NTP Multigenerational Study of Environmental Estrogens

Nearly 10 years ago, researchers with the National Toxicology Program (NTP) and the National Center for Toxicological Research (NCTR) began a complex set of experiments in rats to determine whether exposure to estrogenic compounds throughout life and across generations could cause changes in development or patterns of endocrine-related cancers at doses that cause only subtle reproductive effects. Now, at last, specialists in the field of endocrine-active chemicals are close to getting a first look at the findings from these studies.

The three compounds chosen for study—genistein, ethinyl estradiol, and *p*-nonylphenol—represent a natural estrogenic substance, a drug, and an industrial chemical, respectively. The first experiments for all three compounds focused on determining appropriate dose ranges for later multigenerational studies. Additionally, studies were conducted with genistein and ethinyl estradiol to determine whether and how the carcinogenic potential of these substances changed across generations following long-term chronic exposure.

On 12 June 2006, the first reports based on these experiments will undergo peer review at a one-day meeting at the NIEHS, with final publication expected later this year and additional reports scheduled for review in 2007. The reports to be reviewed on June 12 center on genistein, an estrogen-like compound found in soy, and detail the results of dose range–finding studies and multigenerational reproductive and carcinogenesis experiments.

Years in the Making

According to John Bucher, deputy director of the NIEHS Environmental Toxicology Program and a member of the group that designed and monitored the studies, the potential for endocrine disruption affecting development has been a topic of interest at the NIEHS since the late 1970s, when the institute held its first conference to examine the matter. Through the 1980s and into the 1990s, accumulating research established solid biological plausibility for the idea that small perturbations in hormonal status triggered by environmental exposures could ultimately affect development.

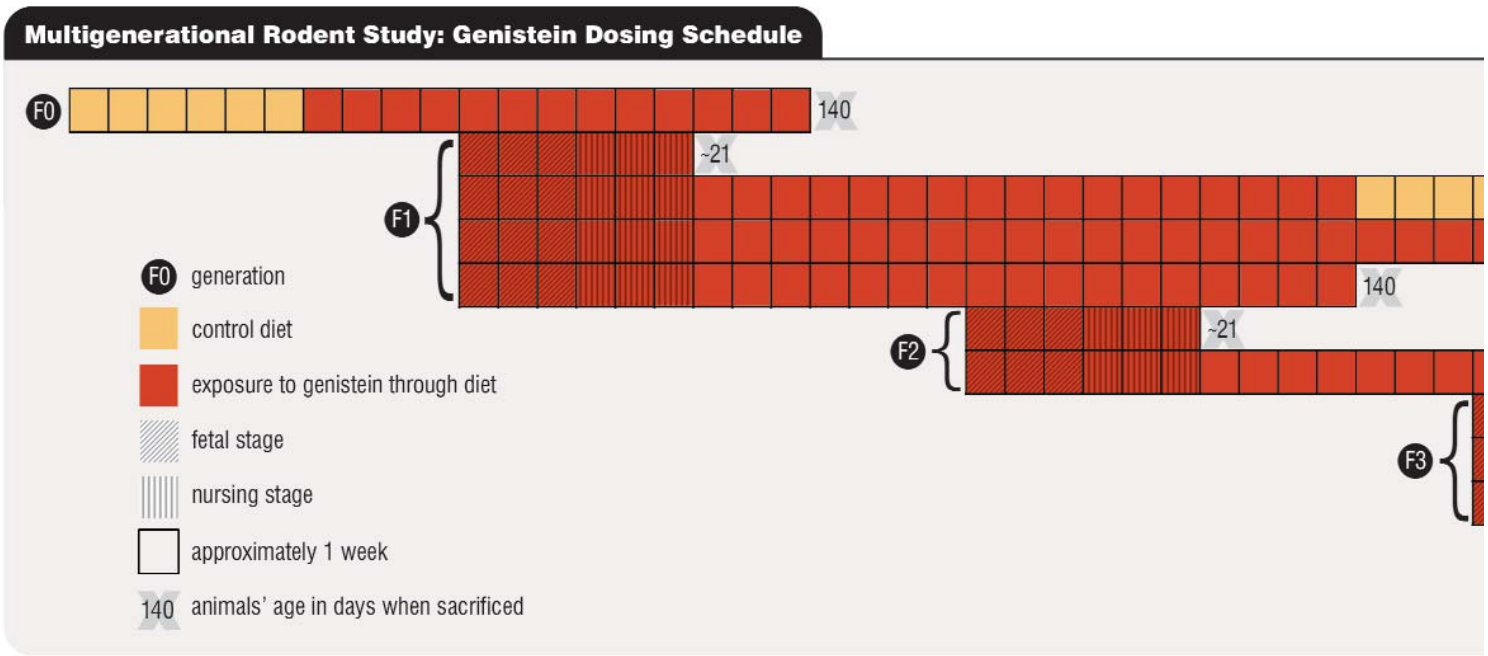
There were still many unknowns, however, according to Robert Chapin, a former NIEHS reproductive toxicologist now at Pfizer. "As is most often the case in science," he says, "there was a whole lot more that was unknown than was known about low-dose exposure to estrogenically active chemicals. There were lots of claims being made about these [chemicals] that were not biologically plausible."

