Vol. 98, pp. 281–286, 1992

Meeting Report

The Third United States–Japan Meeting on the Toxicological Characterization of Environmental Chemicals[1]

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This report summarizes the discussion of the Third U.S.–Japan Meeting on the Toxicological Characterization of Environmental Chemicals held under the auspices of the U.S.–Japan cooperative in research and development in science and technology. Recent data on the interrelationships between toxicity, cell proliferation, and carcinogenicity are presented.

Introduction

The third joint meeting between scientists from the National Institute of Environmental Health Sciences (NIEHS), Research Triangle Park, North Carolina, and the National Institute of Hygienic Sciences (NIHS), Tokyo, Japan, was held February 4–6, 1991. The purpose of these meetings, held under the auspices of the U.S.–Japan Agreement on Cooperation in Research and Development in Science and Technology, is to exchange information on the toxicological characterization of environmental chemicals of mutual interest (*1,2*). The emphasis of this third meeting was on the interrelationship between toxicity, cell proliferation, and carcinogenicity and on mid-term assays and assays for initiation and promoting activities.

Evaluation of Chronic Carcinogenicity in Rodents

The first part of the meeting focused on the strengths and weaknesses of the 2-year chronic carcinogenicity tests in rats and mice. Meeting participants discussed several of the recent criticisms made by Ames et al. (*3*) on the scientific value of long-term chemical carcinogenesis experiments in laboratory animals as reliable predictors of human cancer risk. These authors assert that testing chemicals for carcinogenicity at high (maximum tolerated) doses in rodents does not provide adequate information to predict the excess numbers of human cancers that might occur at low-dose exposures. Ames et al. contend that testing at the maximum tolerated dose can cause chronic cell injury, resulting in increased cell proliferation, which may ultimately result in carcinogenesis (*4*). These authors consider agents that cause cell proliferation to be the most numerous and important class of human carcinogens and argue that many chemicals that cause increases in tumor incidence in rodents do so because they are toxic at the maximum tolerated dose. Thus, the risks from exposures to lower levels in humans is greatly exaggerated, and many chemicals are being falsely identified as carcinogens.

However, a number of studies (*5–10*) offer little evidence to support this theory. An analysis of the U.S. National Toxicology Program (NTP) database (nearly 400 long-term rodent carcinogenicity studies) does not support the

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view that carcinogenesis mechanisms at high exposure levels produce results not found at lower levels (*11*). In 75% of the rat studies (50% of the mouse studies) in which chronic liver toxicity was observed, there was not a corresponding increase in the incidence of liver tumors. Similarly, ethyl acrylate, administered by gavage to F344 rats for 14 days, produced cell proliferation, hyperkeratosis, ulceration, and inflammation of the forestomach; however, when this compound was administered to rats for 13 weeks and the animals were held for 22 months, the forestomachs appeared normal. Evidently, the forestomach lesions produced after 13 weeks of exposure to ethyl acrylate were not sufficient to elicit a carcinogenic response. An analysis of existing data shows that some chemicals can induce cancer without significant toxic effects and cellular proliferation, whereas others induce toxicity and cellular proliferation without inducing cancer (*12*). Unless it can be shown more definitively that enhanced cell proliferation resulting from exposure to nongenotoxic carcinogens is responsible for their carcinogenicity, it is inappropriate to overemphasize the use of cell proliferation data in the risk assessment for human cancer. Procedures used to extrapolate animal carcinogenicity data to human risks from exposure to lower doses of nongenotoxic chemicals should not be amended until the carcinogenic mechanisms of these compounds are more clearly understood.

The experimental design (duration and frequency of dosing) of carcinogenicity studies was also discussed. In the NTP, rats and mice are exposed for 2 years. The guidelines of the Organization for Economic Cooperation and Development (OECD), the Environmental Protection Agency (EPA), and Japan recommend 2 years in rats, and 1.5 years or more in mice. A 1–3 month recovery or withdrawal period after 2-year treatment is also recommended in the Japanese guidelines. During this withdrawal period, a nonneoplastic hyperplasia related to treatment may regress, whereas hyperplasia regarded as preneoplastic will persist. For extremely toxic chemicals, it may be difficult to expose at high doses for 2 years due to high mortality. To address this problem, Japanese scientists proposed a "discontinued protocol" design, which was used in the induction of renal cell tumors in rats by potassium bromate (*13*) and of liver and urinary bladder tumors by 2-acetylaminofluorene (*14*). In both instances, a higher dose given for a shorter period of time induced more tumors than a lower dose given for a longer period. Participants concluded that the duration/frequency/rate of dosing needs to be evaluated on a chemical-by-chemical basis. Additional data are needed before making generalized changes in experimental design.

