoxyuridine (BrdU) (Sigma-Aldrich) was intraperitoneally administered at a dose level of 100 mg/kg body wt. The rats were killed on day 35 by CO<sub>2</sub> asphyxiation. No significant change in body weight was seen among the groups of treated animals, at the completion of the experiment.

### Processing of Mammary Tissue for Immunohistochemistry

Mammary glands were dissected, and the tissues were placed in cryomolds containing Optimum Cutting Temperature (OCT) embedding medium (VWR Scientific, Suwanee, GA). Samples were flash frozen by immersing in a metal container filled with 2-methyl butane (Sigma-Aldrich) pre-chilled in a larger metal container containing liquid nitrogen. Frozen blocks were wrapped in aluminum foil, stored in a –80°C freezer and were later sectioned on a cryostat. 5  $\mu$ m sections (for BrdU labeling) and 15  $\mu$ m sections (for fluorescence *in situ* hybridization) were collected on (+) charged slides (Fisher Scientific, Suwanee, GA) and were fixed just before drying in methanol/acetone (3:1) for 30 min on

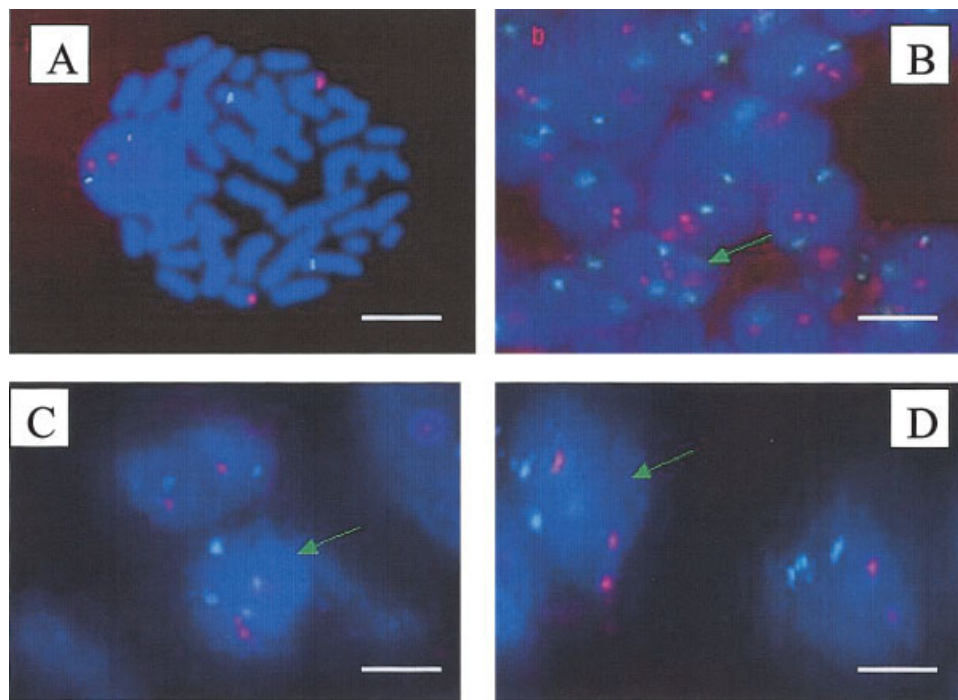
ice. Samples were then briefly rinsed in phosphate-buffered saline (PBS) and processed for immunofluorescent labeling.

