The Pesticides Endosulfan, Toxaphene, and Dieldrin Have Estrogenic Effects on Human Estrogen-Sensitive Cells

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Estrogenic pesticides such as DDT and chlordecone generate deleterious reproductive effects. An "in culture" bioassay was used to assess the estrogenicity of several pesticides. The E-screen test uses human breast estrogen-sensitive MCF7 cells and compares the cell yield achieved after 6 days of culture in medium supplemented with 5% charcoal-dextran stripped human serum in the presence (positive control) or absence (negative control) of estradiol and with diverse concentrations of xenobiotics suspected of being estrogenic. Among the organochlorine pesticides tested, toxaphene, dieldrin, and endosulfan had estrogenic properties comparable to those of DDT and chlordecone; the latter are known to be estrogenic in rodent models. The E-screen test also revealed that estrogenic chemicals may act cumulatively; when mixed together they induce estrogenic responses at concentrations lower than those required when each compound is administered alone. *Key words*: bioassay, estrogens, pesticides, xenobiotic. *Environ Health Perspect* 102: 380–383(1994)

Environmental xenobiotics are suspected to play a causative role in alterations of sexual development in wildlife species (*1*). Some pesticides, such as DDT and chlordecone (Kepone), were found to have estrogenic properties long after they were released into the environment (*2–4*). The use of these pesticides has since been restricted in the United States. It should be noted that the estrogenic activity of chemicals cannot be deduced solely from their molecular structure; therefore, it is uncertain whether pesticides currently used are estrogenic. To explore this possibility, we tested a group of pesticides using a bioassay, the E-screen test, that measures the proliferative effect of estrogens on their target cells (human breast cancer MCF7 cells) (*5,6*).

Materials and Methods

Human breast cancer estrogen-sensitive MCF7 cells were obtained from the Michigan Cancer Foundation (Detroit, Michigan) (*7*). For routine maintenance, cells were grown in Dulbecco's modification of Eagle's Medium (DME; Gibco, Grand Island, New York) supplemented with 5% fetal bovine serum (FBS; Hyclone, Logan, Utah) in an atmosphere of 5% CO_2/95% air under saturating humidity at 37°C.

We prepared plasma-derived human serum from outdated plasma by adding calcium chloride to a final concentration of 30 mM to facilitate clot formation. Blood-derived serum was obtained using blood from healthy adult volunteers; we allowed blood to clot in glass centrifuge tubes for 2–4 hr to obtain serum. Plasma- and blood-derived serum were clarified by centrifugation (3000 rpm × 10 min), heat-inactivated (56°C for 30 min), centrifuged, and stored in glass tubes at -20°C until use.

Charcoal (Norit A, acid washed, Sigma Chemical Co, St. Louis, Missouri) was washed twice with cold sterile water immediately before use. A 5% charcoal–0.5% dextran T70 (Pharmacia-LKB, Uppsala, Sweden) suspension was prepared. We centrifuged charcoal-dextran suspension aliquots of a volume similar to the serum aliquots to be processed at 2500 rpm for 10 min. Supernatants were aspirated, and serum aliquots were mixed with the charcoal pellets. This charcoal-serum mixture was maintained in suspension by rolling at 4 cycles/min at 37°C for 1 hr. This suspension was centrifuged at 2500 rpm for 20 min. We then filtered the supernatant through a 0.45-µm Nalgene filter. More than 99% of serum sex steroids were removed by this treatment (*8*). CD sera were stored at -20°C until needed; samples kept for 1 year in the freezer maintained their inhibitory properties toward the proliferation of human estrogen-sensitive breast tumor MCF7 cells; plasma- and blood-derived sera were equally effective.

