Estrogenic and DNA-damaging Activity of Red No. 3 in Human Breast Cancer Cells

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Exposure to pesticides, dyes, and pollutants that mimic the growth promoting effects of estrogen may cause breast cancer. The pesticide DDT and the food colorant Red No. 3 were found to increase the growth of HTB 133 but not estrogen receptor (ER) negative human breast cells (HTB 125) or rat liver epithelial cells (RLE). Red No. 3, β-estradiol, and DDT increase ER site-specific DNA binding to the estrogen response element in HTB 133 cells and increase cyclin-dependent kinase 2 activity in MCF-7 breast cancer cells. Site-specific DNA binding by p53 in RLE, HTB 125, HTB 133, and MCF-7 cells was increased when they were treated with Red No. 3, which suggests that cellular DNA was damaged by this colorant. Red No. 3 increased binding of the ER from MCF-7 cells to the estrogen-responsive element. Consumption of Red No. 3, which has estrogenlike growth stimulatory properties and may be genotoxic, could be a significant risk factor in human breast carcinogenesis. — *Environ Health Perspect* 105(Suppl 3):625–632 (1997)

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In the United States, breast cancer is the most commonly diagnosed cancer and the second leading cause of cancer deaths (*1*). It has been estimated that one in eight U.S. women will develop breast cancer (*2*). Further, the incidence of breast cancer in the United States is increasing 1% per year in the 1990s (*2*). The personal and economic impacts of breast cancer make this disease a serious national health care concern. The total cost of breast cancer burdens the U.S. economy with direct and productivity losses of approximately $3.8 billion per year (*3*).

Great progress has been made recently in determining the molecular basis of familial forms of breast cancers (*4*). However, only 5 to 15% of the total number of breast cancers can be traced to an inherited familial defect (*4*). Although the etiology of the majority of breast cancers remains undetermined, epidemiologic studies have indicated that a dietary component contributes to the risk of developing the disease (*5,6*). High-fat diets may be a risk factor for breast cancer (*5,6*). However, the focus on fat as a major risk factor has shifted to the effects of hormones and hormone-mimicking chemicals (*2*).

Compounds that mimic the effects of estrogen are popularly referred to as xenoestrogens or environmental estrogens (*7,8*). Pesticides such as DDT, dyes (phenol red), and polychlorinated biphenyls are estrogenic (*9–11*). It has been suggested that exposure to xenoestrogens increases the risk of breast cancer for women in industrialized countries (*8,14*). However, the role of DDT and other environmental estrogens in the etiology of breast cancer in humans is controversial (*7,12–14*).

Recently, it has been shown that phthalate plasticizers from plastic food-packaging materials exhibit estrogenic activity (*15*). Butylated hydroxyanisole, a commonly used food preservative, also has weak estrogenic effects (*15*). Phenosulfo-thiazine, a red dye used in tissue culture media as a pH indicator, is a weak estrogen that stimulates the growth of human breast cells and binds to the estrogen receptor (ER) in MCF-7 human breast cancer cells (*9*). Because some food dyes are carcinogens (e.g., Red No. 4), we hypothesized that the dietary component that increases the risk of breast cancer in U.S. women might be xenoestrogenic food dyes. We examined the ability of food colorants to stimulate ER-positive and negative cells to enter the cell cycle. We also examined dye-treated cells for effects indicating damage to genetic material.

Materials and Methods

Chemicals

17β-Estradiol (E_2) and ICI 182,780 were gifts from J. Wimalasena of the University of Tennessee Medical Center, Knoxville, Tennessee. DDT (Sigma Chemical Co, St. Louis, MO) and estradiol were made up as concentrated solutions in absolute ethanol. An equal amount of ethanol was added to all control cells. Red No. 3 (erythrosin bluish CI 45430) (Pfaltz and Bauer, Waterbury, CT) was dissolved in sterile, distilled water. DNA-damaging chemicals and chemotherapeutic agents (Sigma) were used as previously described (*16*). All other food colorants were obtained from Pfaltz and Bauer, Aldrich Chemicals (Milwaukee, WI), Kodak Fine Chemicals (Rochester, NY), or local suppliers.

Cells

The ER-positive MCF-7 and HTB 133 human breast carcinoma cell lines were gifts from J. Wimalasena of the University of Tennessee Medical Center. HTB 125 ER-negative cells were obtained from the American Type Culture Collection (Rockville, MD). Breast cells were maintained in Dulbecco's modified minimal essential medium (DMEM)/Hams F12 1:1 without phenol red. Both cell lines were incubated at 37°C in a 5% CO_2 atmosphere with 10% fetal bovine serum (FBS). Prior to studies on cyclin-dependent kinase 2 (Cdk2) activation, MCF-7 cells were growth arrested by removal of serum and

transfer into methionine-free medium for 72 hr before exposure. During xenoestrogen exposure, breast cells were maintained in methionine-containing DMEM/F12 (phenol red and FBS free).

Rat liver epithelial (RLE) cells were a kind gift from J. Trosko of Michigan State University, East Lansing, Michigan. RLE cells, which are ER-negative (C Dees, unpublished data), were maintained in Richter's medium.

Proliferation Assays

Proliferation assays were performed by placing the cells in serum-free phenol red and methionine-free medium for 24 hr. Before adding xenoestrogens, cells were returned to media containing methionine but without serum or phenol red. Cells were incubated with xenoestrogens for 72 hr and then released by trypsinization. Cell counts were performed manually or with a Coulter model S cell counter.

