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Estrogenic pesticides: binding relative to estradiol in MCF-7 cells and effects of exposure during fetal life on subsequent territorial behaviour in male mice

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Abstract

Numerous chemicals released into the environment by man are able to disrupt the functioning of the endocrine system by binding to estrogen receptors in estrogen-responsive cells. The ability of *o,p'*-dichlorodiphenyl trichloroethane (DDT) and methoxychlor to compete with estradiol for binding to estrogen receptors in MCF-7 cells (relative binding affinity; RBA) was examined in both serum-free medium and 100% serum; this is referred to as a relative binding affinity-serum modified access (RBA-SMA) assay. RBA's ranged from 0.04% for *o,p'*-DDT (which showed enhanced access to cells in serum relative to serum-free medium) to 0.004% for methoxychlor (which did not show enhanced access in serum). Based on these findings, these pesticides, along with diethylstilbestrol (DES) as a positive control, were fed to pregnant mice from days 11–17 of pregnancy. When the male offspring were examined in adulthood for their rate of urine marking in a novel territory (territorial behaviour), the rate of urine marking increased dramatically with low doses of DES (relative to controls) and then decreased significantly at the highest dose administered prenatally. Relative binding in MCF-7 cells accurately predicted the doses of *o,p'*-DDT and methoxychlor that produced the same results, providing support for the hypothesis that effects on behaviour were mediated by binding to estrogen receptors in the developing brain.

Keywords: Estrogen; Pesticides; DES; DDT; Methoxychlor; MCF-7 cells; Territorial behaviour

1. Introduction

The widespread presence in the environment of chemicals with the capacity to disrupt the functioning of the endocrine system is now established [1]. Chemicals that have the capacity to disrupt the endocrine system act via many different mechanisms [1]. One category of endocrine disrupting chemicals that has generated considerable interest consists of chemicals that are able to bind to estrogen receptors and thus have the capacity to act as either estrogen agonists or antagonists.

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1.1. Estrogenic properties of pesticides

Dichlorodiphenyl trichloroethane (DDT) is one of the most commonly detected environmental pollutants in human tissues. DDT cannot now be legally used in the United States, but its analog, methoxychlor (bis-*p*-methoxy DDT; 1,1,1-trichloro-2,2-bis(*p*-methoxyphenyl)ethane) is in current use and is also commonly detected [2]. The known health effects of these chemicals in man and laboratory animals have recently been reviewed and summarized [1]. All of these organochlorine compounds are present in rain water, suggesting that these compounds are volatilizing to the atmosphere after application [3]. Thus, chemicals such as DDT which are banned in some, but not all countries, may pose a potential risk to health of humans and wildlife throughout the world. While concentrations are often low in water, the lipophilic nature of these compounds causes them to concentrate in lipids, leading to dramatic biomagnification at each level of the food chain, with the greatest danger posed to organisms at the tertiary level of the food chain [2].

The *o,p'*-isomer of DDT is a contaminant (11–29%) of technical grade DDT and is the estrogenic component of technical grade DDT [4,5]. While some studies had suggested that methoxychlor was estrogenic (produced a uterotrophic effect in female rats) only after being metabolized [4–6], recent findings have shown that methoxychlor stimulates estrogen-responsive MCF-7 cell proliferation in vitro (A.M. Soto, personal communication), suggesting that metabolism is not required to render methoxychlor estrogenic. Exposure to organochlorine pesticides, such as DDT and methoxychlor, during early life can cause effects similar to those seen as a result of fetal or neonatal exposure to the synthetic estrogen analog diethylstilbestrol (DES) in female mice and rats. These include acceleration of the first (pubertal) ovulation, as well as acceleration of the loss of fertility, which is referred to as the delayed anovulatory syndrome [4,7]. While the mammary glands, uterus, cervix and vagina are the primary target organs for estrogen action in mammalian females, there is also considerable evidence for estrogen responsiveness of prostate and seminal vesicles in male rodents and other mammals [8], and exposure to DDT during early life can lead to marked impairment of fertility and reduced weight of seminal vesicles and prostate in male rats [9,10].

