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Estrogen Can Protect Splenocytes from the Toxic Effects of the Environmental Pollutant 4-*tert*-Octylphenol*

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Four-*tert*-octylphenol (OP), an environmental pollutant, exerts apoptotic effects on cultured mouse splenocytes. Although OP binds to estrogen receptors, these apoptotic effects are not exerted by 17β-estradiol (E). It remained possible that OP might bind to estrogen receptors and subsequently exert apoptotic effects not exerted by E after it binds to the same receptors. It also remained possible that E-primed splenocytes might respond to OP differently than splenocytes not exposed to E. Thus, we investigated OP and E interactions on the viability of mouse splenocytes in culture. The total number of splenocytes (cells stained and not stained with trypan blue) was not altered or altered slightly after incubation with any agent for 24 h. Incubation of splenocytes in medium containing 5×10^{-5} or $5 \times 10^{-7}M$ OP decreased the percentage of viable cells by approx 47% and 25%, respectively. The addition of 0.8×10^{-5} to $0.8 \times 10^{-9}M$ E to cultures was without effect or decreased the percentage of viable cells by only approx 5%. The addition of these concentrations of E simultaneously with or at 2 h after the addition of $5 \times 10^{-5}M$ or $5 \times 10^{-7}M$ OP to cultures did not interfere with the OP- induced decreases in cell viability. By contrast, incubation of splenocytes in medium containing E for 2 h prior to the subsequent addition of either dose of OP blocked the OP-induced decreases in cell viability in a dose-response manner. There was a marked reduction in the percentage of viable cells (70%) when splenocytes were incubated with $0.5 \times 10^{-5}M$ dexamethasone. The addition of $0.8 \times 10^{-5}M$ E at 2 h prior to the addition of dexamethasone did not prevent the decreased cell viability. Incubation of cells in medium with $0.8 \times 10^{-5}M$ testosterone caused a small decrease in splenocyte viability similar to that observed with E. However, unlike E, the addition of testosterone at 2 h prior to the addition of $5 \times 10^{-5}M$ OP did not prevent the OP-induced decrease in cell viability. These data suggest the presence of estrogen receptors in some splenocytes. They also suggest that if OP binds to these estrogen receptors or other receptors in the absence or initial presence of E, the resulting effect is toxic to the cells. By contrast, exposure of splenocytes to E prior to their exposure to OP can prevent the toxicity of OP.

Key Words: Octylphenol; estrogen; splenocytes; toxicity; testosterone; dexamethasone.

Introduction

Alkylphenols, especially mixtures of nonylphenol isomers and 4-*tert*-octylphenol (OP), have been manufactured for several commercial purposes *(1–3)*. The majority of these manufactured alkylphenols have been used as intermediates to synthesize alkylphenol polyethoxylates (APEOs), nonionic surfactants employed as agricultural and industrial cleaners, and as emulsifiers for many products. In fact, approximately a half billion pounds of APEOs were produced in 1990 in the US alone *(4)*. Aqueous APEO solutions generally are used and usually are discharged into wastewater. They biodegrade slowly *(5)*, at least in part, to the alkylphenols from which they were synthesized *(6,7)*. The alkylphenols themselves also are slowly biodegradable *(8,9)*. Thus, APEOs and alkylphenols accumulate in the environment.

The presence of APEOs and alkylphenols in the environment is a concern because of their toxicity to animal cells. In addition to the biodegradation of APEOs to alkylphenols in the environment, alkylphenols have been found to be breakdown products of APEOs administered to male rats (10). This indicates that consumption of APEOs may increase an animal's exposure to alkylphenols. Lethal thresholds for 4-nonylphenol for shrimp or salmon were approximately $10^{-6}M$, whereas that for OP for shrimp was approximately four times higher *(11)*. It