The meeting participants reiterated and supported the concept that in the absence of epidemiological data, long-term rodent studies are still currently the best available method for evaluating a chemical's carcinogenic potential and for extrapolating the results to humans. They agreed with the conclusions of the International Agency for Research on Cancer (IARC) that in the absence of adequate data for humans, it is prudent to regard agents for which there is sufficient evidence of carcinogenicity in experimental animals as if they presented a carcinogenic risk to humans. It was also recommended that in certain instances, ancillary tests to determine mechanisms of action or answer specific scientific questions might also be incorporated into the 2-year tests. Examples might include "stop-start" exposure studies to evaluate progression, regression of induced lesions; intermittent exposure studies; cellular proliferation studies; studies of oncogene activation; and measurements of DNA damage and adduct formation. Carcinogenesis is a multistage process, and, ultimately, it is the underlying mechanism of carcinogenesis in animals and its relevance to humans that must be determined.

Cell Proliferation

As was mentioned previously, the role of cell proliferation in chemical carcinogenicity has been the focus of considerable debate recently. Cell proliferation has long been implicated as having a critical role in both the initiation and promotion stages of chemically induced cancer. Recently, however, chemically induced, sustained cell proliferation has been implicated as leading to preneoplasia or neoplasia by increasing the probability of spontaneous mutations and has been implicated as the major cause of cancer by nongenotoxic chemicals in animal carcinogenicity studies (*4*). Contrary to this view, cell proliferation per se is not carcinogenic (*15*). A review of the literature on the relationship between chemically induced cell proliferation and carcinogenesis reveals that very few cell proliferation studies have been conducted over extended exposure periods. Moreover, the proliferation response resulting from exposure to many nongenotoxic carcinogens is not well sustained, whereas the elicitation of a carcinogenic response by these chemicals often requires a prolonged exposure duration. Thus, adequate data are not available to support the hypothesis that chemically induced cell proliferation causes cancer.

The NTP has established staining techniques to identify cells undergoing replicative DNA synthesis in histologic tissue sections. Using osmotic minipumps to deliver the labeling agent bromodeoxyuridine, labeling indexes have been measured in several studies. While a tentative relationship between enhanced replicative DNA synthesis (cell proliferation) has been documented in 90-day studies with some carcinogenic versus noncarcinogenic isomers, these studies must be interpreted only as preliminary evidence of an association between enhanced cell proliferation and carcinogenesis in rodents. In the ethyl acrylate studies, dramatic enhanced cell proliferation sustained for 90 days is insufficient to lead to induction of forestomach neoplasia. No enhanced cell proliferation was found in the subchronic and chronic methylene chloride inhalation studies, which would explain the excess liver tumors induced by this chemical. These results are interpreted as evidence that factors other than sustained enhanced cell proliferation must be invoked to explain the observed carcinogenicity for many rodent carcinogens. It is also apparent from these studies that a variety of biological factors must be considered in the design, conduct, and interpretation of cell proliferation studies in rodent bioassays.

Toxicity As a Predictor of Carcinogenicity

There is a lack of adequate data on the relationships between chemically induced toxicity, cell proliferation, and carcinogenicity. The analysis of 2-year carcinogenicity studies of 99 chemicals in rats and mice by Hoel et al. (*11*) revealed that only 7 of the 53 positive animal carcinogens showed a clear link between organ toxicity and tumor development. For the other 46, cancers occurred in organs with no apparent toxicity, and visibly damaged organs had no tumors. More recently, NTP investigators found similar results when analyzing another set of chemicals to determine whether subchronic and chronic toxicity can predict carcinogenicity in the same organ. Subchronic toxicity, chronic toxicity, and carcinogenicity, with respect to site and morphology, were reviewed from a group of 31 chemicals that were recently evaluated by NTP. This group of 31 chemicals (22 carcinogens and 9 chemicals with no evidence of carcinogenicity) was chosen from studies evaluated by the NTP over a specified period of time but was otherwise not a preselected group. In this review, a variety of subchronic and chronic toxic lesions were identified with 30 of these chemicals in at least 18 different organs or tissues. The administration of both genotoxic and nongenotoxic chemicals resulted in toxic injury at a number of sites in which no carcinogenic response was observed. Furthermore, for some genotoxic chemicals, carcinogenic responses occurred at sites in which no subchronic or chronic toxicity was observed. Although subchronic toxicity and subsequent carcinogenic response in the kidney was more often seen with nongenotoxic chemicals, both genotoxic and nongenotoxic chemicals resulted in toxicity at a number of organ/tissue sites in which no carcinogenic response occurred.