Cdk2 Assays

Cdk2 assays were performed as described previously by Foster and Wimalasena (17). Cells for Cdk2 analysis were exposed for 20 hr. After incubation, MCF-7 cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed by the addition of cold lysis buffer (Tris 20 mM, pH 7.5, NaCl 250 mM, NP-40, 0.1% NaF 10 mM, NaVO 1 mM, phenylmethylsulfonyl fluoride [PMSF] 1 mM). After 15 min on ice, the lysates were centrifuged at $20,000 \times g$ for 15 min (4°C). Cdk2 was precipitated from equal amounts of cell extracts using purified rabbit anti-Cdk2 (Santa Cruz Biotechnology, Santa Cruz, CA) and Protein A/G agarose. Immunoprecipitates were washed (three times) with lysis buffer and twice with kinase buffer (Tris 40 mM, pH 7.5, MgCl2 10 mM). The immunoprecipitates were suspended in 30 μl of kinase buffer supplemented with 400 μg/ml histones (Sigma type II-SS [HH1]), 5 μM ATP, 0.5 μM dithiothreitol, 0.5 mM EGTA, and 5 μCi γ-[32P]-ATP for 20 min at room temperature. The reaction was stopped using gel electrophoresis sample buffer, and the reaction products were separated on a 14% polyacrylamide gel (Novex, San Diego, CA).

ER–ERE Mobility Shift Procedures

Cells were cultured in 175-cm² flasks in DMEM/F12 (phenol red free) supplemented with 10% FBS. The medium was replaced with fresh medium without serum 24 to 48 hr prior to adding compounds for

growth promoting effects. Cells were then incubated for 1 hr with xenoestrogens or exposed to electromagnetic fields (EMF) for 1 hr. Cell extracts were prepared by removing the medium and washing the monolayers three times with PBS, pH 7.4. Cells were lysed by the addition of binding buffer (20% glycerol, 0.4 mM KCl, 2 mM DTT, 1 mM PMSF, in 20 mM Tris–HCl buffer, pH 7.5) using a glass Dounce homogenizer. The lysate was centrifuged at $10,000 \times g$ for 15 min and the supernatants retained for testing. Total protein content of the extracts was determined using BCA protein assays (Pierce Biochemicals, Rockford, IL). Protein content for all samples was equalized prior to performing the binding assay. The estrogen responsive element (ERE) (GTCCAAAGTCAGGTCA CAGTGACCGATCAAGTT) as described by Kumar and Chambon (18) and the complementary strand were synthesized, prepared in double-stranded form, and end-labeled with [32P]-ATP using T4 kinase. Binding reactions consisted of 5 μl of protein (approximately 5 ng), 0.5 ng 32P-labeled oligonucleotide, 1 μl of a 1.9-μg/ml Poly dI, dC solution (Sigma Chemical, St. Louis, MO), and 25 μl binding buffer. Binding reactions were incubated at room temperature for 20 min. The entire reaction mixture was then separated on 6% nondenaturing polyacrylamide gels (Novex, San Diego, CA) and visualized by autoradiography.

Competitive binding studies using the ER from MCF-7 were performed as described previously (11) with modifications to accommodate the use of cultured cells. To confirm visual interpretation of ER–ERE mobility shift increases stimulated by xenoestrogens, autoradiographs were scanned using a Hewlett Packard ScanJet IIcx. Densitometric evaluation was performed using SigmaScan software.

p53 Mobility Shift Procedures

Procedures for p53 mobility shift assays were similar to those described previously (16,19,20). RLE cells were cultured in 175 cm² flasks in Richter's medium supplemented with 0.5% newborn calf serum. The medium was replaced prior to adding compounds for testing with fresh medium containing test compounds without serum. Cells were then incubated for 2 hr with DNA-damaging agents or exposed to EMF. Untreated control cells were also examined. S9 homogenate (Molecular Toxicology, Annapolis, MD) was prepared from rats treated with Aroclor 1254. The

S9 mix components were 8 mM MgCl2, 33 mM KCl, 5 mM glucose 6-phosphate, 4 mM NADP, 100 mM sodium phosphate, pH 7.4, and S9 at 10% (v/v) of mix. Food colorants were added to 1-ml S9 mix and then added to cultured cells.

Nuclear extracts from the cells were prepared as described (13). Briefly, the medium was removed from the cells and the monolayers washed with PBS, pH 7.4. Cells were lysed by the addition of 2.5 ml buffer (20% glycerol, 10 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, and 0.1% Triton X-100 in 20 mM HEPES buffer, pH 7.6). The lysate was centrifuged at $800 \times g$ for 4 min; the resulting pellet was diluted with three volumes of 500 mM NaCl in buffer (see previous description), then incubated at 4°C for 30 min with agitation. The mixture was centrifuged at $35,000 \times g$ for 10 min and the supernatants containing p53 were removed for immediate analysis. The total protein content of the extracts was determined using BCA protein assays (Pierce Biochemicals). Protein content for all samples was equalized prior to performing the binding assay. The consensus p53 binding sequence (GGACATGCCCGGG CATGTCC) was synthesized, prepared in double-stranded form, and end-labeled with [32P]-ATP). A 21mer randomized at each base was synthesized and used as a nonspecific competitor control. Binding reactions consisted of 20 μg nuclear protein, 0.5 ng 32P-labeled oligonucleotide, and 0.5 μg salmon sperm DNA (Sigma Chemical) with buffer (without Triton) in a final volume of 25 μl. Binding reactions were incubated at room temperature for 20 min; 8 μl of the reaction mixture was separated on 6% nondenaturing polyacrylamide gels (Novex) and visualized by autoradiography.

