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THE CHALLENGE POSED BY ENDOCRINE DISRUPTING CHEMICALS

by

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SUMMARY

Rapid regulatory developments in the area of environmental endocrine disruption present a series of potential problems that are identified and illustrated with examples taken from the recent literature. A list of priorities is provided, including the need for additional epidemiological and wildlife studies, for the derivation of a harmonized testing strategy, for agreement on the toxicities expected of endocrine disrupting agents, and for acceptance that whole animal assays will be uniquely critical in this area of toxicology. The intrinsic difficulty of attempting to study all aspects of endocrine disruption simultaneously indicates the need to reduce the scope of the problem, which can be achieved by studying toxicities mediated by sex hormone receptors first.

INTRODUCTION.

It has been proposed that humans and wildlife have suffered adverse effects on reproductive health as a result of environmental exposure to chemicals that interact with the endocrine system[1-4]. Mindful that an hypothesis is an idea that has not been sufficiently tested[5], many independent efforts have been undertaken to evaluate the scope and veracity of the problem. As one of these undertakings, the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC) convened the present study group with the aims of discerning the degree to which synthetic chemicals are implicated in this issue, and advising toxicologists and ecotoxicologists on appropriate testing methods and hazard identification strategies. After compiling a list of currently available test methods[6] we turned our attention to the identification of practical tests for endocrine disruption. In order to do that, the enabling assumption was made that a range of endocrine disruption hazards are posed to wildlife and humans by environmental chemicals. We recognized the importance to any future regulatory initiatives in this area of demonstrating dose-response relationships, of establishing the relevance of experimental models to humans and wildlife, and of assessing exposures; but these were considered to be beyond our initial remit.

In parallel with attempts to develop an appropriate hazard identification strategy for endocrine disrupting chemicals, several regulatory initiatives have been taken, the most specific being a mandate by the United States Congress that the US EPA should have a regulatory framework on endocrine disruption in place by 1998[7]. Such a condensed timeframe carries with it the potential for the premature endorsement of unvalidated assays and unrefined testing strategies. The present article outlines some of these potential problems with a view to their clear recognition and circumvention.

THE POTENTIAL PROBLEMS.

Kavlock et al[8] recently conducted a definitive review of the data cited to support links between a range of human health effects and exposure to endocrine disrupting chemicals. The key conclusions of that analysis are presented in Table 1. These authors concluded that there are no clear relationships between endocrine effects in humans and exposures to xenobiotics. These conclusions are further dissected in

Table 2 according to the criteria recommended by Hill[9] for distinguishing between epidemiological association and causation, as recommended by Kavlock *et* al[8]. For comparative purposes, the data supporting causation for the 56 established human carcinogens are also presented. That analysis reveals only tentative associations between human exposure to chemicals and the observation of any of the endocrine toxicities listed in Table 1. With the exception of the clinical use of diethylstilbestrol (DES), there are presently no proven causations in humans. There are also no data to support the assumption[1] that synthetic chemicals, as opposed to naturally occurring chemicals (and in the case of humans, dietary constituents, lifestyle, etc) are the most important etiologic contributors to the projected problem. A similar assumption, now considered to be incorrect[10], was made in the early stages of the study of environmental carcinogenesis. These collected considerations emphasize that any moves to regulate chemicals showing endocrine-disrupting properties should be cautionary and based on confirmed evidence, all of which currently derives from either wildlife or experimental studies. Specifically, the justification for any future regulatory actions should not be based on the presumption that such moves will automatically alleviate the human health effects discussed by Kavlock et al[8].

In contrast to the situation in humans, several etiological links between exposure to synthetic endocrine-disrupting chemicals and adverse effects on wildlife have been established, mainly in contaminated environments[1-4]. Nonetheless, the data supporting some of the suspected environmental links are as fragile as those noted by Kavlock et al[8] for human effects. An example of this is provided by the predominant role played by the natural hormones estrone and 17β-estradiol, as opposed to synthetic xenobiotics, in the partial feminization of fish exposed to effluent of some municipal sewage treatment plants in the United Kingdom[11-16].