In some instances (e.g., 1-4-dichlorobenzene), target organ toxicity may occur with carcinogenicity. However, it is an oversimplification to assume that toxicity can account for all carcinogenicity. The many different sites and types of carcinogenic effects observed, coupled with the diverse structures of chemicals, makes it impossible to make global statements about mechanisms of action or generic statements about structure–activity relationships.

Mid-Term Assays

The latter half of the meeting was devoted to a discussion of additional approaches for detecting carcinogenic effects without conducting the conventional 2-year bioassay studies. A number of studies have defined distinct stages in the carcinogenic process, and several experimental models have been developed in an attempt to identify agents that may affect these different stages. The status of a number of these experimental models and their usefulness in carcinogenicity testing were reviewed. It was concluded that although these assays are limited to effects on single organs or involve administration in conjunction with other chemical substances, they can measure the potential contribution of the test substances to the carcinogenic process. Thus, they are very useful to elucidate the mechanisms of carcinogenicity and setting priorities for further testing needs.

Limited Screening Model Systems

The utility of the strain A mouse pulmonary tumor assay and IV or SC injection assays were evaluated. It was concluded (*16*) that the strain A results were discordant with 2-year carcinogenicity test results for detecting carcinogenic activity of aromatic amines and several other miscellaneous chemicals. Data on the usefulness of IV and SC routes of exposure for detecting the carcinogenic potential of nitrosamines (*17–19*) and the ability of adriamycin to induce mammary tumors (*20*) was also presented. An analysis of the *IARC Monographs* has also revealed a high concordance between distant tumors from SC injections and distant tumors from other routes of exposure.

Assays for Initiating and Promoting Activities Including Early Preneoplastic Lesions

The fate of preneoplastic lesions cannot be predicted unequivocally and thus preneoplastic lesions alone are not definitive indicators of carcinogenicity. The detection of such lesions, however, may be useful for helping to elucidate the mechanisms of carcinogenicity, for predicting potential target organ neoplasia, and for determining which chemicals need more extensive testing. Putative preneoplastic lesions in several organ systems were discussed briefly to illustrate current research efforts and identify future research needs.

Skin

Data were presented on the initiating activities of a variety of chemicals in the two-stage mouse skin carcinogenesis model (*21–24*) using 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as the promoter. All compounds were topically applied twice weekly on the dorsal skin of male mice, and then followed by similar TPA administration for 2 weeks. The compounds, 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (AF-2) and butylated hydroxyanisole showed an initiating potential, but butylated hydroxytoluene and quercetion did not. Nine heterocyclic amines isolated from amino acid pyrolysates were examined similarly, only Trp-P-2 was found to have an initiating action.

Liver

The liver is a target organ in more than 50% of the chemicals so far evaluated for carcinogenicity by IARC and the National Cancer Institute (NCI)/NTP. Therefore, a medium-term bioassay, the dimethylnitrosamine-partial hepatectomy (DEN-PH) model, has recently been developed in Japan to detect liver carcinogens in a short period using male F344 rats (*25*). Two weeks after IP administra-

tion of DEN (200 mg/kg), test compounds are given orally for 6 weeks; partial hepatectomy is conducted at week 3. The animals are killed at week 8, and the liver sections are subject to the immunohistochemical staining to examine the number and areas of glutathione *S*-transferase placental form (GST-P)-positive foci. To date, 95% of the known liver carcinogens, but only 22% of the nonliver carcinogens, have been found to be positive by this model. The DEN-PH model can detect both hepatocarcinogens and chemicals having an inhibitory effect on liver carcinogenesis, irrespective of their genotoxicity, in a limited time using a small number of animals. Observations on altered hepatocellular foci (AHF) in conventional 2-year NTP carcinogenicity studies also demonstrated that hepatocarcinogens may induce unique types of AHF in rats. Such changes are potentially useful predictors of hepatic neoplasia (*26*).