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has been reported that concentrations of 2,4-di-*tert*-pentylphenol alone were in the 10^{-3} *M* range in adipose tissue of carp in the Detroit River's Trenton Channel *(12)*. This alkylphenol likely accumulated in the fat because fat concentrations were greater than concentrations measured in the river water or sediment. Measurement of APEOs or alkylphenols *(7,12–20)* in rivers and in drinking water has shown concentrations to vary widely. Of particular note are the observations that the concentration of 2,4-di-*tert*-pentyl-phenol approached 10^{-3} *M* in the river sediment of the Detroit River's Trenton Channel *(12)*, nonylphenol concentration approached 10^{-6}*M* in the Aire river, and octylphenol concentration was approx 5×10^{-8}*M* in the outer Tees estuary in England *(20)*, nonionic detergents (primarily APEOs) were hundreds of μg/L in water wells in Israel *(18)*, and ng/L concentrations each of different APEOs were found in drinking water in New Jersey *(17)*. The potential toxicity of these contaminants, which may bioaccumulate *(12)*, to animal cells is suggested by recent experiments performed on mammalian cells. In cell cultures of murine splenocytes, two different octylphenol APEOs killed all cells at 10^{-6} *M* and OP, which killed about 68% of the viable cells at 10^{-4} *M* was still effective in killing about 20% of the viable cells at concentrations as low as 10^{-12} *M (21)*.

Interestingly, OP also exerts considerable estrogenic activity. It has been shown to bind to estrogen receptors and to exert estrogenic actions on piscine, avian, and mammalian cells in vitro *(22,23)*. The results of these in vitro studies showed OP to be approx 1000 times less estrogenic than 17β-estradiol (E). It also has been reported to exert estrogen-like actions when administered to adult male rats in vivo *(24,25)*. However, the apoptotic action of OP on murine splenocytes was not shared by E *(21)*.

We concluded that OP did not exert estrogen-like actions to kill rat and murine splenocytes in culture *(21)*. This toxic effect of OP was mediated at least in part if not totally through Ca^{2+}- dependent apoptosis *(21)*. Incubation of splenocytes with E at concentrations as high as 1.8×10^{-6} *M* did not cause apoptotic effects *(21)*. Nevertheless, the possibility remained that OP might bind to estrogen receptors and subsequently exert effects not shared with estrogen after it binds to estrogen receptors. As an extension of this, it also was possible that competition between E and OP for the estrogen receptor or that initial occupation of estrogen receptors by estrogen and the sequelae that occur before exposure of splenocytes to OP might be different than the effects produced by exposure of the cells to OP alone. For these reasons, we investigated OP and E interactions on the viability of mouse splenocytes in culture. The results demonstrate a new phenomenon. Estrogen can exert a time dependent effect on cells that protects them from the toxic effects of a molecule that has been reported to bind to estrogen receptors in other cell types *(23)*.

Results

Previous Study: The Effects of a Single Exposure to OP on the Viability of Cultured Balb/c Splenocytes

The results of three cytotoxicity studies previously published *(21)* are given below. The data are expressed as means (± SE) of the percentage of viable cells. The results were: 68.7 ± 0.3 for cells cultured with 0.08% ETOH; 21.7 ± 5.4 for cells cultured with 10^{-4} *M* OP; 52.7 ± 0.3 for cells cultured with 10^{-8} *M* OP; 54.7 ± 1.5 for cells cultured with 10^{-12} *M* OP; and, 61.3 ± 0.9 for cells cultured with 10^{-16} *M* OP. The values for 10^{-4} *M* OP, 10^{-8} *M* OP, and 10^{-12} *M* OP differed from the 0.08% ETOH value at $P < 0.01$.

Study 1: The Effects of Prior Exposure to E on OP (5×10^{-5} M)-Induced Toxicity of Splenocytes

Incubation of cells in medium containing 0.08% ETOH did not affect the percentage of viable cells at 24 h of culture (Table 1). The addition of various concentrations of E to cultures tended to cause small decreases in cell viability, but the decreases were not statistically significant. In contrast, OP (5×10^{-5} *M*) markedly reduced the percentage of viable cells to 35%. This represented a 47% decrease in the percentage of viable cells from that observed in cultures containing ETOH vehicle. The toxicity of OP was prevented in a dose-dependent manner when the cells were incubated with E for 2 h prior to and during their exposure to OP (Table 1).