### BrdU Labeling and Micronucleus Analysis

BrdU labeling was done by denaturing the cellular DNA in 0.07N NaOH (Sigma-Aldrich) followed by neutralization with PBS. The slides were then incubated with an anti-BrdU antibody (Sigma-Aldrich), diluted with 0.5% Tween-20 in PBS, in a humidified chamber for 30 min. The antibody was then detected by incubation with Texas Red-conjugated goat anti-mouse IgG (10  $\mu$ g/ml) (Molecular Probes, Eugene, OR) in a humidified chamber for 30 min. After washing the slides in PBS, the DNA was counterstained with 4,6-diamidino-2-phenylindole (DAPI; 1  $\mu$ g/ml) (Molecular Probes) in antifade mounting medium. The cells were analyzed at the University of Alabama at Birmingham (UAB) Imaging Facility. The microscope used was a Leitz Orthoplan with epifluorescence and Hoffman Modulation Contrast optics equipped with a Photometrics CH250 liquid-cooled CCD, high resolution, monochromatic camera (Leica, Wetzlar, Germany). For visualization of green, red, and blue fluorochromes, the 83,000 Pinkel filter set from Chroma Technology (Brattleboro, VT) was used. Image acquisition software, IPLab Spectrum, was from Scanalytics (Fairfax, VA).

### Fluorescence *In Situ* Hybridization

The chromosome 4- and 19-specific DNA probes [Hoebee and de Stoppelaar, 1996] were a generous gift from Dr. Barbara Hoebee (Bilthoven, The Netherlands). They were amplified and labeled with digoxigenin-dUTP and biotin-dUTP respectively (Boehringer-Mannheim, Indianapolis, IN) by nick translation in one of our laboratories (University of California, Riverside). Previously described methods were used to perform the FISH experiments [Eastmond and Pinkel, 1990; Trask and Pinkel, 1990; Hoebee and de Stoppelaar, 1996], with some modifications. In brief, the slides were brought to room temperature and the area containing the tissue sections were marked with a diamond pencil. The sections were dipped into three changes of xylene at room temperature for 30 min each to remove the embedding medium. The slides were then dehydrated with absolute alcohol for 5 min two times and air-dried. The target DNA was denatured in 70% formamide (in 2XSSC) for 5 min at 68°C and dehydrated through an ethanol series. To facilitate penetration of the FISH probes into the nuclei, the slides were incubated for 5 min in PBS (pH 7.0) followed by treatment with proteinase K solution (2  $\mu$ g/ml in PBS; pH 7.0) for 5–7 min at 37°C. The slides were rinsed twice with PBS (pH 7.0) and dehydrated through an ethanol series. At this point, the slides were maintained on a 37°C slide warmer. The DNA probe cocktail for each slide consisted of 1  $\mu$ l each of the digoxigenin-labeled and biotin-labeled DNA probes, 1  $\mu$ l sheared herring sperm



**Fig. 1.** FISH with chromosome-specific DNA probes. The dual color FISH technique using locus-specific probes for chromosomes 4 (green) and 19 (red) are illustrated in (A)–(D). (A) Normal interphase and metaphase from fibroblast showing two copies each of chromosomes 4 and 19. (B) Hyperdiploid cell with four copies of chromosome 19 and three

copies of chromosome 4. (C) Hyperdiploid cell with four copies of chromosome 4 and normal copy number for chromosome 19. (D) Polyploid cell with three copies each of chromosomes 4 and 19. Images were taken from DDT- or DMBA-exposed rats. Arrow indicates an aberrant cell. Bar = 10  $\mu$ m.

DNA (1 mg/ml; Sigma, St. Louis, MO), 7  $\mu$ l of MM2.1 hybridization mix (to give a final concentration of 55% formamide, 1XSSC, 10% dextran sulfate) (see Trask and Pinkel [1990] for additional details). The DNA probe cocktail was denatured at 68°C. The denatured probe was applied to the tissue sections, covered with a glass cover slip, and the borders sealed with rubber cement. The slides were incubated overnight at 37°C in a humidified box. Post-hybridization washes were performed with three changes of 50% formamide (in 2XSSC) for 5 min each at 42.5°C. Following a brief rinse in PX-buffer (0.1M phosphate-buffer, 0.2% w/v Triton X-100) at room temperature, the digoxigenin-labeled probe was detected using a fluorescein isothiocyanate (FITC)-conjugated sheep anti-digoxigenin antibody (20  $\mu$ g/ml in PX-buffer with 5% non-fat dry milk supernatant (PXM); Boehringer-Mannheim, Indianapolis, IN) and the biotin-labeled probe was detected with Alexa 555-streptavidin antibody (20  $\mu$ g/ml in PXM buffer). DAPI (0.5  $\mu$ g/ml) in a diphenylene-diamine antifade mounting medium was used to counterstain the DNA.