Given that there is significant conservation among animal species in the mechanisms that control sexual reproduction and development, it is suggested that it will be possible to adopt, at least initially, a common strategy for the identification of wildlife and human endocrine disruptors. For example, the fact that a chemical can induce the production of the female-specific protein vitellogenin in male fish contributes an alert to its potential to cause corresponding effects in other wildlife and in appropriately exposed humans. Likewise, the activity of an agent in a rodent uterotrophic assay contributes to assessment of its potential to cause endocrine toxicities in wildlife. Differences between the assessment of wildlife and human toxicities will most likely be encountered at the level of the nature, timing, duration and magnitude of exposures, i.e., at the level of risk assessment. On some occasions, differences in the physiology or biochemistry of the reproductive process between species, or differences in the accumulation of chemicals between the major biological and geographical compartments, may become important risk modifying factors. However, it is suggested that such differences (e.g., the possibly unique effects of chemicals on metamorphosis or moulting) should be specifically studied to determine their importance, rather than being used to fragment the study of endocrine disruption at this early stage.

DEFINITIONS AND TERMINOLOGY

A surge of studies to define chemical endocrine disruptors, coupled to attempts to solve problems created by preliminary studies, is leading to a confusion of terms. For example, agents are already being labeled as estrogenic based on their activity in the MCF-7 assay (the E-screen[17]), despite the fact that a range of non-estrogenic factors can stimulate these cells to divide[18], and the fact, common to all of biology, that not all activities observed *in vitro* are realized *in vivo.* Alternatively, the absence of agreed definitions could lead to unrelated activities of a chemical being linked with the implication of a mechanistic association. For example, it is superficially attractive to assume that the activity of butyl benzyl phthalate (BBP) in the MCF-7 assay[17] is directly predictive of its reported ability to reduce testis weight in rats[19]. However, BBP and its principal metabolites are inactive in immature and ovariectomized rat uterotrophic assays[20], and its activity as a testicular toxin remains to be confirmed, and explained. Similarly, the observation that continuous subcutaneous infusion of nonyl phenol is capable of stimulating cell division in female rat mammary tissue[21] was suggested[21] to be unrelated to its uterotrophic activity in the rat following intraperitoneal injection[22], and the ability of the anti-estrogen raloxifene to counter bone density loss in ovariectomized rats[23] was recently shown not to involve the DNA-binding domain of the estrogen receptor with which this chemical is known to interact[24]. These recently described examples illustrate the complexity of the biological issues being approached, and they serve to warn against the precipitate adoption of simple definitions and testing strategies.

It is therefore suggested that there is a need for a set of *toxicological* definitions that will serve this new area. For example, there are two current definitions of an estrogen - a compound that binds to isolated estrogen receptors, or a compound that produces trophic effects on the female reproductive tract. However, what is required is a definition of the toxic effects expected of exposure of a whole organism to such a chemical, a definition that may differ between species and sexes. In the absence of such guidance it will be easy to drift into hypothesis-fulfilling conclusions. For example, an agent may show evidence of potential estrogenic activity by virtue of its activity in one of the many available *in vitro* assays, and then be administered to animals to confirm the expression of this toxic potential *in vivo*. However, if there are no agreed expected toxicities, the chemical's ability to affect kidney weights or thyroid gland function, for example, may be taken as automatic confirmation of the original prediction. Such empirical associations may mislead, as illustrated by the three examples cited above.

The collective term *an endocrine disruptor* is coming into general use, but it has yet to be defined. It is suggested here that an endocrine disruptor should be defined in reference to an intact endocrine system, i.e., as an agent that can induce adverse health effects in an intact organism, secondary to disruption of the organism's endocrine system. Other potentially relevant properties of the chemical, including any effects observed *in vitro*, can only contribute to its definition as a potential endocrine disruptor. Specifically, the activity of a chemical in any of the available *in vitro* assays does not define it as an endocrine disruptor, no more than does the demonstration of a chemical's ability to inhibit the enzyme aromatase *in vitro.*

RODENT TOXICITIES OF CONCERN AND REFERENCE CHEMICALS.

A pre-condition for the framing of toxicological definitions is the existence of a list of sentinel rodent toxicities related to each type of endocrine disruption, together with a databank of reference positive control chemicals for the toxicities named, as developed in other branches of toxicology[25,26]. Despite their clear importance, neither of these lists has yet been agreed by the scientific community. In the case of estrogenicity, estradiol or DES are available as positive control agents, but even in these cases the estrogen-specific toxicities expected are not universally agreed.

In the absence of such an agreed range of reference endocrine disruptors it will be difficult to assess the *general* sensitivity of existing or emerging tests. Equally, the current absence of agreed chemicals that are inactive as endocrine disruptors makes it impossible to evaluate the specificity of emerging *in vitro* assays, or to discern the lower level of activity in any predictive assay that would be expected to lead to significant endocrine toxicities *in vivo*. This absence of in *vivo* toxicity data for a range of toxic and non-toxic reference chemicals must be remedied in order to facilitate a sound research foundation upon which to build an effective regulatory strategy. Individual research groups or regulatory authorities may know of such reference chemicals, but unless these can be shared with the general scientific community, they effectively do not exist.

