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# Development of Screening Tests for Aneuploidy Induction by Environmental Pollutants

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**When legally required mutagenicity testing of chemicals is undertaken, the important genetic end point of aneuploidy is not included because validated test methods are lacking. Therefore, the Commission of the European Communities (CEC) has funded a research program to develop and validate tests for aneuploidy induction. Ten chemicals, selected on the basis of their ability to interact with cell organelles relevant for aneuploidy induction, were tested in 11 laboratories. The assays ranged from *in vitro* tubulin assembly studies to *in vivo* germ-cell tests.**

**The results allow several conclusions: *a*) Fungal aneuploidy tests are not capable of detecting inhibitors of mammalian tubulin polymerization such as colchicine and vinblastine. Therefore, they will not play a role in screening for aneuploidy but are of value for studying the relationship between induced aneuploidy and recombination. *b*) Chemicals that induce aneuploidy in mammalian germ cells are readily detected in the *in vitro* mammalian cell systems. Some chemicals such as thiabendazole and thimerosal induce aneuploidy *in vitro* but do not appear to be very effective *in vivo*. *c*) Cell division aberrations induced in mammalian cells *in vitro* seem to be predictive for aneuploidy induction in the same cell type. Likewise, c-mitotic effects and cell cycle delay *in vivo* in mitotic and meiotic cells correlate with aneuploidy induction in the respective tissue. A second CEC Aneuploidy Program has started recently to refine the most promising test protocols, to provide understanding of the variety of mechanisms by which chemicals induce aneuploidy, and to establish a data base for aneugens among environmental pollutants.**

## Introduction

Although numerical chromosome changes have been implicated in both somatic and inherited disease, the role of induced aneuploidy is poorly understood. Unlike the induction of point mutations, where the predominant site of chemical action is the DNA, the induction of aneuploidy may theoretically be produced by chemical action upon a wide variety of cellular targets. These targets include synthesis and polymerization of tubulin; synthesis and functioning of tubulin-associated proteins; functioning of cell division spindle fibers; action of centromere and associated kinetochore proteins; replication and separation of polar bodies; fidelity of the events of homologous chromosome pairing and recombination during meiosis.

A number of previous studies supported by the Commission of the European Communities (CEC) Environmental Projects indicated that a diverse range of chemicals (aneugens) were capable of inducing aneuploidy in a number of species in fungi (*1*). However, little information was available as to the relevance of these fungal observations to human health. Neither was there any evidence to indicate to what extent the available fungal test systems were able to detect the overall spectrum of chemicals that induce aneuploidy in mammals.

In 1987, a coordinated program was initiated under the auspices of the CEC with the aim to develop a database that would allow the recommendation of screening tests for the measurement of aneuploidy induction (*2*). Ten model compounds were tested in a series of *in vitro* and *in vivo* aneuploidy assays. These chemicals were colchicine (COL), econazole (EZ), chloral hydrate (CH), hydroquinone (HQ), diazepam (DZ), thiabendazole (TB), cadmium chloride (CD), thimerosal (TM), pyrimethamine (PY) and vinblastine (VBL) (*3*). During the first 4 years, participation of seven laboratories was supported by CEC contracts (A. Kappas, Athens; A. Abbondandolo, Genova; R. Barale, Pisa; A. Carere, Roma; J.M. Parry, Swansea; I.-D. Adler, Neuherberg, A.T. Natarajan, Leiden), and four laboratories collaborated voluntarily (S. Albertini, Basel; B. Hartley-Asp, Helsingborg; M. Kirsch-Volders, Brussels; S.S. Sandhu, Research Triangle Park).

The assay systems used ranged from *in vitro* tubulin polymerization, via mitotic aneuploidy in fungi (Sac-

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charomyces and Aspergillus), cell division abnormalities, micronuclei and aneuploidy in cultured mammalian cells, induction of somatic aneuploidy in plants, c-mitotic effects, mitotic delay, hyperploidy/polyploidy and clastogenicity in rodent bone marrow cells, as well as meiotic delay and hyperploidy in male germ cells. At the time of conception of the project, no assays were available for the detection and assessment of chemical aneugens that could be considered to have reached the level of development and validation that could make them suitable for incorporation in the Guidelines for the Chemical Safety Programs of the CEC or the Organization for Economic Cooperation and Development (OECD).