### Scoring Procedures and Criteria

For the FISH studies, a Nikon microscope equipped with a FITC/DAPI/Texas Red filter set (Chroma Technology) was used to visualize the fluorescent signals at 1250 $\times$ . All slides were coded prior to scoring. Smaller nuclei representing incomplete nuclei resulting from sectioning were disregarded during scoring. For each slide, at least four different areas of the tissue section were scored for hyperdiploidy and polyploidy. In most cases, 1,000 cells were scored per rat (median 1,000 cells, with a range of 190–1,134 cells). Cells with three or more hybridization signals for either chromosome 4 or 19 were classified as hyperdiploid for that chromosome. Cells with three or more copies of both chromosomes 4 and 19 present in the same cell were classified as polyploid

(Fig. 1). Studies from our laboratory have shown that the use of two separate probes can efficiently detect polyploidy [Schuler et al., 1998; Schuler et al., 2003; Olaharski and Eastmond, 2004]. For BrdU incorporation and micronuclei (MN), an average of 2,000 cells were scored from each treated rat. MN were scored according to previously established criteria [Fenech, 1993].

### Statistical Analyses

The effect of chemical treatment on the frequencies of BrdU incorporation, MN, polyploidy, and hyperdiploidy in the mammary epithelial cells of the control and treated rats was determined by ANOVA on log transformed data [ $\ln(1+x)$ ], using Statview statistical software (SAS Institute, Cary, NC). Following a positive result, Fisher's protected least significant difference (PLSD) test was used as a *post-hoc* test to compare the individual treatments.

### RESULTS

To determine the potential cellular and genetic effects of DDT in the DMBA mammary carcinogenesis model, the incorporation of BrdU, the frequencies of MN, and alterations in chromosome number (hyperdiploidy and polyploidy) were evaluated in rat mammary cells following treatment with DDT, DMBA, DDT+DMBA, or DDT+DMBA+genistein.

**TABLE II. Induction of Cell Proliferation in Mammary Cells of Pubertal Rats Treated with DDT, Genistein, and DMBA**

Treatment	Number of animals studied	Mean $\pm$ SD (%)	
		Frequency of micronuclei <sup>a</sup>	Frequency of BrdU labeled cells <sup>a</sup>
Sesame oil	6	0.53 $\pm$ 0.25	0.62 $\pm$ 0.30
Genistein	3	0.26 $\pm$ 0.04	0.43 $\pm$ 0.18
DDT	6	0.69 $\pm$ 0.26	1.16 $\pm$ 0.52
DMBA	5	0.90 $\pm$ 0.34	1.09 $\pm$ 0.43
DDT + DMBA	5	1.09 $\pm$ 0.62	1.91 $\pm$ 0.72 <sup>b</sup>
DDT + DMBA + Genistein	6	1.09 $\pm$ 1.06	0.98 $\pm$ 0.72

<sup>a</sup>An average of 2,000 cells was scored from each animal.

<sup>b</sup> $P = 0.0005$ .

### Micronuclei

The micronucleus assay was used to determine the chromosomal breakage and loss occurring in rat mammary cells. Although micronucleus frequencies were somewhat elevated in several of the treatments, significant increases were not seen in any of the treatment groups (Table II).

