Toxicology Testing Requirements and the U.S.-Japan Collaborative Study on \textit{In Vitro} Tests for Chromosomal Aberrations

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Introduction

A meeting was held April 2–6, 1990, between scientists from the U.S. and Japan (see the appendix for list of participants) as part of the U.S.–Japan Agreement on Cooperation in Research and Development in Science and Technology. Japanese and American scientists met in North Carolina to exchange information on toxicology testing requirements and guidelines in the two countries and to review progress in a collaborative study on detection of chemically induced chromosomal aberrations in cultured mammalian cells.

Regulatory Requirements for Toxicological Studies

The first 2 days of the meeting focused on regulatory requirements and on continuing efforts to assess the value and role of short-term \textit{in vitro} and \textit{in vivo} genetic toxicity tests to identify chemicals that might present a health hazard to humans.

U.S. testing requirements and guidelines were presented by D. Jacobson-Kram. It was pointed out in his talk that the different regulatory agencies in the U.S. take different approaches to the use of short-term tests; the tests required or recommended, as well as the structures of the testing schemes, vary among agencies as well as among divisions within an agency. An encouraging activity, albeit somewhat complicating at the present, is the review and restructuring of testing guidelines by some regulatory agencies.

M. Ishidate, Jr., presented the genetic toxicity tests and test schemes required by various ministries in Japan; Y. Kurokawa gave a similar overview of all toxicity testing requirements in Japan. The situation in Japan is very similar to that in the U.S. in that a variety of public health laws have led to a range of required tests and testing schemes among the ministries responsible for public health.

Despite some differences in the required or recommended tests in the two countries, the underlying concerns for human health are the same. Cancer and genetic disease are the primary concerns to which the application of genetic toxicity tests are directed.

Evaluation of Chemical Genetic Toxicity

In the session on evaluation of chemical genetic toxicity, E. Zeiger reviewed the course of short-term testing in the U.S. National Toxicology Program (NTP). Current testing activities \textit{a}) employ a smaller number of \textit{in vitro} tests than the four previously used, \textit{b}) include the prudent use of short-term \textit{in vivo} tests, particularly the micronucleus test, and \textit{c}) place increased emphasis on integrated studies designed to characterize and understand the genetic toxicity of specific chemicals rather than the mass screening of chemicals. M. Ishidate stated that, in Japan, expert committees review mutagenic potency information to help categorize the level of risk that might be associated with a mutagenic chemical and to determine additional testing needs.

Results of mutagenicity studies using new strains of Salmonella that have been engineered to have increased levels of nitroreductase and acetyltransferase were presented by T. Nohmi. The new strains have proven very sensitive to low levels of aromatic amines and nitroaromatics. M. Shelby presented comparisons of \textit{in vitro} and \textit{in vivo} short-term test results from NTP-sponsored studies. With regard to rodent carcinogens, levels of con-
cordance of short-term in vivo tests were similar to the concordances of in vitro short-term tests, i.e., 60 to 75%. Shelby suggested caution in attempting to predict carcinogens based strictly on the results of routine short-term testing and pointed out the importance of conducting chemical-specific studies where routine tests do not present clear evidence of genetic toxicity.

The final two presentations in this session were on the topic of nongenotoxic carcinogens. Y. Kurokawa presented the results of a series of experiments on the formation of 8-hydroxydeoxyguanosine in the DNA of rodents treated with carcinogens, particularly those carcinogens that are thought to act through oxidative damage to DNA. He emphasized the need for studying the relationships between oxidative DNA damage and cell proliferation. Kurokawa noted, based on the results of recent NTP cancer studies and the criteria currently used to select chemicals for such studies, a substantial increase in the number of nongenotoxic rodent carcinogens is inevitable. R. Tennant reviewed activities and plans in the NTP to develop methods for detecting chemicals that might be nongenotoxic carcinogens. He discussed the variety of chemical structures contained within this category of carcinogens and reviewed a range of mechanisms by which they might cause neoplasia, mechanisms such as cell proliferation, alterations in transcription, or oncogene activation.

### CHL-CHO Collaborative Study

The session on the CHL-CHO (Chinese hamster lung-Chinese hamster ovary) Collaborative Study started with presentations of the protocols that are being used in the CHL-CHO chromosome aberration collaborative study. T. Sofuni presented the details of the protocol used to test for induction of chromosomal aberrations in CHL cells in Japan. B. Anderson presented details of the CHO chromosomal aberration assay used by the NTP.

The progress in a collaborative study to investigate the differences in test results obtained in the in vitro chromosomal aberration test required in Japan (CHL system) and the CHO test system used by the NTP was reviewed by Sofuni. Results of a testing comparison carried out by the NIH (National Institute of Hygienic Sciences) and the NTP on 25 chemicals (Table 1) have been published (2). Following submission of that manuscript, other laboratories in the U.S. and the U.K. expressed interest in participating in further efforts to define the reasons why the two test systems do not always yield the same results. The goal is to devise a more internationally acceptable system for detecting the induction of chromosomal aberrations in cultured mammalian cells.