Cloned MCF7 cells were trypsinized and plated into 12-well plates (Costar, Cambridge, Massachusetts) at initial concentrations of 20,000 cells per well (*5,6*). We allowed cells to attach for 24 hr; then we removed seeding medium (5% FBS in DME) and replaced it with the experimental medium (5% charcoal-dextran stripped human serum supplemented to phenol red-free DME). To this medium, a range of concentrations of the test compound was added. The bioassay was terminated on day 6 (late exponential phase) by removing the media from the wells, adding a cell lysing solution [10% ethylhexadecyldimethylammonium bromide (Eastman Kodak Co., Rochester, New York) in 0.5% Triton X100, 2 mM $MgCl_2$, 15 mM NaCl, 5 mM phosphate buffer pH 7.4], and counting the nuclei in a Coulter Counter Apparatus (model ZM; Coulter Electronics, Hialeah, Florida).

Estradiol-17ß was obtained from Calbiochem (Richmond, California). Toxaphene, technical grade, and endosulfan, technical grade, were obtained from Chem Services (West Chester, Pennsylvania). Endosulfan α and ß isomers, *o,p'*-DDT, *p,p'*-DDT, *p,p'*-DDD, *p,p'*-DDE, 2,3,4,5 tetrachlorobiphenyl, 2,2',3,3',6,6'-hexachlorobiphenyl, methoxychlor, and dieldrin were from Ultra Scientific (North Kingstown, Rhode Island). Estradiol was stored as a 1 mM stock solution in ethanol at -20°C. Pesticides were dissolved in ethanol to a final concentration of 10 mM, except endosulfan mixed isomers, dieldrin and toxaphene which were dissolved in dimethyl sulfoxide (DMSO); they were all diluted to desired concentrations in phenol red-free DME immediately before using. The final solvent concentration in culture medium did not exceed 0.1%; this concentration did not affect cell yields.

Results are expressed as means ± SEs. We repeated proliferation yield experiments conducted in duplicate wells a minimum of five times. Mean cell numbers from each experiment were normalized to the steroid-free control (100%) to correct for differences in the initial plating density. We assessed differences between the diverse steroid treatment groups by analysis of variance and student Newman-Keuls tests. A p-value of ≤ 0.05 were regarded as significant.

Results

The E-screen test is based on the following premises: a human serum-borne molecule specifically inhibits the proliferation of human estrogen-sensitive cells (*8,9*); and estrogens induce cell proliferation by neutralizing this inhibitory effect (*8,9*). Nonestrogenic steroids and growth factors do not abolish the proliferative inhibition by human serum (*8–10*). The assay compares the cell yield achieved by similar cell inocula harvested simultaneously during the exponential phase of proliferation (*5,6*).

The estrogenic activity of xenobiotics was assessed by 1) determining the relative

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proliferative potency (RPP); this is the ratio between the minimal concentration of estradiol needed for maximal cell yield at 6 days and the dose of the test compound to achieve a comparable proliferative effect, and 2) measuring the relative proliferative effect (RPE); this is 100× the ratio between the highest cell yield obtained with the chemical and with estradiol. The RPE indicates whether the compound being tested induces a proliferative response quantitatively similar to the one obtained with estradiol; that is, if it is a full agonist (RPE = 100), or a proliferative yield significantly lower than the one obtained with estradiol; that is, if it is a partial agonist.

o,p′-DDT and chlordecone are pesticides with well-documented estrogenic activity in several vertebrate species (*2–4,11*). Their RPP measured by the E-screen test was 0.0001% that of estradiol (Table 1); although clearly estrogenic, the cell yield obtained with these chemicals was somewhat lower than that of estradiol (o,p′-DDT, RPE = 86%; chlordecone, RPE = 84%). Technical grade endosulfan and α and ß endosulfan isomers were estrogenic at concentrations of 10–25 µM (Fig. 1); higher concentrations were cytotoxic. It should be noted that the RPE of all of these chemicals is lower than that of estradiol (endosulfan mixed isomers, RPE = 81%; α isomer, RPE = 77%; ß isomer, RPE = 78%); the fact that these compounds have toxic effects at concentrations one order of magnitude higher than those needed to evoke a proliferative response precluded assessing whether higher concentrations would attain full estrogenic activity. Dieldrin and toxaphene were estrogenic at 10 µM (Fig. 2); higher concentrations were toxic. The RPE of these compounds were lower than those of endosulfan (dieldrin, RPE = 55%; toxaphene, RPE = 52%; Table 1).