In evaluating the results of the first phase of the project, certain questions were asked: *a)* What proportion of those chemicals that were capable of inducing aneuploidy in mammalian cells could be detected using simple fungal screening tests? *b)* To what extent were fungal results predictive of aneuploidy induction in mammals? *c)* Were there assay systems with the potential to be developed as recommended procedures for the detection of aneugenic chemicals? *d)* Do aneugens represent somatic and/or germ cell hazards, and what are the relationships between the two events? The results of the first phase of experiments with the 10 chemicals are presented in summary, and the points of interest are discussed.

## Results of *in Vitro* Testing

A summary of the results of the *in vitro* testing is presented in Table 1. The tubulin polymerization assays gave clear positive responses with COL, CH, TB, TM, and VBL (*4*). In contrast, the results with EZ, HQ, DZ, CD, and PY were of borderline significance or positive in one and negative in the other laboratory (*5*). These observations have two possible explanations. Either the test protocol requires further modification to unequivocally detect the latter group of compounds as positive, or the primary target of some of these chemicals is not tubulin but are other cell organelles as indicated above.

In the fungal tests, four chemicals were clearly positive (EZ, CH, TB, and TM), four chemicals gave equivocal results (HQ, DZ, CD, and PY), but most strikingly two chemicals were clearly negative [COL and VBL (*6–7*)]. These latter two chemicals were chosen as positive controls for the project because they are known mammalian spindle inhibitors. It is of particular relevance for the capabilities of detection by fungal systems that these spindle poisons were missed.

In the mammalian cell assays, all 10 chemicals induced aneuploidy and division aberrations including cells with multipolar, monopolar, or lacking spindles, dislocation of chromosomes from the spindle, and lagging chromosomes at anaphase or telophase (*8*). The induction of micronuclei in cytochalasin-B arrested cells was observed with all 10 chemicals when human fibroblasts were used, but could only be detected for four of the chemicals in human lymphocytes [COL, CH, HQ, and VBL (*9,10*)]. Two chemicals (CD and TM) gave contradictory results in the two laboratories (*9,10*). In primary Chinese hamster cells, all the chemicals but EZ and HQ induced micronuclei (*11,12*).

Micronuclei containing centric and acentric moieties were discriminated by the use of immunofluorescent labeling for the presence of centromeric protein by CREST antibodies (*13*). These autoantibodies to kinetochore proteins are present in the serum of patients with the calcinosis/Raynauld's phenomenon/esophageal dysmotility/sclerodactyly/teleangiectesia (CREST) variant of scleroderma. An excess of CREST-positive micronuclei over the control range was observed with COL, CH, TB, and VBL, indicating that the majority (51–94%) of the micronuclei induced by these compounds was derived from whole chromosomes lagging during mitosis. For DZ and CD, the CREST-positive micronucleus frequencies were slightly

**Table 1. Synopsis of *in vitro* data: Athens, Basel, Brussels, Genova, Pisa, Rome, and Swansea.[a]**

| | Chemical | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | COL | EZ | CH | HQ | DZ | TB | CD | TM | PY | VBL |
| Tubulin | + + | −(+) | +(+) | −(+) | − + | + + | −(+) | + + | (+) | + + |
| Aneuploidy | | | | | | | | | | |
| Yeast | − − | + − | + + | + − | (+)− | + + | + − | + + | (+ +) | − |
| Aspergillus | − − | + + | + + | +(+) | − | + + | − | + | − + | nt |
| Mammalian cells | + | + | + | + | + | + | (+) | + | + | + |
| Division aberrations | | | | | | | | | | |
| Immortal cells | + | + | + | + | + | + | + | + | + | + |
| Primary cells | + | + | + | + | + | + | (+) | + | + | + |
| Micronuclei | | | | | | | | | | |
| Primary Chinese hamster cells | + | − | + | − | + | + | + | + | + | + |
| Human lymphocytes | + + | − − | + + | + + | − − | − − | + − | + − | − − | + |
| Human fibroblasts | + | + | + | + | (+) | (+) | (+) | + | (+) | + |
| % CREST positive micronuclei (controls, 34–41) | 84–92 | 28–43 | 51–85 | 34–40 | 54 | 54–86 | 35–57 | 38 | 40 | 94 |

Abbreviations: COL, colchicine; EZ, econazole; CH, chloral hydrate; HQ, hydroquinone; DZ, diazepam; TB, thiabendazole; CD, cadmium chloride; TM, thimerosal, PY, pyrimethamine; VBL, vinblastine. CREST, autoantibody to kinetochore proteins present in the serum of patients with the calcinosis/Raynauld's phenomenon/esophageal dysmotility/sclerodactyly/teleangiectesia (CREST) variant of cleroderma; nt, not tested.