The last 2 days of the meeting were chaired by M. Ishidate and S. Galloway and were conducted as roundtable discussions of several issues related to the conduct of in vitro aberration assays. These issues included the determination of cytotoxicity and selection of test doses, treatment and harvest times, metabolic activation systems, negative and positive controls, and scoring and classification of aberrations.

### Metabolic Activation

S. Galloway discussed results of her metabolic activation studies using CHO cells to compare the S9 conditions from the CHL assay and CHO assay protocols. With regard to treating cells in serum-free or serum-containing medium, she noted that the protein concentration in medium containing 10% serum could be as much as seven times that in serum-free medium containing only S9 mix. In tests for sister chromatid exchange (SCE) induction with dimethylnitrosamine (DMN), benzo(a)pyrene (BP), and cyclophosphamide (CP), the presence of serum did not change the responses to DMN or BP but markedly suppressed the level of SCE induced by CP. Serum suppressed the induction of chromosomal aberrations by BP and CP but did not affect the response to DMN.

Galloway also pointed out that in current studies, she uses S9 induced with phenobarbital plus β-naphthoflavone. With this S9, the response of the positive control is not different from that obtained with Aroclor 1254-induced S9 used for the NTP studies. In her laboratory, the incidences of control cells with aberrations was the same with or without S9 (about 2% cells with aberrations) regardless of whether the enzyme inducer was Aroclor or phenobarbital plus β-naphthoflavone.

### Amount of S9 and Cofactors

With regard to the volume of S9 mix used in treatment medium (15 μL/mL is typically used in the CHO system, while the CHL protocol calls for 50 μL/mL). S. Galloway found that 15 μL/mL is more effective than 50 μL/mL with CP, whereas for DMN, 50 μL/mL gives a higher response than 15 μL/mL; the effect of BP was slightly higher with 50 μL/mL. Her studies with S9 cofactors indicated that only small differences resulted from the differences in cofactors or their concentrations used with CHO and CHL protocols. The volume of S9 appears to be more important than the concentrations of cofactors but, as noted above, no single volume can be considered optimal for all chemicals.

### Table 1. Chemicals tested for induction of chromosomal aberrations in CHL and CHO cells (2).

| Chemical name                       | CAS no.      |
|-------------------------------------|--------------|
| 1. 2,3,4-Trichlorophenol            | 15950-66-0   |
| 2. 2,3,6-Trichlorophenol            | 933-75-5     |
| 3. 3,4,5-Trichlorophenol            | 609-19-8     |
| 4. 2,3,4,5-Tetrachlorophenol        | 4901-51-3    |
| 5. 2,3,4,6-Tetrachlorophenol        | 58-90-2      |
| 6. 2,3,5,6-Tetrachlorophenol        | 935-95-5     |
| 7. ω-Phenyleedianiline              | 95-54-5      |
| 8. m-Phenyleedianiline              | 108-45-2     |
| 9. 2,6-Toluenediamide dibrochloride | 1548170-6    |
| 10. N,N-Dimethyl-p-phenylenediamine| 99-98-9      |
| 11. N,N,N',N'-Tetramethyl-p-phenylenediamine | 100-22-1 |
| 12. N,N-Diethyl-p-phenylenediamine  | 93-05-0      |
| 13. N,N'-Di-sec-butyl-p-phenylenediamine | 101-96-2 |
| 14. N,N'-Diphenyl-p-phenylenediamine| 74-31-7      |
| 15. N,N'-Di-2-naphthyl-p-phenylenediamine | 93-46-9 |
| 16. N-Phenyl-1-naphthylamine        | 90-30-2      |
| 17. N-Phenyl-2-naphthylamine        | 135-88-6     |
| 18. p-Isopropoxydiphenylamine       | 101-73-5     |
| 19. 4,4'-Dimethoxydiphenylamine     | 101-70-2     |
| 20. 4,4'-Dicytodiphenylamine        | 101-67-7     |
| 21. N-Nitrosodiphenylamine          | 86-30-6      |
| 22. Tris(2,3-epoxypropyl)isocyanurate| 245-15-9     |
| 23. Triallyl isocyanurate            | 1025-15-6    |
| 24. Chromium carbonyl                | 13007-92-6   |
| 25. l-(1,2-Dibromoethoxy)-3,4-dibromocyclohexane | 3322-93-8 |
Exposure Time

S. Galloway compared two incubation times with S9, 6 hr as in the CHL protocol, and 3 hr (the CHO NTP protocol used a 2 hr treatment with S9). In this comparison, S9 exposure was in medium containing serum. With DMN, higher SCE frequencies were obtained with 6 hr exposure, but for CP and BP there was little improvement in SCE yield, and toxicity and cell cycle delay were more marked at 6 hr than at 3 hr, particularly for BP. There was general agreement that treatment with S9 should be longer than the 2 hr used in the NTP protocol and perhaps not as long as the 6 hr used in the CHL system. She also concluded that at least for some chemicals (e.g., triallyl isocyanurate), the difference in results with S9 between CHL and CHO tests could be attributed to the length of time between treatment and cell harvest rather than differences in S9 exposure conditions.

