Assay Validation Studies in Progress in the U.S. Endocrine Disruptor Screening Program

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Good afternoon. I would like to thank the Ministry of the Environment for the opportunity to talk about the status of the U.S. Endocrine Disruptor Screening Program. We appreciate the opportunity to share our information in an international forum such as this.

Before describing the current status of our assay validation studies, I'd like to review the validation process we use, to provide a context for understanding how the assays will be used.

We begin with a literature review of the candidate assays that we intend to validate. We do some "prevalidation" work to standardize and optimize the assays, and to make sure that we can make the assays as sensitive as possible. We then go through a process of interlaboratory validation to make sure that several laboratories can get the same answers for the same chemicals, and that performance criteria can be established. Through this process we know the reliability of the data that we get from different sources. Testing known positives establishes the relevance of the assay. We subject the studies to peer review to make sure that independent scientists agree with the work that we have done.

This validation process was developed by the U.S. Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), which will be discussed in more detail by the following speaker.

When putting together the Endocrine Disruptor Screening Program, the USEPA asked for advice from an independent Advisory Committee. The Advisory Committee suggested that the screening program be composed of two levels:

1) Tier 1 assays would help determine the ability of a chemical to interact with the endocrine system (specifically, the estrogen, androgen and thyroid systems but not other endocrine systems for now).
2) Tier 2 assays would confirm that interaction and provide information that can be used in risk assessment, namely, dose response information and information about the adversity of the effects on the endocrine system.

The Advisory Committee suggested the following assays be included:

Tier 1: estrogen and androgen receptor binding and/or transcriptional activation; uterotrophic; Hershberger; female pubertal; frog metamorphosis, fish reproduction screen, steroidogenesis; and as potential alternatives to certain combinations of Tier 1 assays: a male pubertal, an aromatase, and an adult male assay. Tier 2: mammalian 2-generation reproductive toxicity with additional endocrine endpoints; avian 2-generation; amphibian chronic; invertebrate chronic; fish chronic. The Advisory Committee also recommended development of a Tier 1 assay that incorporates in-utero-through-lactation exposure. We are trying to validate each of these assays. This does not mean that all of these assays will be part of the final Screening Program, but we are trying to validate each of them so that we can choose which set of assays is the best to use.

The literature review for each of the assays has been completed and we are well into prevalidation for most of these assays. I'll talk about the validation work that we are doing on each of these assays. However, I will not talk about the uterotrophic and the Hershberger assays. You heard earlier from Dr. Koëter about the status of those assays.

Estrogen Receptor Binding Assay

We have recently initiated a laboratory study to optimize certain parameters of this assay. The assay had already been considered nearly optimized but based on the advice of an expert panel convened by ICCVAM we agreed to consider some minor adjustments. Specifically, separation of bound from free estradiol using charcoal versus hydroxyapatite is being examined. We will also look at the use of protease inhibitors to prevent the degradation of the protein. Finally, we will also optimize the concentration of the radiolabeled estradiol. We do not think these optimization steps will take very much time.

We will then move into studies of interlaboratory variability. We will be using five laboratories. Most will use Good Laboratory Practices (GLP), although there are a couple of laboratories that are not certified as Good Laboratory Practice participants for these kinds of studies.

Preparation of cytosol is a relatively difficult step in doing the receptor binding assay, so we are separating this step from the others. This will allow us to examine the difficulty in standardization across laboratories of that particular step separately from the other steps of the assay.

We will be testing a range of binding affinities during this interlaboratory comparison step. Later, we will be testing the assay using a larger number of chemicals, probably within the range of 20-30 chemicals of widely different binding strengths and chemical structures, to make sure that the estrogen receptor binding assay which is finally standardized is sensitive to the chemicals that are of interest.

Finally, we will have a transferability study to make sure that the written protocol can be followed by a laboratory which is unfamiliar with the assay. Such a laboratory is not likely to be totally naive to assays using receptor systems, but it will not be familiar with the estrogen receptor binding assay itself.

Androgen Receptor Binding Assay

Validation of the androgen receptor binding assay is somewhat behind that of the estrogen receptor binding assay. We are repeating an initial demonstration of the feasibility of the androgen receptor binding assay which we did last year. We recognize, based on the expert panel's recommendations, that the rat prostate cytosol which we intend to use, may not be the best source of receptor, so we will be examining other (recombinant) sources. However, in the United States there are some patent issues associated with the human recombinant androgen receptor and it may not be possible to include such an assay in the Screening Program.

Pubertal assays

We are depending on success of the validation of the pubertal assays, which are the next assays in the list of prospective Tier 1 assays. These assays have been developed by the EPA to examine various endpoints associated with endocrine disruption. The endpoints are not all associated with specific mechanisms; these are apical assays.

We have done a study in a contract laboratory to show that a non-EPA, non-research lab can run the protocol correctly. That study was done with single, high doses of a range of chemicals. We are now looking at the sensitivity of the protocol using lower doses of methoxychlor, vinclozolin, and phenobarbital.

We are also looking at a wider range of chemicals, in a different study. In the female pubertal, we are examining the response of the assay to atrazine, propylthiouracil, ketoconazole, fenarimol, methoxychlor, and bisphenol A. In the male pubertal, we are looking at atrazine, propylthiouracil, vinclozolin, linuron, p,p'-DDE, ketoconazole, methoxychlor, and phenobarbital. We have tried to cover several different modes of endocrine disruption by including both estrogenic and androgenic chemicals and some thyroid disrupting chemicals. We do not have all possible mode of action covered,

particularly for the thyroid system, but there are many different modes of disruption of the endocrine system and it is not feasible to cover them all.