There was a small, but statistically significant decrease in the percentage of viable cells when the highest concentration of E employed (0.8×10^{-5} *M*) was added to the cultures at 2 h prior to the addition of 5×10^{-5} *M* OP. The percentage of viable cells (62.1 ± 0.7%) in this group was not different from that observed when this concentration of E was added to the cultures at 2 h prior to the addition of ETOH (63.1 ± 0.6%). The total number of splenocytes (cells stained and not stained with trypan blue) was not altered significantly by any treatment (Table 1).

Study 2: Further Investigation of the Effects of E Alone on the Viability of Splenocytes

As observed in Study 1 (Table 1), incubation of cells in medium containing 0.08% ETOH did not affect the percentage of viable cells at 24 h of culture (Table 2). Also similar to the results observed in Study 1 (Table 1), the addition of various concentrations of E to cultures tended to cause small, nonstatistically significant decreases in cell viability, and no change in the total number of splenocytes.

Study 3: The Effects of Prior Exposure to E on OP (5×10^{-7}M)-Induced Toxicity of Splenocytes

Incubation of cells in medium containing 5×10^{-7}*M* OP decreased the percentage of viable cells to 49.9% (Table 3). This represented a 25% decrease in the percentage of viable cells from that observed in cultures containing

Table 1
The Effects of Prior Exposure to E on OP ($5 \times 10^{-5}M$)-Induced Toxicity of Splenocytes

Treatment at 0 h, 10 μL each	Treatment at 2 h, 10 μL each	Percentage of viable cells at 24 h	Total cell no., $\times 10^7$/mL
Med	Med	68.1 ± 1.4^a	1.02 ± 0.03^a
ETOH	ETOH	$66.5 \pm 0.8^{a,b}$	1.06 ± 0.05^a
E ($0.8 \times 10^{-5}M$)	ETOH	$63.1 \pm 0.6^{b,c}$	1.00 ± 0.01^a
E ($0.8 \times 10^{-6}M$)	ETOH	$64.2 \pm 1.3^{b,c}$	0.94 ± 0.03^a
E ($0.8 \times 10^{-7}M$)	ETOH	$65.2 \pm 0.8^{a,b,c}$	1.01 ± 0.06^a
ETOH	OP ($5 \times 10^{-5}M$)	35.0 ± 0.6^f	1.05 ± 0.06^a
E ($0.8 \times 10^{-5}M$)	OP ($5 \times 10^{-5}M$)	62.1 ± 0.7^c	1.03 ± 0.06^a
E ($0.8 \times 10^{-6}M$)	OP ($5 \times 10^{-5}M$)	53.1 ± 2.6^d	1.03 ± 0.09^a
E ($0.8 \times 10^{-7}M$)	OP ($5 \times 10^{-5}M$)	41.3 ± 0.5^e	0.89 ± 0.09^a

Data are the mean (± SE) results of three cytotoxicity experiments. Values with different superscripts are significantly different from each other at $P < 0.05$.

Table 2
Further Investigation of the Effects of E Alone on the Viability of Splenocytes

Treatment at 0 h, 10 μL each	Treatment at 2 h, 10 μL each	Percentage of viable cells at 24 h	Total cell no., $\times 10^7$/mL
Med	Med	68.9 ± 0.6^a	0.89 ± 0.03^a
ETOH	ETOH	68.3 ± 0.8^a	0.88 ± 0.02^a
E ($0.8 \times 10^{-7}M$)	ETOH	64.0 ± 1.9^a	0.89 ± 0.04^a
E ($0.8 \times 10^{-8}M$)	ETOH	65.0 ± 2.0^a	0.94 ± 0.09^a
E ($0.8 \times 10^{-9}M$)	ETOH	65.3 ± 1.6^a	0.87 ± 0.05^a

Data are the mean (± SE) results of three cytotoxicity experiments. Values with different superscripts are significantly different from each other at $P < 0.05$.