In summary, the period of early organ differentiation is critical in development. During this time organs are particularly susceptible to the disruptive effects of chemicals which have hormonal or antihormonal activity [8]. The focus of our research is on chemicals which have the capacity to bind to intracellular estrogen receptors, which mediate the effects of endogenous estrogen. While critical exposure to estrogenic chemicals may occur during fetal life, the consequences of such exposure are typically not recognized until well into adulthood when problems relating to the functioning of the reproductive system become apparent [7,10].

We conducted 2 sets of experiments with DES (Sigma, St. Louis, MO) and 2 pesticides: *o,p'*-DDT and methoxychlor (both 99% pure; Radian Corp., Austin, TX), using both in vitro and in vivo techniques. We will begin by describing a rapid (overnight) method to determine the capacity of environmental chemicals to compete with estradiol for binding to estrogen receptors using estrogen-responsive MCF-7 cells. We have used these cells in other kinds of assays to determine xenobiotic estrogenic activities [11].

The method we have developed, which we refer to as a relative binding affinity-serum modified access (RBA-SMA) assay, allows large numbers of chemicals to be screened in both a serum-free medium and in 100% serum. This is important because during pregnancy in women and many other mammals, such as rats and mice, only a very small fraction of circulating estrogen is not bound to plasma proteins (< 1%, referred to as the free, biologically active fraction) and is thus able to freely diffuse into cells. However, many man-made chemicals that have been examined do not bind significantly to plasma hormone-binding proteins potentially, up to 100% of these chemicals circulating in the blood could enter cells and exert biological effects [8]. The potency relative to estradiol of chemicals that do not bind to plasma proteins will thus be substantially underestimated, with in vitro experiments conducted without the presence

of the high levels of the plasma hormone binding proteins that the compounds would encounter in blood.

1.2. Urine-marking behaviour

Marking the environment with urine is a common behaviour in the house mouse, and all surfaces in their home territory become smeared with urine. Urine marking influences social and reproductive behaviours in the house mouse. Male mice are particularly active in urine-marking behaviour, which is influenced by the male's social status and testosterone levels, both in terms of qualitative (different urine composition) and quantitative (intensity rate of urine deposition) differences. Male mouse urine contains olfactory cues (pheromones) that affect both behaviour and physiology of other mice; for instance, male urine elicits inter-male aggressive behaviour, is attractive to females, and contains primer cues that accelerate puberty, induce estrus, block pregnancy and stimulate inter-female aggression [12,13].

Communication through urine marking has several potential functions in maintaining social status and interaction between conspecifics. Males may mark to advertise their agonistic dominance over same sex conspecifics, including both intruders and other residents. Male marking likely plays a role in advertising territorial defense against potential intruders. The rate of urine marking in response to social stimuli can thus be a useful indicator of a male's social rank and territory defense potential, and we thus examined this behaviour in the male offspring of treated females.

In the first experiment, the capacity for estrogenic chemicals to reach intracellular estrogen receptors from human serum was examined in intact MCF-7 cells, which are an estrogen-responsive, human breast cancer-derived cell line. This was measured by the ability of the unlabeled chemical estrogens to compete with the binding of [^{3}H]estradiol, which is referred to as RBA analysis. We also conducted an in vivo experiment in which pregnant mice were fed the oil vehicle, DES, *o,p'*-DDT or methoxychlor (all dissolved in oil) during the period of fetal sexual differentiation. A 5-log range of doses of DES, *o,p'*-DDT and methoxychlor were fed to pregnant female mice. The doses used were based on findings concerning binding affinity relative to estradiol of these chemicals using MCF-7 cells. The offspring of DES- and pesticide-treated mothers were raised and examined for changes in behaviour relative to the control animals whose mothers were fed only oil. The behavioural approach to the study of effects of endocrine disrupting chemicals is one of the most sensitive biomarkers of exposure.