Humans and wildlife are simultaneously exposed to a variety of chemicals (*1,12*). Residues of diverse estrogenic xenobiotics coexist in fat and body fluids of exposed individuals (*12*); hence, it is likely that they may become bioavailable. At such time, these xenobiotics may act cumulatively; that is, when each of them is present at levels lower than those needed to express overt estrogenicity. To explore this concept, we administered a mixture of 10 estrogenic chemicals to MCF7 cells at concentrations 10-fold lower than those required to produce an estrogenic effect when given alone; the resulting cumulative effect is illustrated in Figure 3. This combination resulted in a significant proliferative effect.

Discussion

Several xenobiotics such as polychlorinated biphenyls, chlordecone, and methoxychlor, were shown to be estrogenic in animal models; however, their lower estrogenic potency was interpreted as having none or weaker deleterious effects on humans exposed through the food chain. Occupational exposure to chlordecone, on the other hand, resulted in overt estrogenicity manifested as oligospermia and sterility (*4*). Because most organochlorine contaminants bioacummulate and remain preferentially in fat, concern about their long-term effects has been raised in the public health community. Recent reports suggest that these chemicals are interfering with the reproductive success of wildlife (*13*). In addition, humans may also be at risk. For example, an epidemiological study showed a positive correlation between breast cancer and serum levels of DDE, a DDT metabolite (*14*). This finding suggests, but does not prove, that organochlorines may be an important etiological factor on breast cancer, probably because of their estrogenicity. In addition, it has been postulated that the increased incidence of cryptorchidism, testicular and prostate cancer, as well as the decrease in human semen quality over the last 50 years, may be due to environmental estrogens (*15,16*). Also, other synthetic estrogens such as diethylstilbestrol, used for a perceived therapeutic purpose, induced reproductive tract malignancies in the offspring of exposed women (*17–19*).

The present study shows for the first time that the pesticides dieldrin and toxaphene, which have been restricted from use in the United States since 1974 and 1982, respectively, are estrogenic (Table 1). These compounds are highly lipophilic and bioaccumulate through ecosystems; they are still found in wildlife, coincident with signs of reproductive impairment. Toxaphene is a main airborne pollutant in North America; its residues appear in regions where it has never been used, like the Arctic and Scandinavia (*20*). Toxaphene accumulates in soil (*21*), bioaccumulates preferentially in fat where it persists (*20*), has been linked to reproductive damage in seals (*22*), and is present in Arctic and Baltic salmon muscle fat at concentrations of 700–7,000 ppb (*22*); this concentration is well within those producing estrogenic effects in human breast MCF7 cells (10 µM = 4800 ppb; Fig. 2).

Endosulfan was first introduced in 1954; it is currently used for agricultural purposes in the United States and other countries (*23*). The worldwide production of this chemical was estimated to be 10000 metric tons in 1984 (*24*); recent production data were not available. In a study conducted in Southern Ontario, Canada, 78% of the milk samples analyzed contained 1–10 ppb (*25*). The α isomer was detected in leafy vegetables up to a level of 93 ppb; the ß isomer was detected up to a level of 150 ppb. We now report proliferative, estrogenlike effects in MCF7 cells at doses of 10 µM (4060 ppb). Endosulfan produced testicular atrophy in male rats fed a diet containing 10 ppm (*26,27*); it also lowered gonadotrophin and testosterone plasma levels (*28*). These results are consistent with its estrogenicity revealed by the E-screen test.