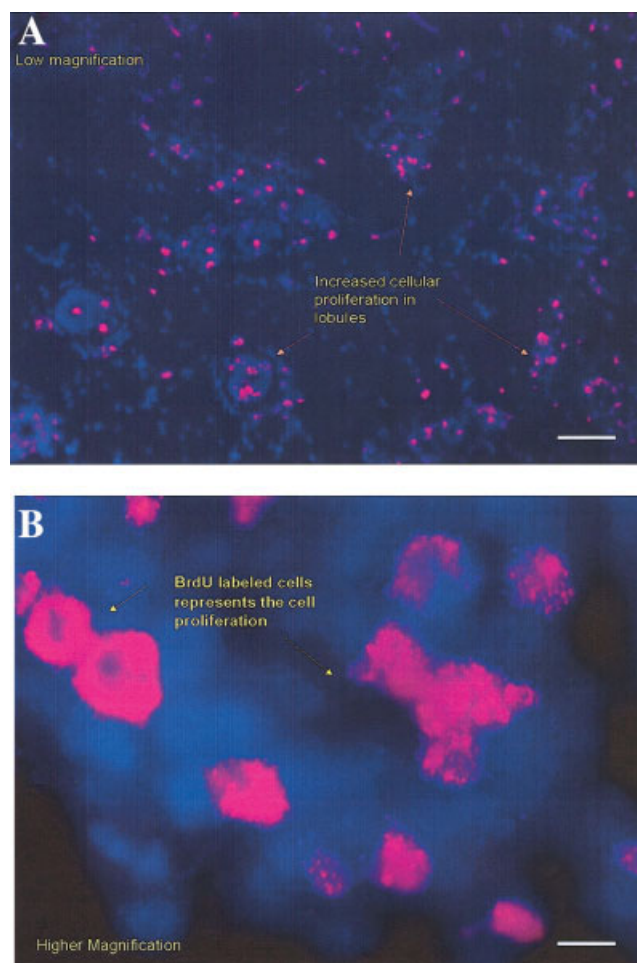
### BrdU Incorporation

BrdU labeling was used to examine cell proliferation in mammary cells. BrdU incorporation was significantly increased in rats treated with DDT+DMBA (Table II,  $P = 0.0005$ ). The increases seen in the DDT+DMBA treatment group were significantly greater than those seen in the rats treated with DDT or DMBA alone ( $P = 0.02$ ).

None of the other treatment groups differed significantly from the control, although some elevation in BrdU labeling was seen in rats treated with DDT or DMBA alone. Rats treated with DDT+DMBA+genistein exhibited significantly reduced BrdU labeling, compared with those treated with DDT+DMBA ( $P = 0.01$ ). As illustrated in Figure 2A and B, proliferating cells were observed in many of the major structures of the mammary gland.

### Hyperdiploidy and Polyploidy

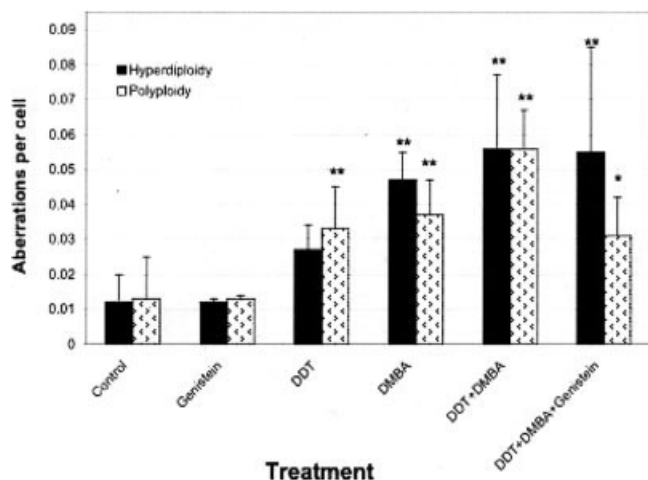
We used FISH with dual color DNA probes for rat chromosomes 4 and 19 to determine the effects of DDT and DMBA on chromosome number. Significant increases in nuclei containing three or more hybridization regions (representing a combination of both hyperdiploid and polyploid cells) were seen in animals treated with DDT, DMBA, DDT+DMBA, and DDT+DMBA+genistein (Figs. 1 and 3). In contrast, no increase was seen in the genistein-treated animals. While the frequency of total numerical aberrations was significantly higher in the DDT+DMBA treatment as compared to the DDT treatment ( $P = 0.014$ ), the same comparison with the DMBA-



**Fig. 2.** A: Cell proliferation as measured by BrdU incorporation in dividing cells. A section of DDT+DMBA-treated mammary gland showing proliferating cells in major structures. Bar = 100  $\mu$ m. B: A is magnified. Bar = 10  $\mu$ m.

treated animals, while elevated, did not quite attain statistical significance ( $P = 0.0673$ ).