Table 3
The Effects of Prior Exposure to E on OP ($5 \times 10^{-7}M$)-Induced Toxicity of Splenocytes

Treatment at 0 h, 10 μL each	Treatment at 2 h, 10 μL each	Percentage of viable cells at 24 h	Total cell no., $\times 10^7$/mL
ETOH	ETOH	66.8 ± 0.3^a	1.17 ± 0.08^a
ETOH	OP ($5 \times 10^{-7}M$)	$49.9 \pm 1.1^{c,d}$	0.98 ± 0.05^b
E ($0.8 \times 10^{-7}M$)	OP ($5 \times 10^{-7}M$)	61.2 ± 0.3^b	$1.07 \pm 0.02^{a,b}$
E ($0.8 \times 10^{-8}M$)	OP ($5 \times 10^{-7}M$)	51.9 ± 1.6^c	$1.03 \pm 0.02^{a,b}$
E ($0.8 \times 10^{-9}M$)	OP ($5 \times 10^{-7}M$)	47.9 ± 1.3^d	$1.03 \pm 0.06^{a,b}$

Data are the mean (±SE) results of three cytotoxicity experiments. Values with different superscripts are significantly different from each other at $P < 0.05$.

ETOH vehicle. This decrease was not as dramatic as that observed after incubation of cells in medium containing $5 \times 10^{-5}M$ OP (Table 1). As observed in Study 1 (Table 1), this toxicity of OP was prevented in a dose-dependent manner when the cells were incubated with E for 2 h prior to and during their exposure to OP (Table 3). However, less E was needed to reverse the toxicity of $5 \times 10^{-7}M$ OP (Table 3) than was needed to reverse the toxicity of $5 \times 10^{-5}M$ OP (Table 1). A small but statistically significant decrease in the total number of splenocytes was observed after treatment with OP. However, this decrease was no longer statistically significant when cells were exposed to E prior to OP (Table 3).

There was a small, but statistically significant decrease in the percentage of viable cells (61.2 ± 0.3%) when the highest concentration of E employed ($0.8 \times 10^{-7}M$) was added to the cultures at 2 h prior to the addition of $5 \times 10^{-7}M$ OP (Table 3). This decrease was similar to that observed (64.0 ± 1.9%) when cells were incubated in medium containing $0.8 \times 10^{-7}M$ E in the absence of OP (Table 2).