Following the 1994 NIEHS-sponsored meeting "Estrogens in the Environment III," Bucher, Suzanne Snedeker (a former NIEHS scientist now at Cornell University), Chapin, and others at the NTP decided to put together a new series of experiments. The goal: to evaluate the potential of estrogenic influences during development to change developmental patterns for sexually related characteristics or hormonally mediated tumor patterns in animals as they aged. "We thought the NTP would be able to do this in a way that few other groups could, in a very comprehensive and thorough manner," says Bucher.

The NCTR's expertise in such large-scale studies and its interest in the area made it the ideal partner. This branch of the FDA conducts toxicological research to determine the human exposure to and risks of products that are regulated by that agency. With that, the NTP entered into an interagency study with K. Barry Delclos, the principal investigator at the NCTR, and Retha R. Newbold, the principal investigator at the NIEHS.

Selecting the Candidates

The researchers originally selected five chemicals for study: methoxychlor, genistein, ethinyl estradiol, *p*-nonylphenol, and vinclozolin. The first four seemed to have estrogenic properties in addition to other, unique characteristics, and their inclusion was expected to provide the opportunity



to tease out which effects could be related to estrogenicity versus the responses specific to the individual chemical.

After dose range–finding studies were completed in 2001, the researchers decided against conducting multigenerational studies on methoxychlor and vinclozolin. There were several reasons for this decision, including the fact that methoxychlor didn't exhibit enough of an estrogenic effect to justify doing the additional studies, and that vinclozolin was the only antiandrogen, with no comparison compounds being tested.

The doses of 5, 100, and 500 milligrams of genistein per kilogram per day were selected very carefully. "What we were interested in was studying a wide range of concentrations," says Bucher. "We wanted to select a top dose for the multigenerational studies that had a clear biological effect but didn't affect the animals to the extent that reproduction would be inhibited. We wanted to put the lower doses in the range of human exposures."

Studies Begin

Exposure for the parental (F_0) generation began when the animals were weaned to feed supplemented with the test compound. The feed did not contain alfalfa or soy, because both contain naturally occurring estrogenic compounds. The subsequent F_1, F_2, and F_3 generations of offspring experienced exposure to the chemicals prenatally but much less so through lactation; subsets of the F_1 and F_2 generations consumed supplemented feed upon weaning, but exposure ceased for the F_3 generation at weaning. The F_4 generation was unexposed. Subsets of animals were examined at each stage of development, and additional subsets of the F_1 and F_3 generations were used for the chronic exposure assays. "It was a long, complicated series of studies," says Bucher.

The complexity was necessary, however, because a major thrust of the studies was to test whether effects worsened over succeeding generations and whether they were reversible if exposure ended. Chapin explains, "From the public policy exposure–decision point of view, if you could show that things started to get better once you stopped exposure, that would mean that . . . if we did something about it, it would reverse any endocrine disruptor–mediated reproductive compromise that might be happening in human populations."