A separate examination of the hyperdiploid and polyploid categories revealed similar, albeit somewhat more varied, results. The frequency of polyploid cells increased almost three-fold in the DDT-treated animals, an increase



**Fig. 3.** Induction of hyperdiploidy and polyploidy in mammary cells of pubertal rats treated with DDT, genistein, and DMBA individually as well as in combination. An average of 1,000 cells was scored from each animal using FISH with region-specific DNA probes for rat chromosomes 4 and 19. Mean frequency of hyperdiploid and polyploid cells was calculated from the transformed data [data were transformed using  $\ln(1+x)$  for each animal]. Final mean (shown in the figure) was calculated by averaging the mean values from five animals. (\* $P \leq 0.05$ ; \*\* $P \leq 0.01$ ; significantly increased as compared with the respective control).

that was significantly higher than that of control frequencies. Similarly, the frequency of hyperdiploid cells (cells exhibiting three hybridization signals for one of the two chromosomes) increased approximately two-fold, which while elevated, was not significantly higher than that of the controls ( $P = 0.12$ ). Significant increases in both hyperdiploid and polyploid cells were seen in the DMBA-, DDT+DMBA-, and DDT+DMBA+genistein-treated animals. A modest but significant decrease in polyploid cells was also observed in the DDT+DMBA+genistein-treated group relative to DDT+DMBA-treated group.

## DISCUSSION

The most extensively studied rodent model for mammary tumorigenesis has been DMBA induction of mammary carcinoma in SD rats. In this model, virtually all of the treated rats develop breast tumors of ductal origin within 3–4 months, provided that DMBA is given during the period of ductal morphogenesis, which occurs at approximately 30–65 days of age [Russo et al., 1990]. A single feeding of DMBA to rats results in a high yield of mammary tumors [Huggins et al., 1961; Gruenstein et al., 1966]. Tumors appearing in this rat mammary model are histologically and biochemically similar to human tumors [Escrich, 1987a; Escrich, 1987b; Russo et al., 1990]. Additional features that make this model the most widely used as an experimental mammary tumor system are tumor induction ease and reliability, organ site specificity,

tumors of ductal origin, tumors of predominantly carcinomatous histopathological characteristics, tumors of varying growth factor and/or hormone responsiveness, and the potential to examine the tumor initiation and promotion processes [Welsch, 1985; Russo and Russo, 1996; Izzotti et al., 1999; Cabello et al., 2001; Kubatka et al., 2002; Solanas et al., 2002; Moral et al., 2003]. Previous work indicates that the induction of mammary carcinogenesis in the rat by DMBA depends on the age of the animals and the extent of differentiation of the mammary gland, at the time the carcinogen is administered [Russo and Russo, 1978; Welsch, 1985]. Perturbations in pathways involved in mammary gland ontogeny, such as morphogenesis, cell proliferation, and differentiation, can lead to neoplasia [Visvader and Lindeman, 2003].

In this study, we have examined both the epigenetic (cell proliferation) and genotoxic (micronucleus induction and hyperdiploidy/polyploidy) effects of DDT and DMBA in pubertal SD rats. A number of chemicals cause cancer through genotoxic mechanisms, by altering DNA and inducing mutations. However, chemicals that do not directly alter DNA or induce mutations and yet induce cancer after chronic administration are designated as non-genotoxic or epigenetic carcinogens. Using the SD rat model, we were able to show that both DMBA and DDT induce numerical chromosomal changes in rat mammary cells following short-term treatment, and that DDT in combination with DMBA stimulates cell proliferation in the mammary glands of the treated rats. To our knowledge, this is the first report of induction of numerical chromosomal changes by DDT or DMBA in the rat DMBA mammary carcinogenesis model. In addition, our data suggest that the tumor-promoting effect of DDT in the DDT–DMBA model may occur through multiple pathways involving both genotoxic and epigenetic mechanisms.