STRUCTURE ACTIVITY RELATIONSHIPS (SAR)

The derivation of SAR in this area would aid the prioritization of chemicals for testing. However, McLachlan[2] and Katzenellenbogan[27] have pointed out the current difficulty of reconciling chemical structure with the wide range of chemical substances reported to have one or other of the several different endocrine disrupting properties. It is anticipated that useful SAR will exist in situations where specific effects are studied within discrete chemical series, using standardized bioassays and clear criteria for activity. One such approach has been recently described[28]. Nonetheless, it is important to acknowledge that SAR derived from *in vitro* studies may differ significantly from SAR derived from studies conducted *in vivo*, and that it is unlikely that any *general* SAR capable of encompassing *all* categories of endocrine disruptor will be developed.

The report that intraperitoneal injection of high dose-levels of the solvents ethylene glycol and dimethyl formamide to rainbow trout results in increased levels of vitellogenin mRNA[29] was unexpected given that these chemicals are structurally remote, on all counts, from the endogenous estrogen receptor agonist estradiol. The data in question could indicate one of three possible things, each of which is pertinent to the construction of SAR databases for endocrine disruption. First, if they are taken as evidence of the estrogenicity of these two chemicals, as stated in the title of the paper, they illustrate the present absence of understanding of the critical chemical features required for endocrine disrupting activity. A second possible explanation was provided by the authors in the text of their paper, namely, that these chemicals had activated the estrogen receptor by inducing a change in its *conformation,* as opposed to binding to the receptor. If this explanation were to be confirmed it would open up a new area of chemical estrogenicity whose toxicological significance is at present

unclear, and which would require the derivation of a separate SAR database. A third possible explanation is that changes to vitellogenin mRNA levels, observed without confirming commensurate changes in protein levels, may not provide a reliable indicator of the estrogenic activity of chemicals. Determination of which of these three conclusions is correct will require the conduct of further studies, and pending the outcome of those, it would be inappropriate to enter either of these two solvents into any SAR database.This discussion therefore reverts to the critical need for agreed definitions of endocrine activities as a necessary precursor to the derivation of SAR and assays for those activities.

IN VITRO ASSAYS.

The crucial problem is that there are no agreed criteria for the selection, development, or grouping of assays into test batteries/tiers, or for assessing their sensitivity, specificity and relationship to each other. Such questions need immediate attention, because already chemicals are showing different qualitative responses between similar *in vitro* assays, between *in vitro* and *in vivo* assays, and between different routes of administration *in vivo*[30]. For example, several yeast assays having the human estrogen receptor stably integrated into their genome are in current use. These assays have subtly different reporter gene constructs and variable numbers of estrogen response elements, and it is currently unclear what effect these differences will have, if any, on experimental outcomes. Similarly, many laboratories are using one or more of a variety of transiently transfected receptor cell lines, and some of these may be difficult to transfer into routine regulatory use (c.f., the level of standardization achieved rapidly, and essentially, for the Salmonella mutation assay). Finally, some of the currently available *in vitro* assays, although potentially valuable, are complex and/or time-consuming to conduct, as illustrated by the fish primary hepatocyte vitellogenin assay[31]. The present proliferation of *in vitro* assays will inevitably continue in pace with revelations of the complexity of normal sexual reproduction[32]. Therefore, prior to the formal (regulatory) adoption of any of these assays, it is vital that:- (1) the differences between existing assays are elucidated and critically examined, (2) robust versions of the preferred and validated assays are developed for routine use, and (3) an agreed framework in which these assays should be used be derived. Failure to meet these needs will lead to the delays in effective implementation similar to those that accompanied the introduction of mutagenicity assays.

Among the *in vitro* assays so far described, with the obvious exception of the fish hepatocyte assay, and the possible exception of the receptor-based yeast assays, none appear to be metabolically competent. The use of *in vitro* mutagenicity assays in the absence of S9 mix would lead to the non-detection of many mammalian mutagens and carcinogens, and a similar problem should be anticipated in this area. One example will serve to illustrate this concern. Shelby *et al*[33] have reported that the *in vivo* xenoestrogen methoxychlor is unable to bind to isolated estrogen receptors, or to activate this receptor in a mammalian cell transactivation assay. This observation led to the independent study of the same sample of methoxychlor in a yeast human estrogen receptor transactivation assay, with a view to confirming its inactivity and establishing the importance of auxiliary metabolism. In fact, it was found to give a

potent 'direct-acting' positive response in the yeast assay[34], presumably reflecting the ability of the yeast cells to demethylate the methoxy groups yielding the active estrogenic phenol derivative. This example confirms that the issue of metabolism *in vitro* has the potential to confound the validation of mammalian cell *in vitro* assays.