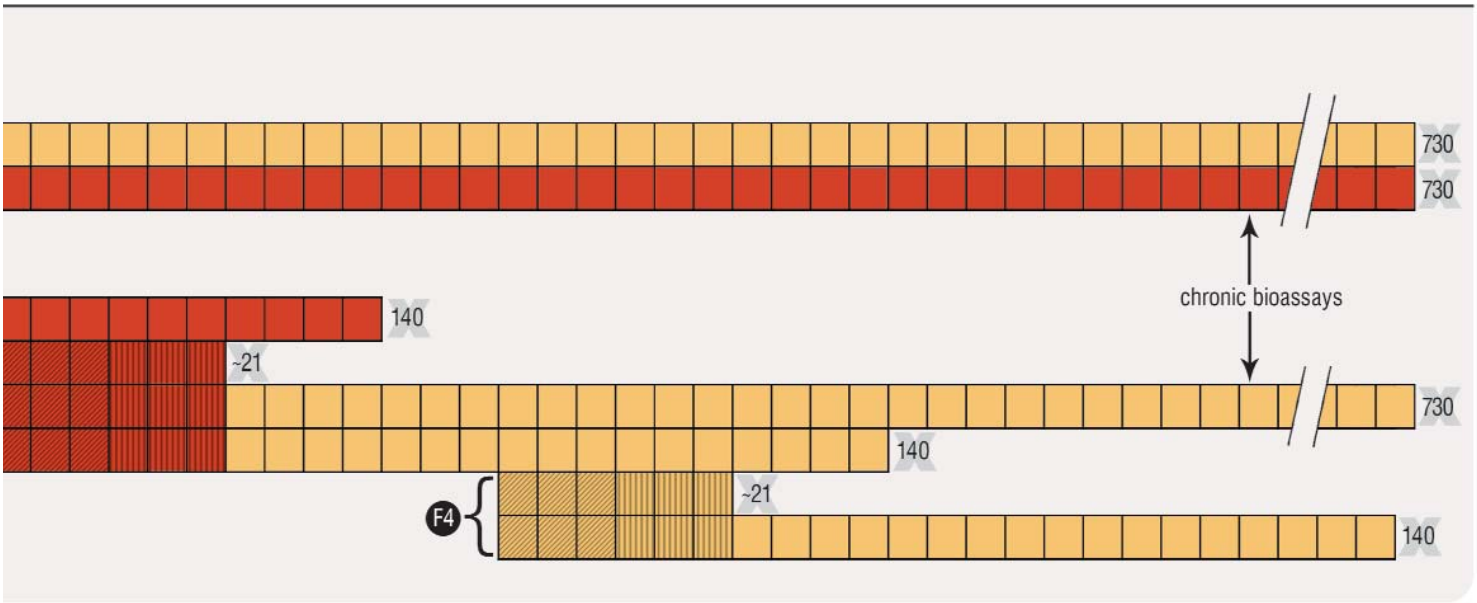
The studies could have been even more complex, according to Delclos. "I can think of a few things off the top of my head that would have been nice to do if resources were unlimited," he says. He speculates that including more dose groups would have allowed for better characterization of the dose–response curve, especially in the lower-dose region reflecting likely human exposure levels. Additionally, no one rodent model is ideal. Although the rats used in these experiments are well suited for multigenerational studies and have low background rates of some reproductive tumors, they do have high background rates of pituitary tumors and, in females, mammary tumors. This makes it more difficult to pick up on subtle changes that might be occurring.

With regard to genistein, Delclos indicates that the use of dosed feed led to very low exposure during the early neonatal period because the transfer of genistein through the milk was minimal; thus, the exposure was too low to have an effect. Overall, comparison of the pure compound with more complex soy extracts would have been of interest, to provide a better sense of the effects of real-life exposure.

Food for Thought

Nevertheless, the studies have yielded in-depth information on how mammals respond across developmental stages and through several generations to estrogenic compounds at exposures relevant to human health. Findings from different sets of experiments may ultimately be compared between test substances to elucidate common responses to estrogenic compounds.