Forestomach

When administered by gavage in corn oil for 2 years, ethyl acrylate induces benign and malignant forestomach neoplasia in both sexes of rats and mice. The initial reaction of the forestomach to such treatment is development of a pronounced and persistent mucosal hyperplasia. This has led to the postulation that the observed forestomach carcinogenicity of ethyl acrylate is secondary to sustained enhanced cell proliferation. In a recently completed study, ethyl acrylate was given by gavage at carcinogenic doses for 90 days, at which time there was clear evidence of mucosal hyperplasia. When rats were held without further treatment for an additional 19 months, the hyperplasia completely regressed and no forestomach neoplasia was observed. It was concluded that sustained, enhanced forestomach mucosal hyperplasia for 90 days was insufficient to induce neoplasia at this target site. Additional studies with this chemical are underway to determine if treatment will lead to induction of forestomach neoplasia.

Pancreas

The morphogenesis of pancreatic carcinomas has been studied extensively in rats and hamsters. Male SD rats were initiated with a single intravenous treatment of 4-hydroxyaminoquinoline-*N*-oxide (4HAQO; 7 mg/kg) and after 1 week were given soybean trypsin inhibitor (SBTI) in the diet (5 and 10%) for 51 weeks. SBTI treatment was found to result in a significant increase in numbers of eosinophilic nodules and a decreased number of basophilic foci. In studies on ductal cell pancreatic carcinogenesis, male hamsters were initiated with five SC injections of *N*-nitroso*bis*(2-oxopropyl)amine (10 mg/kg) and within 4 weeks were simultaneously given 5% SBTI in the diet. After 26 weeks on a basal diet, the numbers of adenocarcinomas and dysplastic foci were significantly suppressed. However, the effect was much weaker when the SBTI diet was given for 37 weeks after initiation. Therefore, SBTI showed an enhancing effect on the promotional stage of acinar cell carcinogenesis and an inhibitory effect on the initiating stage of ductal cell carcinogenesis.

Upper Respiratory Tract

The promoting effect of cigarette smoking was examined using the two-stage laryngo-tracheal carcinogenesis model in hamsters following initiation by a single injection of diethylnitrosamine. The incidences and numbers of papillomas and hyperplasias of the upper respiratory tract and lung lipid peroxidation levels were significantly increased (*27*).

Kidney

A dose-related enhancing effect of potassium bromate on renal tumorigenesis in rats initiated with *N*-ethyl-*N*-hydroxyethylnitrosamine was reported (*28,29*). The mean numbers of kidney dysplastic foci were significantly increased in a dose-related manner in rats treated at doses higher than 30 ppm; the mean number of renal cell tumors was significantly increased at 500 ppm. Potassium bromide, a degradation product, was found to be negative. Studies on five metal compounds (Zn, Hg, Cr, Cd, Ni) using the same two-stage renal carcinogenesis protocol showed that nickel chloride exerted a potential for promotion of renal tumorigenesis (*30*).

Urinary Bladder

Sodium ascorbate, sodium carbonate, and ascorbic acid were found to act as promoters in a two-stage urinary bladder carcinogenesis model in male rats initiated with *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine. An increased incidence of transitional cell carcinomas was observed, and a positive correlation was found between the levels of pH and sodium ion in the urine and the promoting effects of chemicals (*31–33*).

Female Genital, Nervous, and Hematopoietic Systems

Two-stage carcinogenesis assays have not been fully established for the female genital, nervous, and hematopoietic systems because detection of early preneoplastic changes remains difficult, and proper enzymatic markers showing these changes are generally lacking.

Update on National Institute of Hygienic Sciences Activities

The results of the carcinogenicity tests on eight chemicals recently finished at NIHS were presented. Dicofol, an organochlorine pesticide structurally related to DDT, was given to male $B6C3F_1$ mice (50/group) at various doses in feed for 80 weeks. The incidence of liver tumors in groups fed 500, 300, 200, 100, 50, and 0 ppm was, respectively, 85% ($p < 0.01$, 80% ($p < 0.01$), 71% ($p < 0.01$), 34%, 18%, and 25%. Histologically, most of the tumors were diagnosed as hepatocellular adenomas and carcinomas with some cases of hepatoblastomas and hemangiomas. The virtually safe dose (VSD) at risk level of 10^{-6} was 0.191 μg/kg/day by the Weibull model.

Sodium orthophenylphenate (OPP-Na), an antifungal agent, was given at various doses in feed to male F344 rats (50/group) for 104 weeks. The combined incidences of papillomas and transitional cell carcinomas of the urinary bladder were significantly increased in rats fed 1.5 and 2% OPP-Na. The VSD at risk level of 10^{-6} was 147 ppm in the diet by the Weibull model.