Results

Cell Proliferation Studies

We examined a number of synthetic food dyes to determine their potential for growth-promoting activity on ER-positive growth-arrested human breast cancer cells. The effects of Red No. 3 were also tested on ER-negative HTB 125 breast cells and on a RLE cell line. One synthetic food dye (Food Drug and Cosmetics Red No. 3) was found to stimulate the growth of ER-positive human breast cancer cells in proportion to the applied dose (Figure 1A). ER-negative cultured breast cells did not respond to Red No. 3 (Figure 1B), nor did

RLE cells (Figure 1C). However, HTB 125 cells may be myoepithelial in origin, whereas HTB 133 cells are transformed and are probably derived from secretory epithelia. Therefore, HTB 125 cells may differ from HTB 133 cells in many other aspects besides being ER-negative. ER-positive breast cancer cells also respond to DDT in a dose dependent manner (Figure 2A), whereas RLE did not respond (Figure 2B). These two studies suggest that Red No. 3 could stimulate the growth of human breast cells and that effects are mediated

through the ER. Therefore, we examined the effects of the steroidal antiestrogen ICI 182,780 on breast cells treated with Red No. 3, DDT, and β-estradiol. Antiestrogen treatment (10 nM) suppressed the proliferation of breast cells stimulated by DDT (0.3 μM) and Red No. 3 (10 μg/ml) but not β-estradiol (10 nM) (Figure 2C). Higher concentrations of ICI 182,780 (150 nM) suppressed the effects of β-estradiol (Figure 2C). These lines of evidence suggest that Red No. 3 is a xenoestrogen similar to phenol red and DDT.
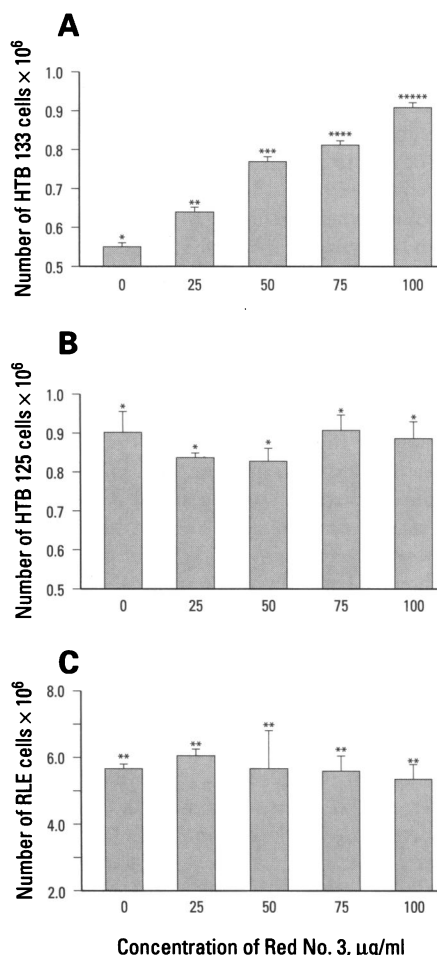
Competitive Binding and ER Mobility Shift Studies

Competitive binding studies (11) were used to confirm that Red No. 3 has estrogenic activity. Red No. 3 successfully competed for the ER from the MCF-7 cells (Figure 3). Red No. 3 (25 μg/ml) increased ER–ERE binding approximately 2.5-fold over that produced by the control (1 times protein control). Doubling the protein in the control reaction mixture (2 times protein control) increased the intensity of the ER–ERE complex approximately 3-fold higher than produced by the 1 times protein control (Figure 4A). The lowest concentration of estradiol that increased ER–ERE binding was 100 pM, which increased ER–ERE intensities approximately 1.3-fold over the 1 times protein control. DDT (300 nM) increased ER–ERE binding nearly 2-fold over the 1 times protein control (Figure 4A).

Red No. 3 increased ER–ERE binding 1.5-fold over the protein control (Figure 4B). Low concentrations of ICI 182,780 (10 nM), when added to the medium of HTB 133 cells containing Red No. 3 (25 μg/ml), completely inhibited increased ER binding to the ERE but only partially inhibited the response stimulated by DDT (0.3 μM) (0.5-fold of the control) and β-estradiol (10 nM) (0.5-fold of the control) (Figure 4B). Complete inhibition of estradiol-induced ER–ERE binding by ICI 182,780 requires 10 to 15 M excess of the inhibitor (17). ICI 182,780, when used at a concentration 10 to 15 times that of estradiol, can inhibit the ER–ERE binding to levels below that of the untreated controls (17). Since ICI 182,780 can inhibit Red No. 3 ER–ERE complex formation (Figure 4B) and stimulation of cell growth (Figure 2), this confirms that the actions of Red No. 3 are mediated through the ER. Figure 4B shows that phenol red (50 μg/ml) simulates the ER–ERE binding as well (1.3-fold increase over control).