These studies are a classic example of the value of the NTP, says Chapin. "They're much larger than any academic or even most contract research organizations would be able to accomplish, [and] they are clearly in the public interest," he says. "It's a perfect reason why we have an organization that does this kind of stuff—to do the kind of studies that are critically important for public health but don't get done anywhere else." **–Julia R. Barrett**



BEYOND THE BENCH

Bringing EXCITEment to the Classroom

Who are the scientists, public health officials, and policy makers who will monitor our relationship with the environment 20 years from now? Right now a lot of them are students in middle and high schools throughout the country. And it's a certainty that these future stakeholders will need to develop the diversity of skills required to tackle the complex issues that arise where environmental and human health intersect—skills that go beyond the practice of simple classroom science experiments. Answering this call to train is Project EXCITE (Environmental Health Science Explorations through Cross-Disciplinary and Investigative Team Experiences), an NIEHS-supported program at Bowling Green State University (BGSU) in Ohio.

Project EXCITE was developed by the Environmental Health Program in the BGSU College of Health and Human Services and the School of Teaching and Learning in the College of Education and Human Development. Under the codirection of principal investigators Chris Keil and Jodi Haney, this seven-year program seeks to raise the bar on training for the next generation of environmental health stewards by focusing on problem-based learning techniques that encourage independent critical thinking skills—or "hands-on, minds-on" learning—for 4th- through 9th-grade students. Teacher and student participants come from schools across northwest Ohio.

The strength of Project EXCITE lies in its two-tiered approach of providing comprehensive training and education to both teachers and students. For teachers, professional development is offered in a two-year "cohort" program. In each cohort, teams of four or more teachers recruited from a variety of disciplines receive training in environmental health content and in research-based best practices for teaching. The teacher teams network with agencies and scientists in their communities as well as BGSU faculty, and spend the first year of the program creating their own "Odyssey"—an interdisciplinary, problem-based curricular unit based on an environmental health science topic—which is then implemented in the classroom the following school year. The teachers receive up to 10 graduate credit hours and a stipend.

For students, learning comes as they travel through the Odysseys their teachers create. Each Odyssey, lasting up to six weeks, is formatted into four steps: Meet the Problem, Investigate and Inquire, Build Solutions, and Take Action. As students follow the steps through an Odyssey, they learn to approach and examine a problem by identifying specific environmental agents and measuring their effects on health. Additionally, students begin to understand how environmental health science research can influence community policy decisions.

"One of the greatest things about Project EXCITE is the real-world context—students explore environmental health issues that are local and are important to them," says Project EXCITE program manager Jennifer Zoffel. "They learn not only that these problems exist, but also that they as students and as community members can build solutions and take actions to minimize the impacts of the issue or educate others about it."

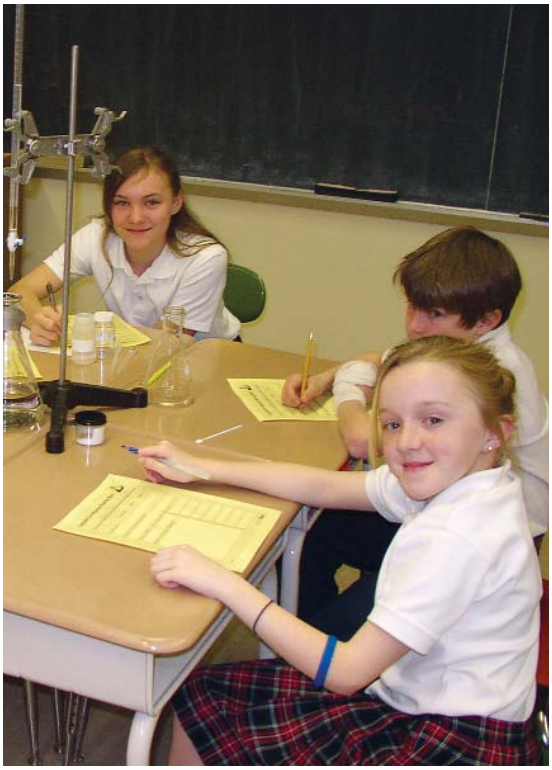
"Sick of School? Odyssey" was inspired by a group of middle school students who investigated the quality of their school's indoor environment as part of the 2001–2003 cohort. The students worked through the first three steps of the Odyssey by researching water damage, bioaerosols, drinking water quality, and elevated carbon dioxide levels in their school building. During the final Take Action step, they delivered recommendations for changes to the district principals and the school board. Two of their recommendations—to change room ventilator filters once per season rather than once per year, and to repair the leaking roof—were accepted.

