Aneuploidy contributes to human disease. Specific numerical chromosome changes in human meiotic cells contribute to embryonic loss and congenital abnormalities such as Downs syndrome, whilst in mitotic cells such changes play an important role in the pathology of some tumours. Genotoxicity testing has tended to concentrate on the potential of a substance to induce gene mutations and structural chromosome rearrangements. There is a perception that the ability of a substance to induce numerical chromosome changes has been a secondary consideration. The objective of the meeting was to assess the current status of aneuploidy testing from an industrial perspective.

Morning session: regulatory and scientific aspects

James Mackay (Macclesfield) described the activities of the aneuploidy task force set up by the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC). The objectives of the group were to review the role of chemically induced aneuploidy in human disease and to evaluate and recommend methods and procedures for testing, hazard identification and risk characterization. Recommendations for assessing aneuploidy vary from not being specifically required (e.g. EC Directive 91/414) to being explicitly required (e.g. the International Conference on Harmonization [ICH]).

Leigh Henderson (Shambrook) reported on the ECETOC recommendations on aneuploidy testing strategy. The ECETOC task force considers that it is prudent to address aneugenic potential of chemicals in the overall safety assessment, since there are sufficient data from animal studies to conclude that chemically induced aneuploidy causes adverse effects and there is no reason to believe that effects in man will be dramatically different.

The assumption that aneugens are not detected in standard assays for mutagenicity was tested by analysis of three data sets. Most (60-70%) polyploidy-inducing compounds also induce structural chromosome aberrations. It was concluded that scoring polyploidy and mitotic inhibition, in association with the analysis of structural chromosome aberrations, would detect most aneugens.

A working hypothesis of the ECETOC group was that chemicals that caused numerical chromosome changes would induce aneuploidy at low doses and polyploidy at high doses. However, polyploidy can be caused by extreme culture conditions and other non-specific effects, such as cell fusion and damage to the membrane. Thus further investigation of polyploidy inducers is required to determine whether they induce aneuploidy.

Since a major target of aneugens is the G2 phase of the cell cycle, it is necessary to expose cells during G2. For the human lymphocyte cytogenetic assay, cell cycle labelling techniques have shown that there are approximately equal numbers of cells at first and second division metaphase at a harvest time equivalent to 1.5 cell cycle durations. Therefore, this exposure time should detect most polyploidy inducers, provided that the compound does not substantially delay the cell cycle.

A tiered screening approach was suggested, with a dose-related increase in polyploidy or increase in mitotic index in the chromosome assay leading to the performance of an in vitro and/or in vivo micronucleus test. Any positive responses could be investigated by kinetochore- or centromere-specific staining, with positive results investigated further for effects in germ cells.

Jim Parry (Swansea) commented that polyploidy is associated with tumour progression in some types, such as those of the thyroid, but is also naturally present in many tissues, such as the liver. He outlined a strategy proposed by the UK Committee on Mutagenicity (COM) for suspected aneugens. The first tier would be an in vitro test, looking for micronuclei with conventional stains. If micronuclei were present, a second set of slides could be probed with kinetochore-specific antibodies or centromere-specific probes to determine if the micronuclei contained chromosomal fragments or whole chromosomes. This would show whether the test compound was inducing principally structural or numerical aberrations. Chemicals which induced micronuclei containing centromeres would be investigated further to determine whether they induced nondisjunction. A positive response should be investigated in an intact animal, because only positive effects that are reproduced in vivo are considered to be significant.

Dick Shillaker (York) said that some of the pesticides that the Pesticides Safety Directorate review affect cell division and so are potential aneugens. An example is the benzimidazole carbasazim, used as a fungicide. Despite this, there is no specific requirement under EC Directive 91/414 to test pesticides for aneuploidy. The results of unvalidated tests should be treated with caution. The methodology suggested by COM for investigating potential thresholds for aneuploidy in vitro may not permit detection of those aneugens requiring metabolic activation. As S9 mix is toxic, only relatively short exposure times are possible.