Figure 1. Red No. 3 induces a dose dependent stimulation of the growth of ER-positive HTB 133 cells (A). Red No. 3 does not stimulate growth in ER-negative human breast cells (B) or in RLE cells (C). Tops of bars indicate the mean of three replicate counts. Error bars represent the standard deviation. Treatment groups were analyzed using analysis of variance followed by a Student's t-test modified to account for pooled variance. Treatment groups with the same number of asterisks are not significantly different from each other where n = 3, α = 0.05.
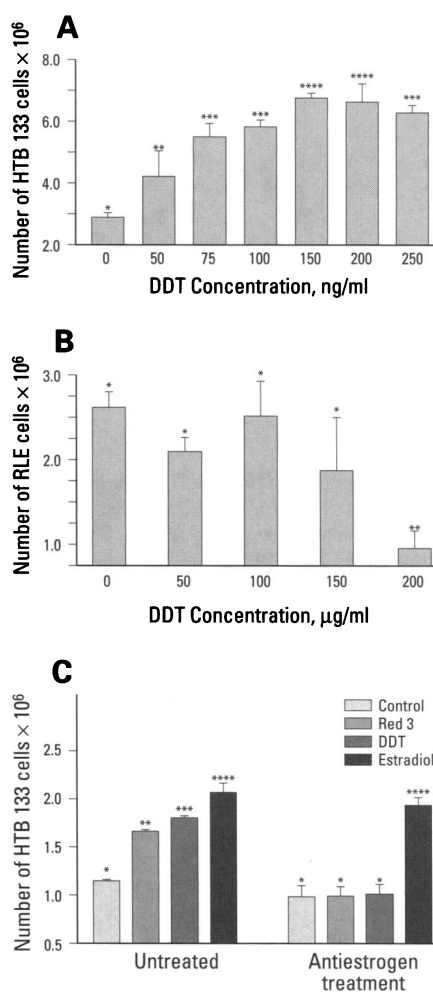
Figure 2. DDT increases the growth of ER-positive HTB 133 cells (A) but ER RLE cells were unaffected (B). DDT and Red No. 3 enhance the growth of HTB 133 cells; the effects of Red No. 3 (10 μg/ml) are significantly less than stimulated by DDT, and DDT (0.3 μM) effects are less than that of estradiol (10 nM). Treatment of HTB 133 cells with the steroidal antiestrogen ICI 182,780 (10 nM) suppresses DDT and Red No. 3 but not estradiol. Suppression of estradiol-induced cell growth required approximately 15 times more ICI 182,780 (150 nM).
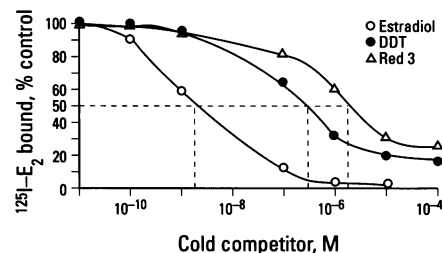
Figure 3. Red No. 3 and DDT compete successfully for the MCF-7 cell ER in competitive binding assays. Affinity for the ER by Red No. 3 and DDT is significantly less than that of estradiol.
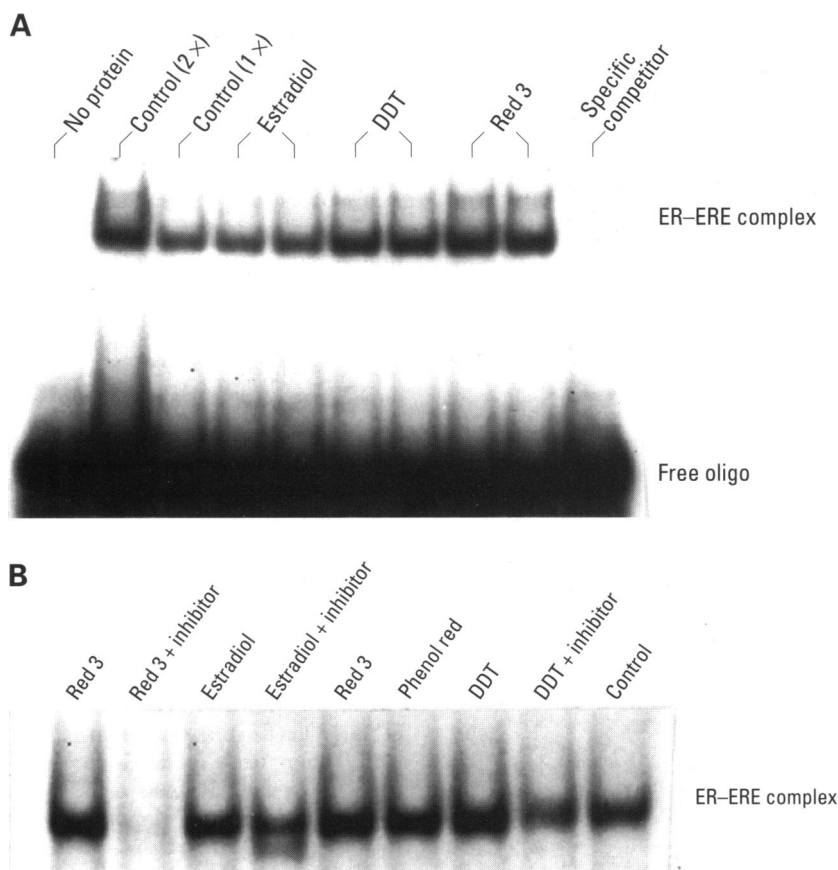
A