An epic learning adventure. Project EXCITE offers science teachers the opportunity to craft interdisciplinary curricular units called "Odysseys," which they then carry back home to their students. Each Odyssey introduces students to a real-world environmental health issue. The students investigate the issue, devise solutions, and then take action, sometimes effecting actual changes in their own environments.

Odyssey programs created by previous cohorts are available for sale at the program website, http://www.bgsu.edu/colleges/edhd/programs/excite/. Besides "Sick of School? Odyssey," other programs currently available include "ZoOdyssey" (based on student illnesses that arise after a trip to the local zoo), "AgOdyssey" (which compares small- and large-scale farming), "Food Odyssey" (a study of food contamination in restaurants), and "ChemOdyssey" (which examines the safety of common chemical cleaners).

Educators who are unable to participate in a full two-year cohort can still take advantage of intensive two-day workshops, or "institutes." There they will receive one hour of graduate credit, funds to purchase classroom supplies, and a completed Project EXCITE Odyssey for classroom implementation.

The program, now in its sixth year, recently received the U.S. EPA's 2006 Children's Environmental Health Recognition Award—one of 30 given, and the only one awarded in the state of Ohio. New Odysseys are also in the works: among others, "GermOdyssey" will allow students to become "disease detectives" by learning about different pathogens and how they infect the body, as well as the mechanisms that the body uses to fight off these illnesses, and "Sick Ship Odyssey" will look at illnesses aboard cruise liners. **–Tanya Tillett**



Left to right: Project EXCITE; National Human Genome Research Institute

Headliners | Neurodevelopment

NIEHS-Supported Research

Genomewide Screen Reveals Candidate Genes for Neural Tube Defects

Rampersaud E, Bassuk AG, Enterline DS, George TM, Siegel DG, Melvin EC, et al. 2005. Whole genomewide linkage screen for neural tube defects reveals regions of interest on chromosomes 7 and 10. J Med Genet 42:940–946.

Neural tube defects are among the most serious and most common severely disabling forms of human birth defects. These defects—which arise from failure of the neural tube to close, an event that usually happens around day 28 after conception—are thought to be caused by a complex interaction between a person's genetic makeup and environmental factors. Now NIEHS grantee Marcy C. Speer of Duke University Medical Center and colleagues from 14 research facilities across the United States report on a nationwide collaborative effort to gain new insights into the possible sites of a neural tube defect gene or genes.

There are three major types of neural tube defects, all with devastating consequences. Nearly all children with anencephaly (the absence of a major portion of the brain, skull, and scalp) die *in utero* or shortly after birth. Children with encephalocele (in which the brain protrudes through an opening in the skull) may survive but are almost always mentally retarded. And children with spina bifida (in which the spine fails to close properly) have varying degrees of muscle weakness and sensory disorders.

The most important environmental risk factor for neural tube defects is insufficient folate consumption by the mother around the time of conception. Folate supplementation reduces the risk of neural tube defect recurrence by 50–70%, but it does not entirely eliminate the risk. This suggests underlying genetic factors, a supposition bolstered by the increased rate of recurrence in siblings and the increased risk of defects in the offspring of a person with a neural tube defect. However, studies of folate-related and other developmental genes in humans have failed to definitively identify a gene causing neural tube defects.

In the current study, the researchers identified 44 families with more than one occurrence of a neural tube defect. They extracted DNA from whole blood samples of the affected individuals and related family members, for a total of 292 samples. Then they performed both parametric and nonparametric genomic linkage analyses. The results pointed to two candidate genes on human chromosome 7 and three on chromosome 10.

The researchers expect these results will help to prioritize future studies of neural tube defect candidate genes, and they plan to add additional families to their analyses. They also want to expand the phenotypic classifications to allow for a greater sample size and integrate other data such as those from mouse models of neural tube defects. The data in the present study bring the medical community closer to the day when individual-level prediction of the risk of a neural tube defect may be possible. **–Jerry Phelps**