2. Materials and methods

2.1. RBA of pesticides in 0% and 100% serum

To estimate access from serum by use of Scatchards for estrogenic chemicals would require that they be available in a radiolabeled form, which, unfortunately, is not the case. Therefore, access to intracellular estrogen receptors from serum was determined for unlabeled chemicals by use of RBA analysis. To determine whether the RBA for the estrogenic chemical *o,p'*-DDT was enhanced in serum compared to serum-free media, we saturated estrogen receptors in MCF-7 cells with [^{3}H]estradiol in either serum-free Modified Eagles medium (MEM) without phenol red or in 100% human serum, and then determined the range of concentrations of *o,p'*-DDT that led to displacement of the [^{3}H]estradiol in both conditions. The ratio of the concentration of non-radiolabeled estradiol required to dissociate 50% of [^{3}H]-estradiol divided by the concentration of (non-radiolabeled) *o,p'*-DDT required to dissociate 50% of [^{3}H]estradiol is the RBA of *o,p'*-DDT.

The modified relative access from serum compared to serum-free medium measured in these experiments was by the ratio of the RBA's measured in serum and in serum-free MEM; all RBA's were relative to the competition of non-radiolabeled estradiol for [^{3}H]estradiol at near-saturating concentrations (1 nM labeled estradiol in MEM, 10 nM in serum). If the RBA for a compound was greater in serum than in MEM, then the compound exhibited enhanced access in serum. In Table 1, the 'Modified access' column is the ratio of the RBA's in serum and MEM, and if it was greater than 1 then there was enhanced access in serum. If the ratio of RBA's was less than 1 then

Table 1
RBA in serum-free MEM compared to 100% human serum

Compound	RBA in MEM	RBA in serum	Modified access
Synthetic estrogen			
DES	21%	70%	3.3
Phytoestrogen			
Equol	0.015%	0.169%	11.3
Pesticides			
o,p'-DDT	0.013%	≅0.040%	≅3.1
Methoxychlor	0.004%	<0.004%	<1
Reference			
Estradiol	100%	100%	—

A value greater than 1.0 indicates increased biological activity relative to estradiol.

there was actually a reduced access in serum compared to serum-free MEM. In this initial experiment we used serum from adult men due to its ready availability, but this serum has lower concentrations of serum sex steroid binding proteins than blood from pregnant women or fetuses. Enhanced access of chemicals that do not bind to plasma proteins would increase as the concentration of steroid binding proteins increases in serum, and thus would be substantially higher in fetal serum than in serum from adult men [8].

2.2. Effects of fetal exposure to estrogenic pesticides on subsequent territorial behaviour

In this study we used CF1 mice (*Mus domesticus*). Adult females were time-mated. The synthetic estrogen, DES, and the estrogenic pesticides, *o,p'*-DDT and methoxychlor, were dissolved in tocopherol-stripped corn oil (Cat# 901415, ICN, Aurora, OH). Each pregnant female received daily administration of 30 μl of corn oil (with or without a chemical) from day 11 to day 17 of pregnancy. Maternal body weights ranged from 45 to 65 g from days 11 to 17. Body weights of fetuses (used in another study) on days 15, 16 and 17 averaged 0.35, 0.55 and 0.80 g, respectively; body weights were not determined prior to day 15. Fetal body weight was thus approximately 1/100 maternal body weight during the time of chemical administration, suggesting that fetal exposure to the test chemicals might have been 1/100 of the dose administered to the mother (assuming no metabolism occurred in the placenta).

An electronic micropipetter (Rainin) enabled delivery of an accurate volume of corn oil into the mouth of an animal. Mice readily consume corn oil, and this procedure did not result in the severe stress associated with gavage. Severe maternal stress significantly alters fetal hormone levels and thus alters the course of fetal development, and maternal stress would be a significant confounding variable in these experiments [14]. The last treatment was on day 17 to reduce the possibility that the higher doses of DES and pesticides would interfere with parturition.