ER–ERE complex

Free oligo

B

ER–ERE complex

Figure 4. (*A*) DDT (0.3 μM) and Red No. 3 (25 μg/ml) stimulate the binding of the ER to the ERE using gel mobility shift assays on extracts from treated HTB 133 cells. Estradiol (100 pM) stimulated ER–ERE binding but to a lesser extent when compared to untreated control cells. No effect was seen when 50 μg/ml of Blue No. 1, Green No. 3, Red No. 40, or Yellow No. 5 were added to ER-positive cells (not shown). (*B*) The addition of ICI 182,780 (150 nM) and Red No. 3 completely prevented ER–ERE binding in Red No. 3-treated MCF-7 cells. ICI 182,780 partially prevented ER–ERE binding in cells treated with estradiol or DDT. Note that another xenoestrogen, phenol red, enhances ER–ERE binding.

Cyclin Kinase Assays

Entrance into the cell cycle requires activation of Cdk2, which is a late event in G_1/S transition (*21,22*). The cyclin E–Cdk2 complex then phosphorylates the tumor suppressor protein pRb105 (*21–23*). We reasoned that if Red No. 3 were capable of stimulating breast cell growth, the activity of Cdk2 must be increased in treated cells. Increased activity of Cdk2 can be demonstrated in ER-positive breast cancer cells using immune complex assays with histone H1 as the target (Figure 5) (*17*). DDT stimulates Cdk2-associated kinase activity in MCF-7 breast cancer cells, but increased Cdk2 activity requires larger amounts of DDT when compared with estradiol (Figure 5A). Increased Cdk2 activity was also stimulated by adding Red No. 3 to MCF-7 cells (Figure 5B). Between 3 to 10 μg/ml of Red No. 3 were required to achieve the same phosphorylation of the

histone target as that produced by 2% (v/v) FBS (Figure 5B). The activation of Cdk2 by Red No. 3 and DDT confirms that these compounds can stimulate human breast cancer cells to enter the cell cycle. Preliminary studies using Red No. 40 on MCF-7 cells have failed to show any stimulation of Cdk2 activity (data not shown).

Effects on p53–DNA Binding

The current paradigm for cancer etiology suggests that two major effects are required for cells to become cancerous: irreversible genetic damage and promotion of cell division. Our studies suggest that Red No. 3 is a xenoestrogen capable of stimulating the proliferation of cultured breast cells. To be a complete carcinogen, Red No. 3 must also be capable of damaging genetic material. We examined the effects of Red No. 3, Blue No. 1, Green No. 3, and Yellow No. 5 for potential effects on p53–DNA

sequence-specific binding in RLE cells and the human breast cell lines HTB 125 (ER-negative, normal human breast, myoepithelial), MCF-7 (ER-positive, human ductal carcinoma) and HTB 133 (HTB 133, ER-positive human ductal carcinoma). DDT and DNA-damaging agents including actinomycin, mitomycin C, and 5-fluorouracil (5-FU), also were tested for effects on p53–DNA site-specific binding.

Figure 6 shows that p53 extracts prepared for DNA binding studies, which were isolated from MCF-7 cells treated with Red No. 3 (100 μg/ml), are intensely red colored. Cells treated with other food colorants do not show any indication of colorant association with the exception of Green No. 3. Extracts from MCF-7 cells treated with Green No. 3 are very slightly colored (Figure 6). Therefore, Red No. 3 appears to penetrate human breast cancer cells *in vitro* and has access to the nuclear compartment including the genetic material and the enzymes that modify it.

After the treatments, p53–DNA site-specific binding increased in cultured HTB 125 breast cells after the application of DNA-damaging chemotherapeutic agents and Red No. 3 (Figure 7A). Titration of Red No. 3 on RLE cells showed that treatment of the cells for 2 hr with 25 μg/ml was sufficient to increase p53–DNA binding. As little as 100 ng/ml of Red No. 3 was sufficient to significantly increase p53 binding after a 4-hr exposure (data not shown). For unknown reasons, DDT failed to increase p53–DNA binding when added to the medium of RLE cells (Figure 7A).

We also examined other commonly used food dyes to determine their effects on p53–DNA binding and if metabolic activation or inactivation might occur by treatment with S9 liver extract. Blue No. 1, Green No. 3, and Yellow No. 5 slightly increased p53 binding in MCF-7 cells after treatment with S9 but not without treatment (Figure 7B). Red No. 3 stimulated the largest increase in p53–DNA binding without S9 treatment (Figure 7B). Although S9 treatment of Red No. 3 had no additional effect, p53–DNA site-specific binding was increased in HTB 133 cells treated with Red No. 3. The effects of Red No. 3 and other dyes on HTB 133 cells were similar to our studies using MCF-7 cells (Figures 7B, 7C). Preliminary studies using Red No. 40 on MCF-7 cells have shown no effect on p53–DNA binding (not shown).