The estrogenic potency of dieldrin, toxaphene, and endosulfan as measured by the E-screen test was comparable to that of DDT and chlordecone, and six orders of magnitude lower than that of estradiol (Figs. 1, 2) (*6*). Studies carried out in rats showed that the estrogenic effect of chlordecone measured as increased uterine wet weight is qualitatively comparable to that of estradiol (*11*); however, to achieve this effect a 1000- to 5000 fold higher dose of chlordecone than that of estradiol was required (*11*). This discrepancy may be due to the rapid metabolism of estradiol and the persistence and bioaccumulation of chlordecone *in vivo*. Differences between results in culture and in live animals reflect the different parameters used as a measure of estrogenicity. On one

Table 1. Estrogenic effect of pesticides[a]

Compound	Concentration	PE	RPE (%)	RPP (%)
Estradiol	10 pM	3.68	100	100
o,p′-DDT	10 µM	3.17	86.14	0.0001
Chlordecone	10 µM	3.09	84.00	0.0001
Endosulfan[b]	10 µM	2.99	81.25	0.0001
DDT[b]	10 µM	2.93	79.61	0.0001
Endosulfan ß	10 µM	2.88	78.26	0.0001
Endosulfan α	10 µM	2.84	77.17	0.0001
Dieldrin	10 µM	2.02	54.89	0.0001
Toxaphene	10 µM	1.91	51.90	0.0001

[a] "Concentration" denotes the dose at which estrogenic effect is detected. PE; proliferative efficiency, measures the ratio between the highest cell number in the presence and in the absence of estrogen. RPE, relative proliferative efficiency; measures the ratio between the maximal cell yield achieved by the xenobiotic and that of estradiol. RPP, relative proliferative potency; measures the ratio between the dose of xenobiotic and that of estradiol needed to achieve a proliferative effect.
[b] Technical grade.

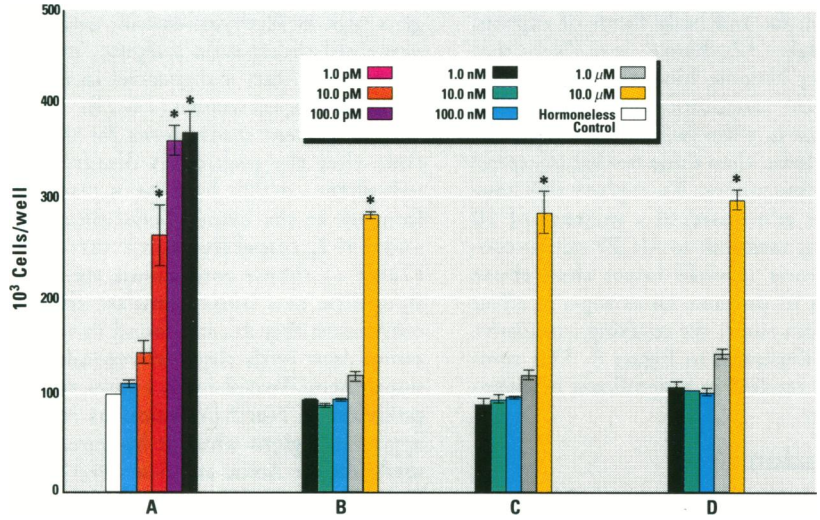

Figure 1. Proliferative activity of (A) estradiol, (B) endosulfan α, (C) endosulfan ß, and (D) endosulfan, technical grade. *Significant differences from hormoneless control (p<0.01).