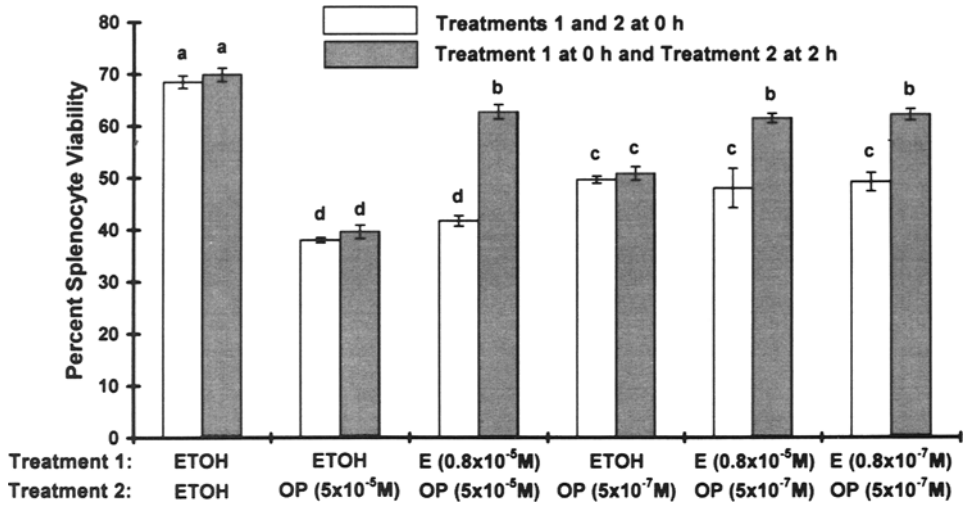

Fig. 1. Comparison of the effects of simultaneous exposure to E and OP with that of exposure to E for 24 h prior to and during exposure to OP on OP-induced toxicity of splenocytes. Data are the mean (± SE) results of three cytotoxicity experiments. Values with different superscripts are significantly different from each other at $P < 0.05$.

Study 4: Comparison of the Effects of Simultaneous Exposure to E and OP with that of Exposure to E for 2 h Prior to and During Exposure to OP on OP-Induced Toxicity of Splenocytes

The addition of OP to culture medium decreased the percentage of viable splenocytes in an apparent dose-response manner (Fig. 1). The addition of E simultaneously with OP did not prevent the toxic effect of either dose of OP. In contrast, when cells were incubated with E in medium for 2 h prior to and during exposure to OP, there was only a small decrease in splenocyte viability compared to cultures containing medium with ETOH vehicle.

Interestingly, $0.8 \times 10^{-5} M$ E was equally as ineffective as was $0.8 \times 10^{-7} M$ E in preventing the toxicity of $5 \times 10^{-7} M$ OP when the two agents were added simultaneously (Fig. 1). It also is of interest that there was still a small, but statistically significant decrease in the percentage of viable cells when cultures were exposed to $0.8 \times 10^{-5} M$ E prior to and during exposure of the cells to $5 \times 10^{-7} M$ OP. This small decrease was not different from that observed when the lower dose of E ($0.8 \times 10^{-7} M$) was added to culture medium at 2 h prior to the addition of $5 \times 10^{-7} M$ OP (Fig. 1). The total number of splenocytes was not altered by any treatment (data not shown).

Study 5: The Effects of Exposure to E After Initial Exposure to OP on OP-Induced Toxicity of Splenocytes

In this study, the small decrease in the percentage of viable cells observed when E was added to the culture medium reached statistical significance (Table 4). As observed in Study 4 (Fig. 1), the addition of OP to culture medium decreased the percentage of viable splenocytes in an apparent dose-response manner. The addition of E at 2 h after the addition of OP to the cultures did not block the OP-induced toxicity. None of the treatments altered the total number of splenocytes when compared to the ETOH controls (Table 4).

Study 6: The Effects of Prior Exposure to E on Dexamethasone (DEX)-Induced Toxicity and the Effects of Prior Exposure to Testosterone (T) on OP-Induced Toxicity of Splenocytes

As observed in Study 1 (Table 1) and Study 4 (Fig. 1), incubation of cells in medium with $0.8 \times 10^{-5} M$ E for 2 h prior to and during exposure of the cells to $5 \times 10^{-5} M$ OP markedly reduced the toxicity of OP (Table 5). There was a marked reduction in the percentage of viable cells when splenocytes from the same mice were incubated with DEX (Table 5). The addition of $0.8 \times 10^{-5} M$ E at 2 h prior to the addition of DEX did not prevent the decreased cell viability. The addition of $0.8 \times 10^{-5} M$ T, like the addition of $0.8 \times 10^{-5} M$ E (Study 5, Table 4), to culture medium caused a small but statistically significant decrease in the percentage of viable cells (Table 5). However, unlike E (Study 1, Table 1; Study 3, Table 3; Study 4, Fig. 1), incubation of cells with T for 2 h prior to and during exposure to OP was ineffective in preventing the OP-induced decrease in the percentage of viable cells (Table 5). The total number of splenocytes was not altered by any treatment (Table 5).