For CHO cells, Galloway is now using a 3-hr treatment time both with and without S9 and a 20-hr harvest as her standard test. She presented data showing that following a 3-hr treatment, the percentage of aberrant cells and the aberrations per cell do not peak at the same time but that a 20-hr harvest reliably detected an effect for a wide variety of chemicals if the doses were properly chosen.

M. Ishidate pointed out that the main differences between the CHL and CHO protocols are treatment time and volume of S9. In the CHL system, continuous treatment with S9 mix for 24 and 44 hr ensures treatment of cells in all stages of the cell cycle and might detect some chemicals for which CHL cells have a low level of metabolic activation capability. The choice of a 6-hr incubation time with S9 in the CHL system was based on the observation that treatment with S9 of monolayer cultures for 6 hr was more sensitive than treatment in suspension for 3 hr.

Preliminary Results of Cell Type Comparisons

R. Marshall presented results of studies at Hazleton Microtest (U.K.) wherein 4 of the 25 NTP/NIHS chemicals were tested in CHL, CHO-WBL (used in NTP tests), CHO-UK, and human lymphocytes for aberration induction. Three of these chemicals were phenylenediamines; the fourth was triallyl isocyanurate. All were positive in CHL and negative in CHO in the NTP/NIHS study (2). In all cases, cells were treated without S9 for 20 and 44 hr and harvested at these times. N,N-diethyl-p-phenylenediamine and N,N-diphenyl-p-phenylenediamine were positive in all four of Marshall’s test systems including the CHO cells. He noted considerable variability in the dose levels that led to mitotic inhibition and aberration induction among the four cell types. N,N-di-2-naphthyl-p-phenylenediamine was negative in all four cell systems, but the doses tested were much lower than those found positive at NIH. Triallyl isocyanurate was weakly positive in human lymphocytes only. The reason for Marshall’s use of lower doses was to remain within the range of solubility of the test chemicals. These results again suggest that a harvest time greater than the approximately 12 hr used by NTP is necessary to detect the induction of aberrations by some chemicals.

Discussions following Marshall’s presentation led to a consensus that tests could be conducted over a dose range that included precipitating doses but that doses without precipitate should be included. Also, T. Sofuni agreed to use a final concentration of dimethyl sulfoxide of 1% (v/v) rather than 0.5% as currently used. This would be consistent with the CHO protocol and should permit more comparable dosing between the two systems. Dimethyl sulfoxide will be the solvent used for all chemicals in the collaborative study.

Sofuni presented preliminary results of cell-stage analyses (M1, M2, etc.) with bromodeoxyuridine on two chemicals using both CHL and CHO cells. His results show remarkable cell cycle delay in cells treated with higher doses of the test chemicals for 24 and 48 hr.

Scoring Aberrations

A major issue of concern has been that the different results obtained with the CHL and CHO systems might arise from the use of different scoring conventions in the U.S. and Japan. To help resolve this issue, photographs and microscope slides were reviewed at the meeting. In the end, it appeared that there is agreement on scoring most categories and major aberrations. The primary difference that exists is in the scoring of acentric lesions (gaps) and chromatid breaks. T. Sofuni and M. Ishidate ignored many of the small unstained regions (less than the width of a chromatid). The other participants record these regions (less than the width of a chromatid) as gaps but do not include them in the evaluation of results. The Japanese record as gaps and include in their evaluation of results many of the large, unstained regions that the other investigators considered chromatid breaks by virtue of their length or slight displacement. Despite the difference in nomenclature, there is apparently only one class of chromosome damage that might be scored differently by investigators in the two countries. Unstained (or very lightly stained) regions of a chromosome that are longer than the width of a chromatid, are not spatially displaced, and may or may not appear to contain chromosomal material would be scored a “gap” by the Japanese investigators and a “break” by the other investigators.

The result of this work session was a much better understanding of the scoring conventions in the two countries, and, although some disagreements still exist, everyone understands the different scoring criteria. It was suggested that in subsequent work in this collaborative study, participating laboratories may want to note on their score sheets breaks or gaps that would be scored differently by another laboratory. This would make comparison of results easier when the testing is completed.

Plans for Collaborative Study

In further comparative studies, four tests will be conducted on selected chemicals; CHL cells using CHL protocol, CHL cells using CHO protocol, CHO cells using CHO protocol, and CHO cells using CHL protocol. Protocol differences that were not discussed extensively at this meeting but which are of importance in the comparative study are listed in Table 2.

| CHL protocol | CHO protocol |
|--------------|--------------|
| Cells seeded 3 days before test | Cells seeded 24 hr before test |
| No change of medium | Fresh medium at time of treatment |
| Medium contains calf serum | Medium contains fetal calf serum |
| 6 hr, -S9 control in +S9 test | No such control |
In other studies, a set of slides to be read by each of the participating laboratories will be circulated to permit an assessment of interlaboratory differences in cell selection and scoring as well as information on how different laboratories score the same cells; bromodeoxyuridine will be used to determine the metaphase (M1, M2, etc.) in which aberrations are scored; and the effects on growth kinetics of calf serum on CHO cells and of fetal calf serum on CHL cells will be investigated to determine if the effects are large enough to justify adjustment of harvest times.

Appendix
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REFERENCES

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