[a]Each plus or minus represents the results of one laboratory; parentheses indicate borderline responses.

elevated above control level and for EZ, HQ, TM, and PY they were in the control range (*9–10,12*).

## Results of *in Vivo* Testing

A summary of the results of the *in vivo* testing is presented in Table 2. In mouse bone marrow cells, the induction of mitotic arrest, c-mitoses, and anaphase decrease was observed for five of the chemicals (COL, EZ, CH, HQ, and VBL), whereas the other five compounds had no significant effects (*3*).

The average cell generation time based on counting the proportions of first, second, and third mitoses in BrdU-labeled bone marrow mitoses was prolonged by COL, CH, HQ, TB, and VBL (*14–16*). Chromosome counts in BrdU-labeled second mitoses of mouse bone marrow cells showed hyperploidy to be induced by COL, CH, HQ, and VBL (*14–17*). The other participating laboratories all found a small increase in hyperploid cells with these four chemicals and additionally with EZ and DZ (*18*).

Polyploidies were only seen with COL and VBL in one of the two laboratories (*14–18*) and also with HQ in the second laboratory (*18*). It should be noted that HQ also readily induced structural chromosome aberrations (*19*). A clastogenic response was also seen with the two spindle poisons and CD in one laboratory (*18*).

Micronuclei were found in mouse polychromatic erythrocytes after treatment with COL, HQ, and VBL by all participating laboratories (*14,16,18,20*). One laboratory also obtained a positive micronucleus response with TB and PY (*14*). Another laboratory (not shown in Table 2) obtained a weak micronucleus induction with CH, DZ, TB, CD, TM, and PY, which was only observed with one of several doses in the lower dose range tested and never exceeded a doubling of the concurrent control values; however, the positive effect was confirmed in a second experiment (*18*).

As expected, the two spindle poisons elevated the frequencies of CREST-positive micronuclei above the control level. However, only 15% of the HQ-induced micronuclei showed a positive CREST response (*21,22*), indicating that at the doses tested the clastogenic action of HQ masked the spindle effects in the micronucleus assay.

Meiotic arrest was induced in mouse spermatocytes with COL, EZ, CH, HQ, DZ, CD, and VBL (*23*). These 7 chemicals also increased hyperploidy in second meiotic divisions of mouse spermatocytes in the same laboratory (*23*). The other laboratory obtained an increase in hyperploid second meiotic divisions with COL, HQ, and TB, but not with CH, DZ, and CD (*14*). The differences in results from the two laboratories are most likely due to different test protocols.

## Conclusions

In response to the questions posed in the Introduction, the following conclusions are drawn based on the data from the first set of tests for aneuploidy.

Fungal test systems are not capable of detecting potent inhibitors of mammalian tubulin polymerization such as colchicine and vinblastine. However, fungal systems uniquely provide the potential for studying the relationship between induced aneuploidy and recombination. They are also a valuable tool for studying structure–activity relationships of some aneugens.

Aneuploidy may be induced by a diverse range of mechanisms. Chemicals that induce aneuploidy in germ cells by mechanisms involving modification of the spindle apparatus are readily detected in the *in vitro* mammalian cell systems. Analysis of the effects of the test chemicals upon the fidelity of mitotic cell division *in vitro* appears to be remarkably predictive for aneuploidy induction in the same system. Likewise, cell cycle delay *in vivo* in mitotic or meiotic cells is obviously predictive for aneuploidy induction in the respective tissue.