Discussion

To our knowledge, the phenomenon of E exerting a time-dependent effect on heterogeneous cells in culture that protects at least some of them from the toxicity of an environmental pollutant has not been reported previously. Our results clearly demonstrate that exposure of cultured

Table 4
The Effects of Exposure to E After Initial Exposure to OP on OP-Induced Toxicity of Splenocytes

Treatment at 0 h, 10 µL each	Treatment at 2 h, 10 µL each	Percentage of viable cells at 24 h	Total cell no., $\times 10^7$/mL
ETOH	ETOH	66.8 ± 0.2^a	$1.04 \pm 0.07^{a,b}$
ETOH	E ($0.8 \times 10^{-5} M$)	63.4 ± 1.3^b	$1.07 \pm 0.10^{a,b}$
OP ($5 \times 10^{-5} M$)	ETOH	34.4 ± 1.2^e	0.89 ± 0.01^b
OP ($5 \times 10^{-5} M$)	E ($0.8 \times 10^{-5} M$)	31.7 ± 1.7^e	$1.05 \pm 0.09^{a,b}$
OP ($5 \times 10^{-7} M$)	ETOH	50.8 ± 1.2^c	1.20 ± 0.10^a
OP ($5 \times 10^{-7} M$)	E ($0.8 \times 10^{-5} M$)	45.5 ± 0.6^d	$1.14 \pm 0.11^{a,b}$
OP ($5 \times 10^{-7} M$)	E ($0.8 \times 10^{-7} M$)	50.3 ± 0.2^c	$1.16 \pm 0.03^{a,b}$

Data are the mean (±SE) results of three cytotoxicity experiments. Values with different superscripts are significantly different from each other at $P < 0.05$.

Table 5
The Effects of Prior Exposure to E on DEX-Induced Toxicity and the Effects of Prior Exposure to T on OP-Induced Toxicity of Splenocytes

Treatment at 0 h, 10 µL each	Treatment at 2 h, 10 µL each	Percentage of viable cells at 24 h	Total cell no., $\times 10^7$/mL
ETOH	ETOH	65.8 ± 1.8^a	1.06 ± 0.06^a
ETOH	OP ($5 \times 10^{-5} M$)	37.2 ± 0.5^c	1.04 ± 0.08^a
E ($0.8 \times 10^{-5} M$)	OP ($5 \times 10^{-5} M$)	57.2 ± 0.6^b	1.06 ± 0.04^a
ETOH	DEX ($0.5 \times 10^{-5} M$)	20.1 ± 2.5^d	0.91 ± 0.08^a
E ($0.8 \times 10^{-5} M$)	DEX ($0.5 \times 10^{-5} M$)	19.9 ± 2.4^d	1.00 ± 0.06^a
T ($0.8 \times 10^{-5} M$)	ETOH	60.3 ± 0.7^b	1.14 ± 0.04^a
T ($0.8 \times 10^{-5} M$)	OP ($5 \times 10^{-5} M$)	35.7 ± 2.3^c	1.04 ± 0.11^a

Data are the mean (±SE) results of three cytotoxicity experiments. Values with different superscripts are significantly different from each other at $P < 0.05$.

mouse splenocytes to E for 2 h prior to and during exposure of the cells to OP can prevent the toxicity of OP. This protective effect of E was not observed when E and OP were added to cultures simultaneously or when E was added to cultures after the addition of OP. In addition, this protective effect of E was not universal because E did not prevent the toxic effects of DEX on splenocytes. It also was specific in the sense that T, another steroid that can be converted to E by some cells, did not prevent the OP-induced toxicity.

Our studies conducted in vitro do not determine whether E and OP might act similarly in vivo. If one assumes that they do, it is not known whether circulating levels of E in males or females would be effective in protecting splenocytes and possibly other cells from the cytotoxic effects of OP that may enter the circulation after environmental exposure. The fact that OP at $10^{-12} M$ was effective in killing rat and murine splenocytes in culture *(21)* and the dose-response effect of E to prevent OP-induced toxicity that is dependent on the dose of OP employed (present study) raise the possibility that E might indeed act to protect cells after exposure of animals to OP in the environment. Although the protective effect of E on the OP-induced cytotoxicity was observed with supraphysiological concentrations of E, it remains possible that physiological concentrations of E would protect cells from sublethal effects of OP. In any case, it should be noted that in these studies, E and OP, a xenoestrogen, did not exert synergistic effects as has been reported for combinations of xenoestrogens *(26)*.