On day 11 of pregnancy, females were randomly assigned to one of 18 groups (n = 6–10 females): Group 1 = unhanded: females were left undisturbed; Group 2 = control: females were administered corn oil alone; Groups 3–8 = DES in 6 doses: 0.001, 0.01, 0.1, 1, 10, 100 μg/30 μl; Groups 9–13 = *o,p'*-DDT in 5 doses: 1, 10, 100, 1000, 5000 μg/30 μl; Groups 14–18 = methoxychlor in 5 doses: 1, 10, 100, 1000, 5000 μg/30 μl.

Only 2 males from each litter were used to study postnatal behaviours to minimize possible litter effects. When they were adults (60 days old), CF1 male mice were individually housed for 4 weeks to eliminate any effects of having been housed with other males [15]. The following prenatal treatment groups were examined for urine-marking behaviour: oil (n = 24); DES = 0.001, 0.1, 10.0 μg/30 μl (n = 18/dose); DDT = 1, 100, 5000 μg/30 μl (n = 10/dose); methoxychlor = 1, 100, 5000 μg/30 μl (n = 10/dose).

Urine-marking tests were conducted for 1 h in clean 30 × 30 × 15 cm cages divided into 2 chambers by a removable wire-mesh barrier. The floor of the cage was covered by a large sheet of Whatman No. 2 filter paper during the 1-h test. Test males were placed into the test cages separated by the wire-mesh barrier from a 90-day-old unhanded female. At the end of the 1-h urine-marking test, the filter paper was removed, and discrete urine marks (which fluoresce under UV light) deposited on it by males were counted.

3. Results

3.1. Access of estradiol to cells in 0% and 100% serum

In these initial experiments we used serum from adult men, since it is readily available. Saturation

binding profiles (Scatchard plots) of the specific binding of [^{3}H]estradiol in serum-free MEM and in 100% human serum yielded apparent Kd's of 0.24 and 5.25 nM, respectively. The ratio of these Kd's was used to estimate the free fraction of estradiol in serum, and indicated that 95.4% of the estradiol was bound in the human serum (for specifics concerning cell culture and media, see [16,17]). Binding to serum proteins of the labeled ligand in 100% serum therefore reduced the access of the [^{3}H]estradiol for estrogen receptors within the MCF-7 cells by approximately 20-fold (5.25/0.24).

3.2. RBA of pesticides and DES in 0% and 100% serum

The results presented in Table 1 show that the RBA of *o,p'*-DDT relative to estradiol was lower in MEM (0.013%) than in serum (0.040%), and *o,p'*-DDT thus showed an enhanced access to cells relative to estradiol in serum compared to the serum-free medium. The RBA of *o,p'*-DDT in serum of 0.040% suggests that a concentration of *o,p'*-DDT between 1000 and 10 000 higher than estradiol would be needed to observe equivalent estrogenic effects, given that *o,p'*-DDT has already been found to be an estrogen agonist rather than antagonist [4,7]; it is important to emphasize that our relative binding assay does not discriminate between chemicals that act as agonists or antagonists after binding to the receptor. We found that methoxychlor competed with estradiol for binding to estrogen receptors. However, we did not find that methoxychlor exhibited an enhanced access to cells in serum compared to serum-free MEM. The binding affinity of methoxychlor was approximately 0.004% that of estradiol in either medium. Methoxychlor was thus about a 10-fold less potent estrogen than *o,p'*-DDT.

The RBA of the synthetic estrogen, DES, was close to that of estradiol, but showed some enhancement in serum. We also examined another estrogenic chemical, equol, which is the major circulating metabolite of isoflavone metabolism when animals consume the dietary estrogens in clovers, and equol is present in humans when the diet contains soya flour [18]. Equol showed an RBA of 0.010% in serum-free MEM, but its RBA in serum was over 10 times greater, at 0.112%.