Some nongenotoxic stresses increase p53–DNA binding, including hypoxia and heat shock (*24,25*). The increases in
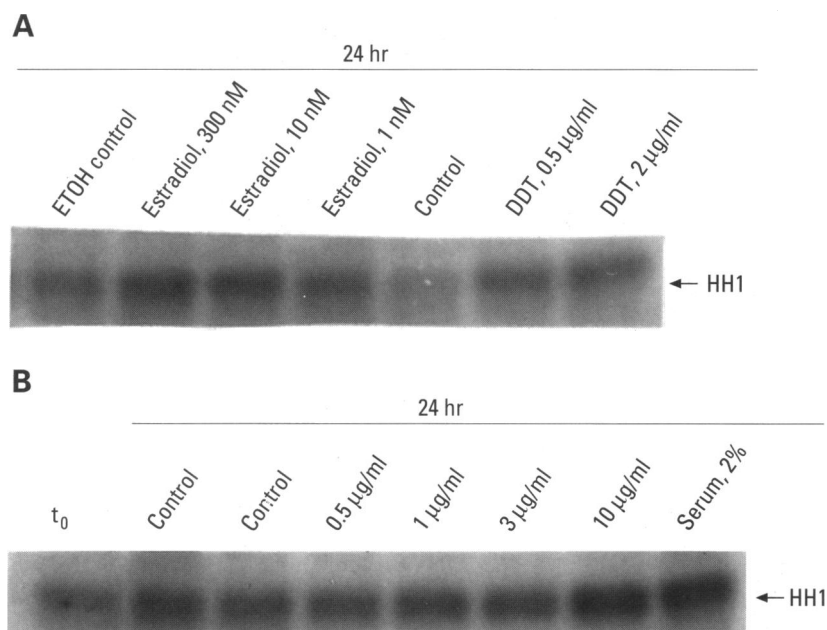
Figure 5. (A) DDT and estradiol increase the phosphorylation of histone H1 (HH1) by Cdk2 immunoprecipitated from treated MCF-7 cells. (B) Red No. 3 also stimulates the activity of Cdk2 when added to starved MCF-7 cells. Approximately 3 to 10 μg/ml of Red No. 3 are required to detect increased phosphorylation of the histone target, which is approximately equal to that stimulated by the addition of 2% (v/v) FBS.
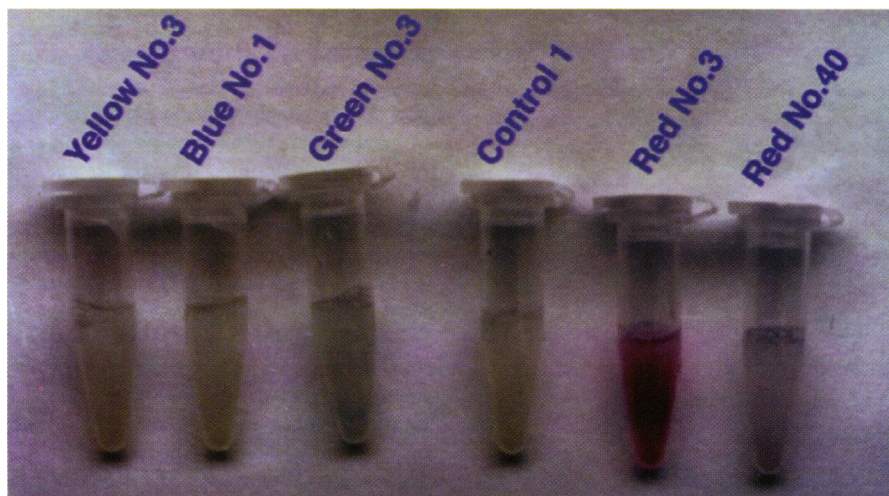

Figure 6. MCF-7 cell extracts that have been prepared for p53–DNA binding studies from cells treated with Red No. 3 (100 μg/ml) are strongly colored. Little or no color is associated with extracts treated with other colorants, including Red No. 40.

p53–DNA binding stimulated in RLE and human breast cells may occur by some mechanism other than DNA damage. However, it has been demonstrated that p53 recognizes damaged DNA (25,26) and it has not been reported that treatment of cultured cells with nongenotoxic agents such as estrogen will stimulate p53–DNA binding. Preliminary studies suggest that treatment of DNA with Red No. 3 prior to modification of the DNA using restriction endonucleases alters the results produced by action of the endonucleases (C Dees, unpublished data). Preliminary studies also suggest that Red No. 3 decreases mutations in the Ames II test (C Dees, unpublished results). These results are consistent with two previous studies that suggest Red No. 3

interacts directly with DNA or affects DNA-modifying enzymes (genointeractive) (28,29). Few other studies have examined the risks of DNA damage that might be associated with food colorants. No studies have specifically investigated the effects of synthetic food colorants on human breast cells. Red No. 3 has been shown to be mutagenic in the *Bacillus subtilus* sporulation assay (28), genointeractive in the *Salmonella typhimurium* assay (Ames test) (29), and carcinogenic in B6C3F1 mice (increased pheochromocytomas in males) (30). In the *B. subtilus* sporulation assay, S9 treatment appeared to decrease the effect of Red No. 3 (28), whereas in our study S9 treatment appears to have no effect. We believe that our results using a p53–gel mobility shift assay are consistent with a conclusion that Red No. 3 is capable of damaging DNA.