These data need to be interpreted with respect to the following. Our splenocyte cell cultures contained mostly lymphocytes and the maximal percentage of live cultured splenocytes killed by OP even at $10^{-4} M$ is approximately 70% *(21)*. Additionally, we have not yet determined the subpopulations of splenocytes susceptible to OP. Octyphenol has been reported to bind to estrogen receptors in hepatocytes in rainbow trout in a competitive fashion with estradiol *(23)*. Because we did observe an effect of estradiol, it is reasonable to postulate that some splenocytes did contain estrogen receptors. There is evidence that only certain subpopulations of lymphocytes contain estrogen receptors *(27)* and we have not determined which cells in our splenocyte culture contain estrogen receptors. It is possible that the splenocytes susceptible to OP also contain estrogen receptors. If the populations are identical or if the population with estrogen receptors exceeds the popula-

tion susceptible to OP, E could then exert its protective effect directly on the cells susceptible to OP. On the other hand, if the population of splenocytes with estrogen receptors is significantly less than the population susceptible to OP, one needs to postulate that E causes the release of a substance into the culture medium or that E causes cell-to-cell interactions, which protect splenocytes from the toxic effects of OP. Thus, until the population of cells susceptible to OP and those with estrogen receptors are identified, no definitive conclusions can be drawn.

The protective effect of E, which clearly requires that splenocytes be exposed to E prior to their exposure to OP, supports the view that the sequelae of events that follow E binding to estrogen receptors is protective against the toxic effects of OP. The cells that were exposed to E for 2 h prior to their exposure to OP were different, at the time of addition of OP to the culture medium, than the cells that were initially exposed to E and OP simultaneously. Our results also demonstrate that whatever this protective effect may be, it is not an all-or-none effect. These observations would argue against E exerting its protective effect by acting in an all-or-none fashion, such as causing a shift in the stage of the cell cycle for splenocytes that might make them resistant to OP. We did not test whether the continuous presence of E in the culture medium during the time period that splenocytes were incubated with OP was also necessary for E to exert its protective effect.

In the absence of direct measurements, it remains possible that E and OP are capable of competing for binding to estrogen receptors in splenocytes, as they compete for cytosolic estrogen receptors in hepatocytes in rainbow trout *(23)*. It also is possible that E and OP bind to different domains of a membrane or intracellular estrogen receptor in a noncompetitive fashion. This could explain the inability of excess estrogen to block the toxic effect of OP when the two compounds were added simultaneously to the splenocyte cultures. The estrogen receptor–OP complex might then follow a secondary pathway different from that of the estrogen receptor–E complex to cause apoptosis regardless of whether or not E was also attached to the estrogen receptor-OP complex. Different secondary pathways have been reported for E and a estrogen receptor agonist, raloxifene, after they bind to the estrogen receptor. The estrogen receptor–raloxifene complex binds to an adapter protein, whereas the estrogen receptor–E complex does not *(28)*. In any event, OP response elements in the regulatory regions of the genes that might be involved in effecting apoptosis would be different from the E response elements.

The tendency for E to cause a small decrease in the percentage of viable cells reached statistical significance in one study. A similar small decrease in the percentage of viable cells also was observed when T was employed. Thus, it is possible that some added steroids or metabolic conversion products may exert such an effect in culture. However, we consider E formed by aromatization of T less likely, because T was not effective in acting like E to prevent the OP-induced toxicity. It also is of interest that the percentage of viable cells observed after addition of E to culture medium was not different from the percentage of viable cells observed when maximal doses of E were added to culture medium before OP to block OP-induced toxicity. Thus, the failure of high doses of E to totally reverse the decrease in the percentage of viable cells caused by OP may represent a small population of splenocytes that are killed by E, rather than a small population of splenocytes that E is unable to protect against the toxic effects of OP.