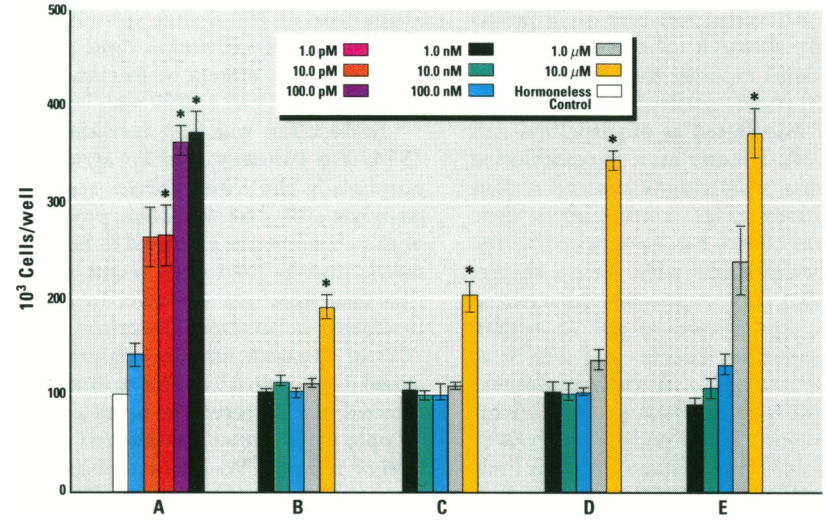

Figure 2. Proliferative activity of (A) estradiol, (B) toxaphene, (C) dieldrin, (D) DDT mixed isomers, and (E) o,p'-DDT. *Significant differences from hormoneless control (p<0.01).

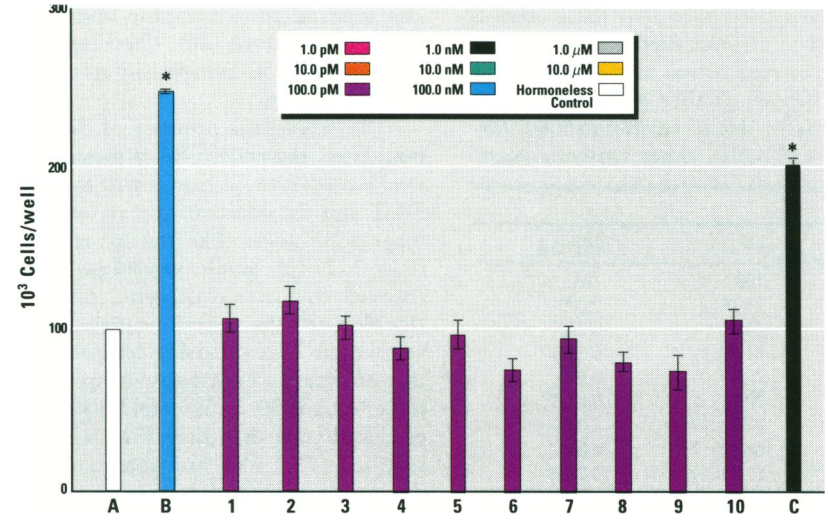

Figure 3. Proliferative activity of (A) hormoneless control, (B) 10 μM endosulfan ß, (1) 1 μM endosulfan ß, (2) 1 μM endosulfan α, (3) 1 μM toxaphene, (4) 1 μM dieldrin, (5) 1 μM 2,3,4,5-tetrachlorobiphenyl, (6) 1 μM p,p'-DDT, (7) 1 μM 2,2',3,3',6,6'-hexachlorobiphenyl, (8) 1 μM p,p'-DDD, (9) 1 μM p,p'-DDE, (10) 1 μM methoxychlor, and (C) mixture of the 10 chemicals indicated as 1–10, each at 1 μM. *Significant differences from hormoneless control (p<0.01).

hand, the rodent assay measures the increase of uterine wet weight; this is only a crude estimate of estrogen action because it represents the combination of three separate effects, namely, water imbibition, hypertrophy, which is also produced by estrogen antagonists, and hyperplasia (*29*). The human E-screen cell bioassay, on the other hand, measures cell proliferation; this is the sole parameter acknowledged to be the hallmark of estrogen action (*30*). In addition, whereas the human E-screen test measures direct estrogenicity at the target-cell level, animal studies are a composite of several pharmacokinetic parameters such as metabolism and clearance.

Environmental estrogens may act cumulatively (Fig. 3) and with endogenous estrogens to disrupt the endocrine system of exposed wildlife and humans. The data in Figure 3 suggest that measuring the total estrogenic burden due to environmental contaminants may be more meaningful than assessing exposure by measuring the levels of each of the known xenoestrogens. The E-screen test may be used to this end once a protocol is developed to separate environmental estrogens from endogenous ones. As a preventive approach, the E-screen test may be used to screen chemicals for their estrogenicity before they are released into the environment.

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