Discussion

Estrogen increases the risk of breast cancer (31), but the importance of xenoestrogens such as DDT in the etiology of breast cancer remains controversial (7,12–14). It is interesting to note that the major route of exposure to a number of xenoestrogens is through the food chain (32). However, while the incidence of breast cancer in the United States is increasing, exposure levels to several estrogenic pesticides and pollutants are decreasing (32).

In contrast, the diet of women in the United States includes processed foods that are increasingly likely to contain food colorants and additives (27). Industrialized countries rely heavily on processed foods; over the last several decades about 80% of their food supply has been processed by the food industry (27,33). Further, the use of food additives continues to increase at a rate of 4 to 5% annually (34). Processed foods are also more likely to be packaged in materials that may contaminate the food with estrogenic plasticizers (15). Therefore, as the diet of industrialized countries becomes more reliant on processed foods, the exposure to estrogenic dyes, preservatives, and contaminants from packaging materials increases. Estrogenic pesticides and pollutants also contribute to the total xenoestrogen exposure via foods.

It is difficult to determine the total dietary consumption of synthetic colorants and only a few studies have examined this issue. As documented by the National Academy of Sciences/National Research Council (NAS/NRC) in 1979, the reported consumption of all food colorants by
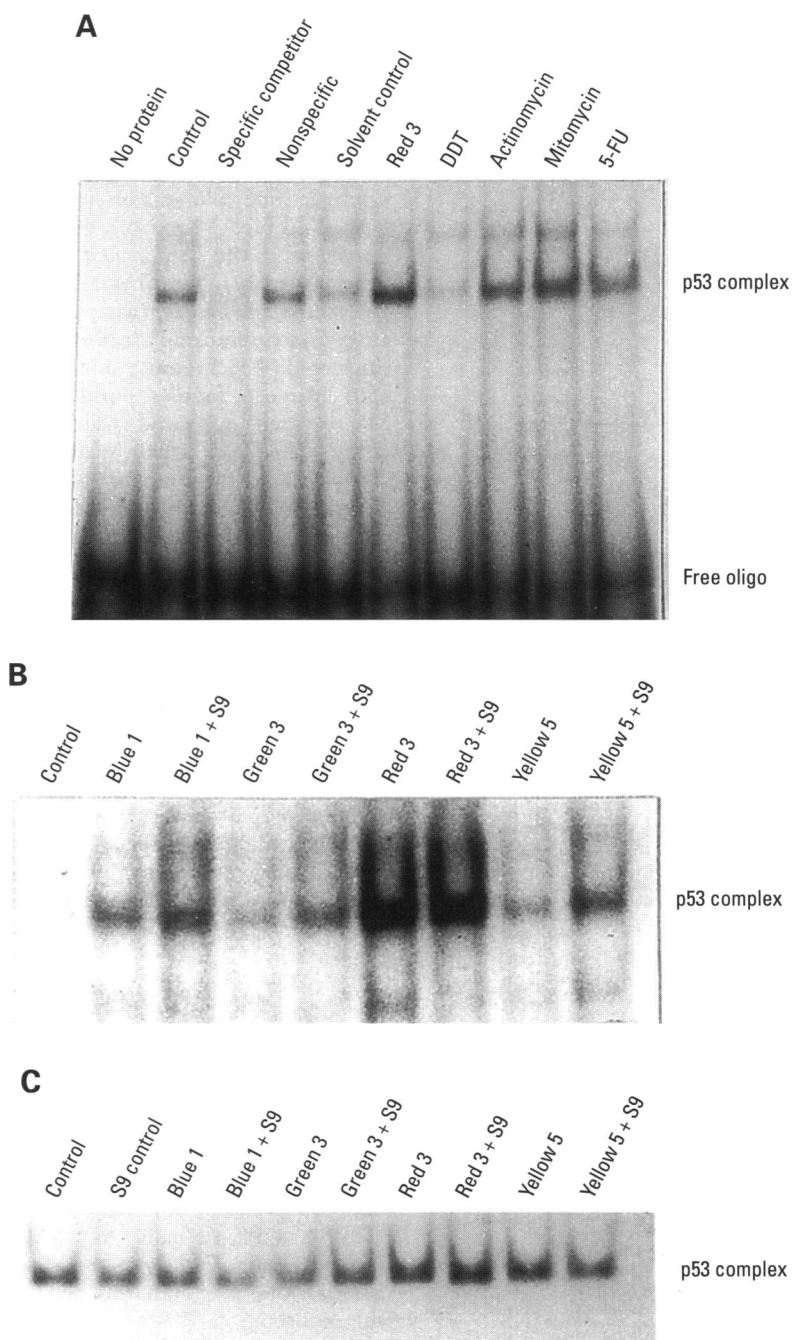
A

B

C

Figure 7. (A) Two hr after Red No. 3 (25 µg/ml) is added to HTB 125 human breast cells, increased levels of p53 can be detected using a gel mobility shift assay. DNA-damaging and chemotherapeutic agents also increase p53 levels in these cells. (B) Red Dye No. 3 increases the p53 levels 2 hr after treatment of MCF-7 cells. Simultaneous addition of S9 rat liver extract failed to affect p53 levels in these breast cancer cells. Minor changes in p53 levels could be detected when other dyes were tested, but p53 levels in MCF-7 cells were the most dramatically increased. (C) The effects of Red No. 3 on HTB 133 human breast cells were similar to those noted when HTB 125, MCF-7, or RLE cells were tested.