In these studies, we used *o,p'*-DDT which has more estrogenic activity than *p,p'*-DDT [Welch et al., 1969; Robison and Stancel, 1982], and the results indicate that the *o,p'* isomer can induce both proliferative and genetic alterations in the mammary cells of DMBA-treated rats. Numerical chromosome alterations were also induced by *o,p'*-DDT itself indicating that this compound can induce significant genetic changes in the mammary gland of treated rats. The consequences of these changes and long-term treatment with *o,p'*-DDT are uncertain, as chronic animal studies using only this isomer have not been performed. Previous carcinogenicity bioassays have employed technical grade DDT, which consists primarily of *p,p'*-DDT (~70–85%), with lesser amounts of *o,p'*-DDT (~10–20%), *o,o'*-DDT, and other chlorinated compounds (~0–4%). These studies involving rodent models have clearly demonstrated that the technical-grade mixture of the DDT isomers is carcinogenic, producing tumors in several tissues. Interestingly, increases in mammary tumors have not been defined in these studies. It should be noted,



however, that the doses of *o,p'*-DDT used in the present study were significantly higher than those administered with the technical grade DDT. Exposure to technical grade DDT at doses as low as 0.26 mg DDT/kg/day produced liver tumors in mice [Tomatis et al., 1972; Thorpe and Walker, 1973]. Increases in pulmonary adenomas and malignant lymphomas also were seen in mice at doses ranging from 1–32.5 mg/kg/day [Kashyap et al., 1977]. In rats, liver tumors were reported at similar doses (34.1 mg/kg/day) [Rossi et al., 1977]. Based on these and other results, several authoritative bodies, including the International Agency for Research on Cancer, the National Toxicology Program, and the US Environmental Protection Agency, have concluded that DDT is carcinogenic to animals and is a possible human carcinogen [IARC, 1991; NTP, 2002; EPA, 2004].

Previous studies with other xenoestrogens such as diethylstilbestrol (DES) have shown that these agents can act through multiple genetic and nongenetic mechanisms [Russo and Russo, 1980]. The role of cell proliferation as a critical event in determining cancer risk has also received increasing attention [Cohen and Ellwein, 1990; Preston-Martin et al., 1990]. A dividing cell is much more at the risk of undergoing both chromosomal and point mutations than a quiescent cell, particularly those induced by carcinogens or mutagens [Cohen and Ellwein, 1990]. Adducts or other types of DNA-damage also can be converted to heritable mutations, during cell division [Preston-Martin et al., 1990]. Furthermore, the loss of a tumor suppressor gene or a rearrangement of an oncogene typically requires cell division. In addition, it is generally accepted that an estrogen-mediated increase in cell proliferation leading to disturbances in the cell cycle may provide the necessary environment for the development of some cancers, particularly breast cancer in humans [Preston-Martin et al., 1990]. In our studies, significant increases in cell proliferation were observed only in the DDT+DMBA group (Fig. 2A; Table II).

MN are formed from chromosomal fragments or whole chromosomes that are not incorporated into daughter nuclei during mitosis. In our studies, micronucleus frequencies were somewhat elevated by several of the treatments. However, significant increases in MN were not seen in any of the treatment groups. It should be noted that because of the small cytoplasmic space in mammary epithelial cells, the formation and detection of MN in these cells is difficult and it is unlikely that the analysis of MN efficiently detects chromosome loss or breakage occurring in this type of cell.

Studies have shown that *o,p'*-DDT competitively inhibits estradiol from binding to the ER. Since estrogens are responsible for the growth of tumors, it is suggested that *o,p'*-DDT influences DMBA-induced tumors through this interaction [Mason and Schulte, 1981]. Estradiol metabolism yields two major metabolites, 2-hydroxyestrone