It will be of importance in future studies to ascertain whether E protects other cell types, including those of the reproductive system, from the toxic effects of OP both in vitro and in vivo. Furthermore, it is of interest to determine whether the protective effects of E extends to other environmental pollutant compounds and if such protection relates to the ability of the compound to bind to estrogen receptors.

Materials and Methods

Animals

Seven-week-old male Balb/c mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA), housed 4–5/cage in a room with controlled lighting (lights on 0700–1900 h daily) and temperature (19–21°C), given free access to Teklad Rodent Diet 8604 and tap water, and used within 4 wk. The mice were maintained and utilized in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Preparation of the Basic Cell Culture

The preparatory medium was RPMI 1640 (Lot No. 19N4358; Life Technologies, Inc.; Grand Island, NY) containing 2% penicillin-streptomycin (Lot No. 23K4363; Life Technologies, Inc.). The culture medium was preparatory medium containing 5% heat-inactivated (50°C for 30 min) fetal bovine serum (Lot No. 91913; Harlan; Indianapolis, IN) and 2 m*M* glutamine.

On each day of the experiment, one mouse was decapitated between 1100 and 1330 h. The spleen was removed and placed in a sterile dish containing 5 mL of preparatory medium. Splenocytes were forced out of the spleen by gently flushing it with preparatory medium through holes made with a 22-gage needle. The cell suspension was centrifuged, and the supernatant was decanted. The red blood cells were lysed by resuspending the pellet in 1.2 mL of 0.16*M* ammonium chloride at 37°C for 3 min. The lysing procedure was terminated by adding 30 mL of preparatory medium followed by centrifugation. The supernatant was decanted, the pellet was resuspended in 30 mL preparatory medium, and the cell suspension was centrifuged; this procedure was repeated. After this last wash, the cells were resuspended in culture medium. The cell concentration and the viability of the cells were determined by exclusion of

trypan blue employing an aliquot of the cells. The volume was adjusted with culture medium so that the cell count was 1×10^7 splenocytes/ml. One-hundred-microliter aliquots of the cell suspension were added to Falcon 12 × 75-mm polypropylene culture tubes (Becton-Dickinson; Lincoln Park, NJ).

Test Agents and Solutions

OP (Lot No. PN07905DN) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Dexamethasone (Lot No. 34H0502), E (Lot No. 120H0126), and T (Lot No. 11H0756) were purchased from Sigma Chemical Co. (St. Louis, MO). Solutions of each of the test compounds were made in absolute ethanol (ETOH) just prior to their use. Gloves and mask were used as protective clothing while handling these compounds. The final ethanolic solutions were diluted 1:200 with culture medium. All solutions were made in polypropylene containers.

Cytotoxicity Studies

Test solutions were added as follows: 20 µL at 0 h or 10 µL at 0 h and 10 µL at 2 h. With a total of 20 µL of the various solutions added to the 100 µl samples of the cell suspensions, the concentration of ETOH in the culture medium was 0.08%. Control cells were cultured in culture medium with or without 0.08% ETOH. Cells were cultured at 37°C in a 95% O_2–5% CO_2 atmosphere for a total of 24 h. The percentage cell viability and total cell number were determined by trypan blue exclusion. Between 440 and 695 cells were counted within 16 grids of a hemocytometer for each sample.

Experiments were conducted three times for each study. Six studies were conducted. The test agents added at 0 and 2 h for each study are specified in the Results section.

Statistics

Data from Study 5 were analyzed by two-way analysis of variance. Data from each of the other five studies were analyzed by one-way analysis of variance. Duncan's multiple range test was used for post-hoc analyses. A probability < 0.05 was considered statistically significant.

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