people over the age of 2 years in the United States during 1977 averaged 70 mg/kg/day. The top 1% of this group consumed 239 mg/kg/day of all food colorants and 1 mg/kg/day of Red No. 3

(35). With the use of food colorants increasing at a rate of 4 to 5% each year since the time of the original survey (34), the amount of Red No. 3 being consumed by the top 1% in 1995 would be estimated

at 2.508 mg/kg/day using +5% each year (2.025 mg/kg/day at +4% each year). Assuming an equal distribution in body water, the highest estimate would represent a daily dose of Red No. 3 that is approximately 30 times greater than the concentration of Red No. 3 required to induce p53 in cultured cells after a 4-hr exposure (100 ng/ml). The NAS/NRC reported that the intake at the 50th percentile was 11%, and the 90th percentile was 41%, of that reported for the top 1% (35). Thus half of the population may consume sufficient Red No. 3 to produce a tissue concentration approximately 3 times greater than that necessary to cause genetic damage to human cells in culture, and 10% of the population consumes approximately 12 times more. Even if there is a 5-fold overestimation in the consumption of Red No. 3, the daily intake of Red No. 3 would still be 6 times greater than that required to produce risk-enhancing effects *in vitro* for the top 1% and approximately 2 times greater for the top 10%. Perhaps more important, the reported intake from young childhood through puberty was actually higher than for the total population: approximately 1.6 to 2.5 mg/kg/day at the 90th percentile (adjusted to 1995 estimates as above). Thus, during growth and development the intake of Red No. 3 may actually be higher than that necessary to induce p53 binding for greater than 10% of the population at a time when developing breast tissues may be most susceptible to xenobiotic challenge.

In addition to inducing p53 binding to DNA, Red No. 3 also causes an induction of cell proliferation (inhibitable by anti-estrogen) at a concentration of 10 µg/ml. This concentration is only approximately 3 times greater than the predicted concentration for the top 1% of the population or the top 10% of young children consuming dye-containing foods. The predicted physiological concentration of Red No. 3 is approximately 33% of the level necessary to enhance cell proliferation *in vitro*. However, it should be noted that the full interaction of growth factors on signal transduction in relation to steroid binding to ER, and the role of growth factors in inducing ER-positive cells to enter and complete the cell cycle, remains to be fully elucidated. For example, the ability of xenoestrogens to induce breast cancer cells to enter the cell cycle is enhanced when other growth factors (e.g., insulin) are present (36). Growth factors such as insulin, insulinlike growth factor, and epidermal growth factor are able to phosphorylate

and activate the ER via kinase activated signaling pathways without the presence of compounds bound to the ER ligand binding site (37,38). Phorbol esters are also able to induce the phosphorylation of the ER (39). When the ER is phosphorylated it can bind to the estrogen responsive elements without a compound bound to the ligand binding site (38). In addition, other investigators have documented that the activity of one xenoestrogen may be synergistically enhanced when another one is present (40). Other factors may greatly enhance the ability of xenoestrogens such as Red No. 3 to induce ER-positive cells to enter the cell cycle. Therefore, a concentration of Red No. 3 in vivo that is 33% of that necessary to cause proliferation in vitro may be sufficient to trigger ER-positive cells to progress to the cell cycle.

Cancer risk from Red No. 3 may be further increased if developing reproductive tissues are exposed. The effects of extra estrogen on developing reproductive tissue has been demonstrated. In laboratory animals the mammary glands of female mouse pups exposed to inappropriate levels of estrogen during development are larger than those of control animals (41,42). Furthermore, the increase in terminal end bud formation observed in these mice increases the likelihood that they will develop breast cancer (41,42). In addition, exposure to estrogens during the development of the mouse reproductive tract permanently estrogenizes cells (43). Two genes that respond to estrogen (lactoferrin and epidermal growth factor) are persistently expressed after exposure to estrogen during development (43). Thus, the greatest risk associated with exposure to xenoestrogens may occur during the period from early childhood through puberty, a period in which the highest consumption of Red No. 3 occurs.

While the role of diet in increasing breast cancer risk of U.S. women is generally accepted, the particular component of the diet that confers the risk is not. Most of the current studies on the factors in the diet of U.S. women that contribute to breast cancer have focused on total fat content. Processed foods contain the highest levels of added fat also and are foods most likely to contain the highest levels of food colorants. Therefore, it is possible that the correlation of high fat foods to increased risk of breast cancer noted in previous studies is actually caused by the presence of xenoestrogenic food additives such as Red No. 3. Xenoestrogenic food additives and other xenoestrogens that are found in foods, including pollutants (e.g., dioxins, polychlorinated biphenyls), packaging contaminants (phthalates), and pesticides, may be in total the dietary factor that contributes to the high breast cancer risk of women in the United States and other industrialized countries. However, many other factors are likely to increase the risk of breast cancer for women living in industrialized countries. Additional risk factors may include high fat diets, poor exercise habits, and high total body fat (6). The age of menarche or menopause, alcohol use, and parity may also increase risk. While our studies suggest that a xenoestrogenic food dye may increase the risk of breast cancer, further studies are required to determine if the estrogenic and genotoxic effects of Red No. 3 on cells in vitro also occur in vivo.

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