3.3. Effects of fetal exposure to estrogenic pesticides on subsequent territorial behaviour

With the exception of DES 10 and 100 μg-treated females, females delivered normally on day 19. One of 6 DES 100 μg and 5 of 10 DES 10 μg-treated females delivered during day 20 of preg-

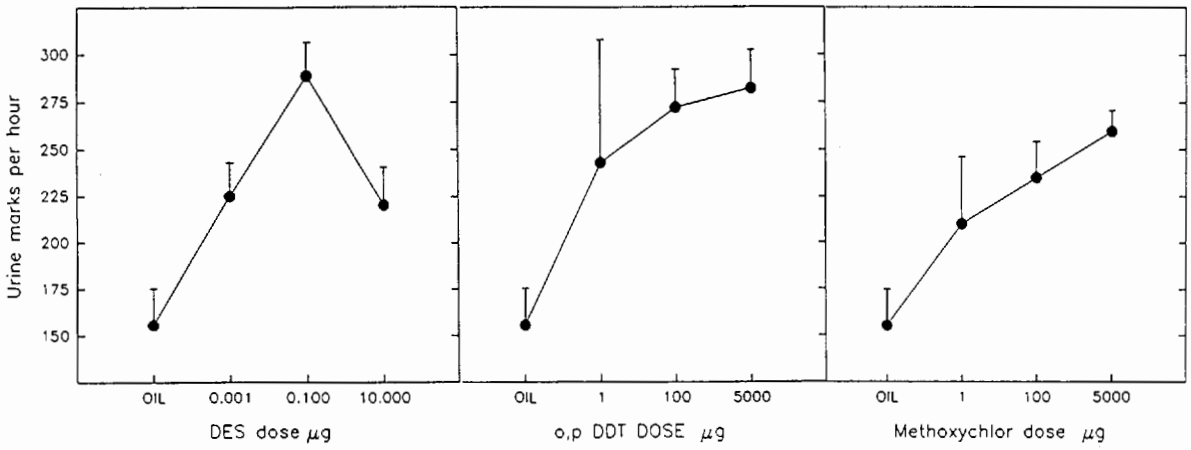

Fig. 1. The number of urine marks deposited by male mice during a 1-h test in a novel environment. Doses of DES, *o,p'*-DDT and methoxychlor refer to the doses that the males' mothers were administered during pregnancy. The pregnant females were fed the chemicals each day (in 30 μl corn oil) between days 11 and 17 of pregnancy. Males in the one oil group (shown as a reference in each graph) were produced by mothers fed only corn oil.

nancy. In the remaining cases for these 2 DES doses, litters with dead pups were found on days 22–23 of gestation.

The results shown in Fig. 1 reveal that the lowest prenatal dose of DES examined (0.001 μg) significantly increased urine-marking behaviour ($P < 0.05$) relative to control males. However, males whose mothers were fed the 10 μg dose of DES showed significantly lower rates of urine marking than did males in the 0.10 μg group. This type of inverted U function is typical in studies of the effects of estrogen on development [8].

Based on the in vitro study with MCF-7 cells, we had predicted similar effects of *o,p'*-DDT at between 1000 and 10 000 times higher dose relative to DES (which was similar to estradiol in binding affinity), while methoxychlor was expected to exert an estrogenic effect at doses between 10 000 and 100 000 higher than DES, which is exactly what we found. The doses of pesticide that resulted in changes in behaviour were thus predicted very closely by our in vitro relative binding assay using MCF-7 cells. These findings show that prenatal exposure to the pesticides *o,p'*-DDT and methoxychlor can markedly alter social behaviour in male mice.

4. Discussion

A major finding from these experiments was that our rapid (18 h) RBA-SMA assay using MCF-7 cells closely predicted the potency relative DES of the estrogenic insecticides, *o,p'*-DDT and methoxychlor, in terms of altering the adult behaviour of the male offspring of pregnant females fed these chemicals. There is a pressing need to screen both previously released and new chemicals for their capacity to act as endocrine disrupters. While this assay addresses only one category of endocrine disruption, namely, disruption as a result of binding to estrogen receptors, it is rapid, inexpensive, and easily conducted with large numbers of chemicals. Thus, the RBA-SMA assay has the potential to be very useful as an initial screen for determining whether a chemical might be an estrogenic endocrine disrupter.