As seen in many other test systems, chemicals such as cadmium chloride, which have a narrow dose window of response are difficult to evaluate. Some chemicals, such as thiabendazole and thimerosal are potent inducers of aneuploidy *in vitro*, yet do not appear to be very effective

**Table 2. Synopsis of *in vivo* data: Neuherberg, Brussels, Rome, Pisa, and Shreveport.[a]**

| | Chemical | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | COL | EZ | CH | HQ | DZ | TB | CD | TM | PY | VBL |
| Bone marrow | | | | | | | | | | |
| c-Mitoses | + | + | + | + | − | − | − | − | − | + |
| Cell cycle delay | + | − | + | + | − | + | − | − | − | + |
| Hyperploidy | + + | − + | + + | + + | − + | − − | − − | − − | − − | + + |
| Polyploidy | + + | − − | − − | − + | − − | − − | − − | − − | − − | + + |
| Structural chromosomal aberrations | − + | − − | − − | + + | − − | − − | − + | − − | − − | − + |
| Micronuclei | + + + | − − − | − − − | + + + | − − − | − − + | − − − | − − − | − − + | + + + |
| % Crest positive micronuclei | 66 | | | 15 | | | | | | 70 |
| Spermatocytes | | | | | | | | | | |
| Meiotic arrest | + | + | + | + | + | − | + | − | − | + |
| Hyperploidies | + + | + − | + − | + + | + − | + − | + − | − − | − − | + |

Abbreviations: COL, colchicine; EZ, econazole; CH, chloral hydrate; HQ, hydroquinone; DZ, diazepam; TB, thiabendazole; CD, cadmium chloride; TM, thimerosal, PY, pyrimethamine; VBL, vinblastine.

[a]Each plus or minus represents the results of one laboratory.

*in vivo*. It is important to determine whether such chemicals and/or their metabolites reach the germ cells and somatic tissue in rodents.

As a preliminary recommendation, a simplified screening procedure can be envisaged consisting of three steps: *a*) Evaluation of cell division aberrations in cultured mammalian cells, *b*) analysis of mitotic arrest and c-mitoses in rodent bone marrow cells, and *c*) determination of meiotic delay in rodent germ cells.

More questions arose from the results of the first set of experiments than could be answered in the study period. Therefore, a new CEC Aneuploidy Program commenced in late 1991 and focuses primarily on six points that address the most prominent issues that arose from the previous program (Table 3). Because the previous studies indicate that lower eukaryotic tests such as those that use fungal cultures are not predictive for the activity of aneugenic chemicals in higher organisms, the new project predominantly uses mammalian cell systems and intact rodents.

***Mechanisms.*** Cellular targets in the mammalian cell of significance in the interaction of aneugenic chemicals will be identified and the relative role of damage to such targets in inducing aneuploidy will be determined. Furthermore, the role of aneugenic chemicals in the progressive stages of tumor development will be determined in the cell transformation assay.

***Metabolism.*** The role of cellular and organ-specific metabolism in the interaction of environmental chemicals with cellular targets of relevance will be studied. Labeled chemicals will be used to determine their distribution in rodents.

***Sex and Tissue Specificity.*** The mechanisms of action and relative activities of aneugenic chemicals in both germ cells and somatic cells will be analyzed in rodents. Research will be undertaken to determine the potential value of transgenic animals in the detection of induced aneuploidy in specific rodent tissues.

***Environmental Significance.*** Chemicals of environmental significance will be tested with the simplified screening procedure, such as noscapine, tear gas, and methyl vinyl sulphone, which have already been found positive *in vitro* (*24–26*), industrial solvents and pesticides, e.g., benomyl, which has been labeled as a potential mutagen by the CEC based on *in vitro* and *in vivo* data (*27,28*).

**Table 3. Second CEC Aneuploidy Program, 1991–1993.**

| |
|---|
| Main topics |
| Mechanisms |
| Metabolism |
| Sex and tissue specificity |
| Environmental significance |
| Optimal test systems |
| Aneugen database |
| Chemicals of environmental significance |
| Noscapine |
| Industrial solvents |
| Pesticides (e.g., benomyl) |
| Tear gas |
| Acrylamides |

***Optimal Test System.*** The refinement of protocols for the detection of induced aneuploidy in cultured mammalian cells and intact rodents will particularly focus on the micronucleus and chromosome counting techniques by using immunofluorescent *in situ* labeling techniques. Emphasis will be placed on the development of molecular probes for the analysis of specific chromosomes and the development of improved methods to analyze cell preparations.

***Aneugen Database.*** The range of chemicals evaluated in the most promising aneuploidy test systems will be expanded with the aim of classifying the particular chemical classes capable of interacting with relevant cellular targets. It is the ultimate goal of the project to establish a database for aneugens and to recommend a reliable set of screening tests for aneuploidy induction.

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