(2-OH E<sub>1</sub>) through the catechol pathway and 16 $\alpha$ -OH E<sub>1</sub> by an alternative pathway [Fishman et al., 1984; Bradlow et al., 1996]. These metabolites have markedly different properties. 2-OH E<sub>1</sub> is weakly antiestrogenic and nongenotoxic [Suto et al., 1993], and has been associated with reduced breast cancer risk [Bradlow et al., 1996]. In contrast, 16 $\alpha$ -OH E<sub>1</sub> is estrogenic, forms covalent bonds with ERs, is tumorigenic and genotoxic, and causes increased cell proliferation [Fishman et al., 1980; Swaneck and Fishman, 1988; Telang et al., 1992]. DDT and *o,p'*-DDE significantly increase levels of 16 $\alpha$ -OH E<sub>1</sub> while lowering levels of 2-OH E<sub>1</sub>. DMBA also increases ratios of 16 $\alpha$ -OH E<sub>1</sub> [Bradlow et al., 1995]. It is possible that DDT and DMBA synergistically alter estradiol metabolism to produce the genotoxic metabolite 16 $\alpha$ -OH E<sub>1</sub>, which results in the initiation process of carcinogenesis. On the other hand, soy isoflavones containing the weakly estrogenic genistein and daidzein increase the metabolism of endogenous estrogens to the protective 2-hydroxylated estrones in women [Lu et al., 2000]. They were also shown to reduce 16 $\alpha$ -OH E<sub>1</sub> levels [Kishida et al., 2000]. Thus in our study, it is possible that genistein is exerting a protective effect on DDT+DMBA-induced genotoxicity, by lowering production of genotoxic metabolites and production of less active metabolites such as 2-OH E<sub>1</sub>. It also is likely that DMBA can cause direct toxicity by producing genotoxic metabolites. DMBA can be metabolized to the genotoxic metabolite DMBA-dihydrodiolepoxide, which can bind to DNA and initiate the carcinogenic process [Izzotti et al., 1999]. DDT could then exert its promoting effects on the initiated cells.

DDT inhibits gap junctional intercellular communications [Kang et al., 1996]. Lack of these junctions can also contribute to the invasiveness of tumor cells.

Chromosomal aberrations are commonly used as an indicator of exposure to genotoxic compounds, and aberrations are associated with increased risk for cancer [Oshimura and Barrett, 1986]. Studies also have shown that aneuploidy is a frequent genetic alteration associated with cancers and cellular transformation [Li et al., 1997]. Chemical estrogens such as DES, estradiol and bisphenol A produce aneuploidy [Schuler et al., 1998; Ochi, 1999], presumably by interfering with the spindle apparatus, during cell division. In addition to aneuploidy, our studies show that DDT and DMBA can increase the frequency of polyploidy in the treated animals. Polyploidy is another type of genetic alteration that has been associated with carcinogenesis [Atkin, 2000; Storchova and Pellman, 2004]. It should be noted that the hyperdiploid cells detected in this type of FISH study may represent polyploid cells that have lost one hybridization signal due to tissue sectioning, inefficient hybridization, or signal overlap. In addition, since the regions targeted by the DNA probe are located on the arms of the rat chromosomes, an increased number of hybridization signals also could

result from a segmental aneuploidy or premature chromosome separation induced by DDT or DMBA. Significant increases in hypodiploidy were not observed in any of the treatment groups. This may be due to the insensitivity of FISH techniques to detect chromosome loss as well as the difficulties in detecting chromosome loss in sectioned tissues where only part of a nucleus may be present. Most previous cytogenetic studies with DDT were conducted in vitro and were largely negative [IARC, 1991]. However, it is unlikely that these in vitro systems were capable of detecting the estrogenic effects of *o,p'*-DDT.

Modulation of chemically induced mammary tumor development by organochlorines may be important in understanding the initiation and promotion phases of carcinogenesis. In the absence of an initiator, organochlorines may not induce the critical effects necessary for mammary tumor development. However, as seen in our studies, *o,p'*-DDT by itself is capable of inducing genetic changes in the rat mammary cells. It is clear that more research is needed to fully understand the DDT–DMBA mammary carcinogenesis model. Using the chemopreventive agent genistein, we were able to demonstrate a modest protective effect on the DDT+DMBA-induced chromosomal aberrations and cell proliferation. In summary, our results indicate that cellular and chromosomal alterations are caused by DDT and DMBA and may represent early events in rat mammary carcinogenesis. These results also support the hypothesis that similar alterations occurring in DDT-exposed women may contribute to increases in incidence of breast cancer.

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