The added important advantage of our RBA-SMA assay over the more common cell proliferation assay using MCF-7 or other cell lines is that it is considerably shorter (18 h vs. 4 days), and we can conduct the assay in both serum-free MEM and in 100% serum, which is not possible with longer-term cell culture experiments. We can thus determine whether the presence of serum proteins alters the RBA of a chemical, since this has a significant impact on the biological response to the chemical in vivo.

In the second study described here we used the ethological approach to the study of behaviour. Ethological analysis relies upon the study of animal behaviour in an evolutionary perspective — that is, taking into account the adaptive significance of the behaviour and the selective pressures that had acted on the behaviour. In the ethological approach, animal behaviour is thus examined in situations that approximate as much as possible the context in which a given behaviour was selected for, while recognizing the reality that, for purposes of experimental control, working in the field is often not practicable.

It is well established that during fetal life, hormones have marked effects on subsequent social behaviours. Evolution has operated on these developmental processes such that fitness is maximized. Therefore, perturbation of systems that differentiate under endocrine control may result not only in the disruption of organ function, but also of an individual's social interactions. These effects on social behaviours may be dramatic. If animals within a population all show changes in social-sexual behaviours, marked disturbance in social structure can occur. The ethological approach to the study of effects of endocrine disrupting chemicals thus may prove to be one of the most sensitive biomarkers of exposure.

It is noteworthy that the initial observations concerning the possibility that some chemicals might have these effects involved studies of wildlife populations experiencing declines due to abnormal behaviour. For example, gulls inhabiting the shoreline of the Great Lakes (in particular, Lakes Ontario and Michigan) showed female-female pairing, supernormal clutches (containing nonviable eggs) and altered nest defense and egg incubation behaviour; this was associated with a marked reduction in reproductive success and

population size [19]. Subsequent studies have shown that treatment of eggs with concentrations of *o,p'*-DDT found in affected eggs led to feminization of gull embryos [20]. In direct contrast to mammals, in birds females are the sex that shows active differentiation, males are the default phenotype, and estrogen acts to induce feminization of embryos [8].

In our experiment we chose to examine a behaviour which plays a major part in determining reproductive success in male mice, namely, urine-marking behaviour. Since marking of a territory is a central feature of a male mouse's reproductive strategy, exposure to environmental chemicals that can alter this behaviour could markedly impact the social structure of this species. A stable mouse population requires that a single male within a small family (deme) of mice be the dominant territory-marking male, while other males are subordinate and neither mark the territory nor mate with females [12]. An increase in urine marking (which we found in response to exposure to DES, *o,p'*-DDT and methoxychlor during fetal life) suggests that an increase in inter-male aggression might also be observed in male mice exposed prenatally to estrogenic chemicals, although this possibility has, as yet, not been examined. An increase in aggression within rodent populations is associated with lower reproduction and a decrease in population size [21].

Our finding that the lowest doses of these chemicals administered to pregnant females (0.001 μg/day DES; 1 μg/day *o,p'*-DDT and 1 μg/day methoxychlor) significantly increased urine-marking behaviour in male offspring, and that the highest dose of DES actually led to a decrease in the behaviour relative to lower doses, is quite important. The lowest dose of DES administered (0.001 μg/day to the mother) led to an average of approximately 0.02 μg/kg maternal body weight/day from days 11–17 of pregnancy. Significant behavioral effects of pre-natal exposure to this low dose of DES were not predictable on the basis of published studies, in which much higher doses have been used (for example, 100 μg/kg [22]). The impact of exposure to estrogenic environmental chemicals on social-sexual behaviour in wildlife, domestic animals and humans needs to be further examined, since these chemicals are distributed so widely in the environment.

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