In addition to the general problem of metabolism, and again based on experience gained with mutagenicity assay development, it will be helpful if investigators can rapidly agree which assays are unreliable, and/or, non-specific, and then share that conclusion openly. As an example, the polyclonal nature of MCF-7 cells[17] and the insensitivity of some of the clones to estradiol[35], coupled to the problems of its low specificity discussed above, suggest that this assay will have limited value for general screening purposes, despite the fact that it can be performed adequately in some laboratories. There is a need for such a clear conclusion to be openly agreed in the scientific community, because in its absence, the assay will continue to be used to define potential endocrine disruptors. All new test systems should be scrutinized by the broader scientific community before they are accepted for general use.

It is proposed that the development of *in vitro* assays for potential endocrine disruptors should be led by the naming of significant toxicities that are secondary to disruption of the endocrine system of intact organisms, followed by attempts to model these effects *in vitro*. When appropriate, such assays should then be refined to produce robust test protocols suitable for general use. This is in contrast to the uncoordinated proliferation of superficially validated assays which would act as a brake on progress and lead to the generation of potentially large amounts of uninterpretable data. The need for scientific caution in progressing this new area of toxicology is illustrated by the failure to confirm[34,36] the recent report by Arnold *et al*[37] of synergism of estrogenic activity observed *in vitro* between a range of environmental chemicals.

IN VIVO ASSAYS.

It is general practice in toxicology to screen for a potential toxic activity *in vitro,* and to then confirm the expression of that activity *in vivo,* before attributing a given toxic property to the test agent. For this, and several other reasons outlined in this article, *in vivo* assays will assume a dominant position in screening strategies and risk assessment processes for endocrine disruption. Further, the trend common to other branches of toxicology, of combining a range of endpoints in a single test protocol, will probably apply equally to *in vivo* assays for endocrine disruption. However, an inevitable corollary to the use of multiple-endpoint assays is that one is forced to rank endpoints, often in the absence of guiding data, when qualitatively divergent responses are obtained among the several endpoints being monitored. This indicates the need for an agreed hierarchy of endpoint sensitivities for studying endocrine disruption in a given organism. In addition, the decision to conduct an assay *in vivo* carries with it a range of decisions regarding the choice of test species/strain, route of administration, and duration of dosing. The rodent uterotrophic assay can be used to illustrate these generic problems, with a view to their discussion before, rather than after, the regulatory protocols for *in vivo* assays are fixed. The uterotrophic assay is often referred to as the gold standard of estrogenic activity *in vivo.* However, the data upon which this conclusion is based were derived using a variety of protocols. The

key variables being the use of rats or mice; immature, hypophisectomized, or ovariectomized animals; the use of subcutaneous, intraperitoneal injection, or oral administration of the test agent, and a duration of dosing of between 3 to 6 days. Further, some investigators recommend concomitant assessment of associated markers of estrogenic activity, such as vaginal opening, vaginal cornification, uterine epithelial cell height or stromal proliferation[38,39]. To decide which of these many variables are important to the overall sensitivity of an assay will require assessment of a range of appropriate positive and negative endocrine disruptors. Similarly, it will be important to study the sensitivity and specificity of new markers of estrogen action before they can replace existing markers. For example, lactoferrin mRNA levels in the immature mouse uterus can be increased several hundred fold upon exposure to estradiol[40], but before this can be developed into a replacement for the uterotrophic assay it will be necessary to evaluate the specificity of this response, and to establish that the estrogen and growth factor response elements in the mouse uterus lactoferrin gene are representative of that in humans. The failure to approach such basic questions in genetic toxicity research has led to the development of a large number of competing *in vivo* techniques, with no general agreement on which of them are complementary to other assays and which are redundant.

MULTIPLE MECHANISMS OF ACTION.