Musk xylol, a synthetic nitro musk, was administered at dietary levels of 0.15 and 0.075% to groups of 50 male and 50 female $B6C3F_1$ mice for 80 weeks (*34*). Combined incidences of malignant and benign liver cell tumors were clearly increased in both sexes, and in males a positive significant trend was also noted for the occurrence of hepatocellular carcinomas. In males, the incidence of Harderian gland tumors was also significantly increased. It was concluded that musk xylol is carcinogenic in mice.

6-Mercaptopurine (6-MP), an anticancer agent, was administered to F344 rats of both sexes (50/group) at dietary levels of 50 and 25 ppm for 104 weeks (*35*). In females, the incidence of C-cell tumors of the thyroid and pheochromocytomas of the adrenals in the 50 ppm group were significantly increased. However, considering the fact that the rates for spontaneous tumors in this test were lower than those of historical controls, it was concluded that the carcinogenicity of 6-MP was very weak or marginal.

5-Fluorouracil (5-FU), an anticancer drug, was given to F344 rats of both sexes (50/group) at doses of 125 and 62 ppm in the drinking water for 104 weeks. There were no significant increases in the incidences of any tumors when compared to those of controls, and it was concluded 5-FU was not carcinogenic in rats. However, because the maximum tolerated doses used in these tests were very close to the clinical doses used, the true carcinogenic potential of 5-FU and 6-MP remains unknown.

Phenytoin, an anticonvulsant, was administered to groups of 50 male and 50 female F344 rats in the diet at doses of 0.05 and 0.25% for 104 weeks. No significant increases were observed in the incidences of any particular type of tumors in the treated groups of either sex. It was concluded that phenytoin was not carcinogenic in rats.

Calcium lactate, a food additive, was given to F344 rats of both sexes (50/group) in the drinking water at the concentrations of 5 and 2.5% for 104 weeks (*36*). The rats were killed after an 8-week recovery period. There were no significant increases in tumor incidence related to the treatment, and it was concluded that calcium lactate was not carcinogenic in rats.

Monosodium succinate, a food additive, was given in the drinking water at levels of 2 and 1% to F344 rats of both sexes (50/group) for 104 weeks, and animals were killed in week 112 (*37*). Although an increase in the incidence of C-cell tumors of the thyroid was found in high-dose group females, it was probably a function of experimental variability and not related to the treatment. It was concluded that monosodium succinate had no carcinogenic activity in rats.

The chemicals currently being tested in rats (F344 or Wistar) at the NIHS are: 2,2,3,3,3-pentafluoropropanol, 2,4,5-triamino-1,3,5-triazine (Melamin); *p*-dichlorobenzene; 2,2′-methylene*bis*-(4-methyl-6-*tert*-butylphenol); diisopropylbenzene; cyclodextrin; stevioside, *n*-paraffin; and potassium iodide.

Update on Chemical Substances Control Act in Japan

Under the Chemical Substances Control Act, adopted in 1973 and revised in 1987, new and existing chemicals have been classified into class-1 specified chemical substances, class-2 specified chemical substances, and designated chemical substances based on their properties of biodegradation, bioaccumulation, and chronic toxicity (*38*). Since the previous report (*1*), *bis*(tributyl)tin oxide has been added to the list of class-1 specified chemical substances. The survey on the mutagenic and toxicological profiles of 218 new chemical substances that were evaluated by the Safety Evaluation Committee of the Ministry of Health and Welfare was presented. Out of the 218 new chemicals, 60 (28%) were classified as designated chemical substances. With respect to mutagenicity, 20 (9%) and 60 (28%) chemicals were positive in the Ames and chromosome aberration tests, respectively. The results of the 28-day repeated dose study with a 14-day recovery period showed that the liver and the kidney were the main target organs. In general, chemicals whose no observed effect levels were lower than 20 mg/kg/body weight and which showed positive mutagenic activity were classified as designated chemical substances.

Reproductive Familiarization Study

The OECD has recently proposed a new guideline, "Combined Repeat Dose and Reproductive/Development Toxicity Screening Test (Repro/Tox)," for rapid screening of high-production-volume chemicals (*39*). NIHS scientists have become familiarized with this novel and unique protocol, designed to detect general toxicological effects simultaneously with reproductive/developmental effects. Studies were conducted on cyclosphosphamide (CP), selected as a reference compound, according to the test guideline. As a result, most of the known systemic and reproductive/developmental toxicological effects of CP were demonstrated by this test. Therefore, Repro/Tox can be considered as a useful rapid screening test for high-production-volume chemicals (*40*).

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