Frog metamorphosis assay

We are developing a frog metamorphosis study to screen for interaction with the thyroid system. This was recommended to us by the Advisory Committee several years ago. The particular protocol that the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) recommended turned out not to be very sensitive and we are trying to develop a different protocol based on earlier stages of metamorphosis than was recommended by the Advisory Committee. A description of the prometamorphosis assay is at our website. (The website address is given at the end of this paper.) Dr. Joe Tietge's presentation to the Endocrine Disruptor Methods Validation Subcommittee summarizes the evidence showing that an earlier life stage is more sensitive than the later life stages which had earlier been recommended to us.

Fish reproduction screen

We are also developing a fish reproduction screen for use in Tier 1. We are doing four different studies: a comparison of vitellogenin measurement methods, a comparison of the vitellogenin assay across species; a comparison of two different durations of the fish reproduction screen (a 21-day protocol versus a 14-day protocol), and a multiple-chemical evaluation of the fathead minnow assay using atrazine, bisphenol A, dibutyl phthalate, p,p'-DDE, perchlorate, and cadmium chloride.

Steroidogenesis assay

We are also developing a steroidogenesis assay. This one is somewhat further behind in standardization and optimization than most of the other Tier 1 assays. Currently we are measuring steroidogenesis expressed through the minced testis, but there are cell lines available which may be able to tell us about steroidogenesis without requiring the use of tissues from whole animals. EPA is looking to develop such a cell-based assay although this development may not fall within our Endocrine Disruption Screening Program per se.

Aromatase assay

Another in vitro assay that we are validating is an assay for aromatase activity. Aromatase activity is not well covered by the pubertal male assay so a separate assay need to be used in conjunction with it. At the moment, we are not sure what source of aromatase to use. The Advisory Committee had suggested using human placental aromatase but the difficulty of working with human tissues (for example, the difficulty of obtaining human placenta, and concern for diseases that might be associated with human tissue) make it an unattractive alternative. We are investigating cow or pig placentas as sources of aromatase. [Note added in proof: Due to the difficulty in obtaining cow and pig placenta and the low yield of aromatase, this approach is being abandoned.] We are also looking at recombinant human aromatase.

We are also doing a separate study to optimize parameters within the aromatase protocol.

Finally, we are doing a study to look at the response of the aromatase assay using different chemicals.

Adult male assay

The next assay we are considering is an adult male assay, which will eventually be compared with the pubertal male assay, the Hershberger assay, and the OECD 407 assay (28-day repeated dose assay) to

determine which is most sensitive to endocrine disruption in males. This assay has been developed outside of EPA, but the protocol has been made available. The developers have published several studies covering many chemicals. There are a few chemicals that we felt should be run in addition to the ones that have already been published so that we can make a better comparison between that assay and the pubertal and the Hershberger and 407. We would like to examine linuron, methoxychlor, atrazine, trenbolone, perchlorate, fenarimol, and dibutyl phthalate. At the moment we have funds to do only linuron and methoxychlor, but in the near future we hope to be able to get to the others.

Mammalian 2-generation reproductive toxicity assay

For the mammalian 2-generation study we finished a study on thyroid endpoints that was done using propylthiouracil. It was completed this past year and the preliminary data are up on our website. We concluded from the study that T4 and TSH, thyroid weight, and histopathology will probably be added to our 2-generation reproduction assay, but we probably will not add T3 as an endpoint.

We are almost finished with a separate study looking at whether one can see additional effects in the F1 generation if more animals are allowed to mature past postnatal day 21, to postnatal day 90 or longer. The 2-generation study usually sacrifices most of the animals (all except one breeding pair) at postnatal date 21 — that is, at weaning. The concern is that effects that were generated in utero may not have a chance to appear by postnatal day 21. We are currently running a study to see if it is possible to detect such effects. If it is possible we may add an appropriate modification to the 2-generation mammalian study.

Avian two-generation assay

We have three studies going on related to the avian 2-generation study. One is a species comparison of Japanese quail versus bobwhite quail to see differences in sensitivity. The Japanese quail is easier to work with. An assay using this species is shorter and has other advantages. With the data from this study we will be able to make an intelligent choice between those two species.

The second study is a dosing study, to examine whether the animals should be allowed to reach sexual maturation before dosing or whether dosing should start before sexual maturation. There are advantages to each of these, but we need data to show which dosing method is more sensitive.

There is also a question as to whether the F1 generation should be treated throughout its lifetime. That question is being studied in the dosing study. The dosing study is expected to begin in January 2003 and is expected to be finished sometime in 2004.

Finally, we are doing an egg injection study to see if this method of exposure can give us data that will be useful in range-finding for the avian 2-generation study. Both the dosing study and the embryo assay study are ad hoc studies that will help decide how to do the avian 2-gen.

Other studies

EPA has additional work on quantitative structure-activity relationships going on, and some on high throughput pre-screening. This is being done through the Office of Research & Development rather than through the Endocrine Disruptor Screening Program. (We did finish, within the Endocrine Disruptor Screening Program, a validation study on two QSAR methods. We hope to make the information public fairly soon.)

The EPA did a restricted-feeding study for the pubertal assays. The amount of feed given to animals was reduced to see what the effect was on the reproductive endpoints in the pubertal assays, particularly preputial separation in the males and vaginal opening in the females. The conclusion was that up to about a 10 or 12% reduction in body weight gain, there is no effect on the reproductive endpoints in the

pubertal assays. We hope to publish the data fairly soon.

We have published our position on the low-dose effect at our website, which I encourage you to visit. (http://www.epa.gov/scipoly/oscpendo/) We try to put as much information and data on all aspects of the Endocrine Disruptor Screening Program there as we can. If you have any questions, please look at our website.