Agreement on a testing strategy to detect significant mammalian and wildlife estrogens would be relatively easy to achieve, and several such proposals have already been made[38,39,41,42]. However, this would not be expected to predict endocrine toxicities associated with disturbances to normal steroid hormone synthesis or metabolism, thyroid gland function, or pituitary/hypothalamic feedback control mechanisms. Such effects will be difficult, if not impossible, to simulate *in vitro*, and this again indicates the need for a high level of reliance on acute or sub-acute whole organism assays. For example, although some of the *in vivo* effects of PCBs may be predicted by *in vitro* assay results, particularly those effects mediated by direct receptor interactions, this will not always be true. As an example, it is unlikely that any cell-based assay could anticipate, at least for the correct reason, the ability of certain PCBs to increase the weight of rat testis[43]. This is because the effect is dependent upon PCB-induced hypothyroidism preventing the cessation of Sertoli cell division on day ~16 *post-partum*, an effect that is probably independent of the weak uterotrophic activity seen for PCBs in the rat[44]. Likewise, the testicular effects reported for BBP[13] are unlikely to be associated mechanistically with its mitogenicity to MCF-7 cells, just as the endocrine toxicities of *p,p'*-DDE are most probably mediated by its anti-androgenic properties, rather than by its initially defined estrogenic properties[45].

DIFFERENTIATION OF TOXICITIES AND EFFECTS.

The present uncertainty regarding the *in vivo* toxicities to expect of a chemical that has shown activity *in vitro* could lead to the measurement of a wide range of parameters in follow-up *in vivo* studies. Such toxicological fishing exercises might sometimes be justified, but to be useful they will require the separate recognition of significant toxicities and transient adaptive effects. For example, a small

chemically-induced change in the levels of sex hormones in a rodent may be devoid of toxic significance. A different example is provided by the measurement of the ano-genital (AG) distance in neonatal rats whose mothers have been exposed to a potential endocrine disrupting agent. This endpoint is a potentially valuable marker of endocrine disruption, but it has been little studied to date, and few control data have been published. Therefore, it is legitimate to interpret with caution alterations in this parameter in cases where the effect resolves by weaning, and the pups show normal sexual development and function. Such effects may be of value to explain the observation of a recognized endocrine toxicity, but they may be of little value when the effects themselves constitute the only evidence for endocrine disruption. This is in contrast to situations where an irreversible change in AG distance is subsequently accompanied by other effects, such as a change in the time of vaginal opening or preputial separation, or reduced fertility of the adult progeny. This situation may change with the acquisition of a larger database for AG distance (and for other relatively new markers of endocrine disruption), but in the meantime it will dangerous to interpret such effects in isolation. A related example would be a transient induction of the mRNA in male trout that was not shown to be accompanied by the synthesis of vitellogenin protein. The distinguishing of toxic responses from transient effects will be particularly important in these early days of the study of endocrine disruption, because it will enhance the rapid recognition of endocrine toxicities of immediate potential relevance to humans or wildlife.

AN INTEGRATED APPROACH

If the current concerns turn out to be justified, the problem posed will not be confined to a few countries. Therefore, it is important that the many initiatives being taken by individual governments and chemical industries to assess this issue should be prioritized and progressed with some level of international coordination (Table 3). The divergent testing strategies and regulatory requirements that resulted from individualistic national approaches to carcinogen screening should act as a particular warning. However, the task faced on this occasion is even more complex, because the chemical disturbance of essentially any aspect of animal physiology is under consideration. The detailed knowledge required to respond optimally to this situation is concentrated in a relatively small number of endocrinologists around the world, but they may not be equipped to advance this broad area of toxicology unaided. Thus, the need for toxicologists, regulators and endocrinologists to pool their differing expertise at the international level.

Key among the priorities suggested in Table 3 are the need to continue to support studies to define better the reality and nature of the hazards posed, and to progress the originally perceived problem of exposure of wildlife and humans to estrogen and androgen receptor agonists/antagonists. When progress has been made in these areas, attention can be given to the development of assays for other mechanisms of endocrine disruption. This will involve the development of assays that measure enzyme or hormone levels/activities *in vivo*. Such techniques may be difficult to refine into robust regulatory tests. This sequential approach to the many issues posed will enable harmonized progress to be made in defined areas. As general confidence

in a core set of assays grows, consideration should be given to integrating endpoints into a reduced number of assays.

Finally, one should always keep in mind that the regulation of synthetic chemicals for endocrine disrupting properties may not alleviate the observed increases in human breast and testicular cancer, or the apparent decrease in human sperm counts and sperm quality reported for some countries. Therefore, while attending to one possible contributor to these problems, synthetic chemicals, we should remain alert to the possible importance of alternative contributory factors, such as diet and lifestyle.

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