Aneugenic activity per se is likely to be a threshold-mediated effect. Dick Shillaker outlined a provisional scheme to determine safe exposure levels based on no observable adverse effect levels seen in standard in vivo toxicity studies. He suggested that, alternatively, in vitro no effect concentrations for aneuploidy could be compared with in vivo tissue concentrations to arrive at an acceptable human exposure level, but stressed that these were personal thoughts.

Jim Parry highlighted the paucity of studies linking chemical exposure with an increase in congenital abnormalities, an exception being the effects of trichlorfon on women in Hungary (Czeizel et al., 1993). It is not known whether unique germ cell aneugens exist.

Substances such as benomyl and nescapine induce numerical chromosome changes only and are not directly reactive with DNA.

Proteins such as topoisomerase II, phosphotyrosine phosphatase and tyrosine kinase C are connected with cell division. Chemically induced changes in such proteins may modify the fidelity of normal cell division, chromosome segregation and disjunction.

When considering the in vivo potential of in vitro aneugens organ-specific effects must be taken into account. For example, the nervous system is susceptible to tubulin-reactive chemicals. Also, tissues most likely to be exposed should be examined.
e.g. the gastrointestinal tract for an oral route of administration or the skin for accidental exposure.

Afternoon session: practical aspects of aneuploidy testing

Gill Clare (Loughborough) presented the results of a questionnaire that had been circulated among members. Most laboratories considered that they could detect potential potent aneugens/polyploidy inducers using the current battery of tests. New cytogenetic methods were being used for research and to address substance-specific issues. None of the respondents were currently working with meiotic cells. For aneugens reproducibility and determining an effective dose range could be difficult.

Liz Parry (Swansea) gave an overview, updated from a previously published paper (Parry et al., 1995).

Aneuploidy can be detected at metaphase by chromosome counting (e.g. during performance of the in vitro chromosome aberration assay) and at interphase by the micronucleus assay. Most methods are well established but in need of validation. Standardized protocols are unavailable for many assays but may not be desirable because the targets for induction of aneuploidy are complex. Chromosome counting is the most definitive end-point, as other assays may miss non-disjunction events. It is important to use cells with low frequencies of spontaneous aneuploidy and diploid numbers. Treatment for the entire cell cycle is important. The observation of a diploid chromosome number without chromosome identification does not always mean that the set is complete. Chromosome gain has been considered more meaningful than chromosome loss. In situ hybridization offers the greatest potential for future development of aneuploidy assays.

Each human chromosome can be probed with different colours (spectral karyotyping). At present it is most practical to paint three chromosomes in different colours, although this begs the question of which are the most relevant chromosomes to consider, since individual chromosomes may not be equally susceptible to displacement from the spindle. The potential exists for automation in the micronucleus assay, where chromosome loss and breakage can be distinguished by the use of kinetochoore or pancentromeric probes.

Whilst it is possible to count large numbers of cells in the in vitro micronucleus test, there are some concerns about the use of cytochalasin B. If the block is incomplete cells may escape. All cell types must be calibrated for their sensitivity to cytochalasin B. The timing of exposure must be adjusted to avoid the appearance of multinucleate cells. There is the possibility of interaction of the test chemical with cytochalasin B.

There is an emphasis on using mitotic cells, with an implicit assumption that this will allow mitotic cell aneugens to be detected, even though meiosis is fundamentally different. Germ cells contain additional targets specific to meiosis. Non-disjunction in human oocytes is a primary source of chromosome imbalance. Methods exist for looking at mouse and human germ cell chromosomes. However, a drawback is that tests using mitotic cells are more expensive, difficult and time-consuming.

Clare Bourner (Sharnbrook) described results generated in an in vitro chromosome assay using human peripheral blood lymphocytes and assessing cell cycle time. Colchicine, carbendazim, griseofulvin and cytochalasin B induce polyploidy at a time equivalent to 1.5 cell cycles. 6-Mercaptopurine, phorbol 12-myristate 13-acetate and chloral hydrate were negative at a time equivalent to 1.5 cell cycles and 24 h later, even when tested up to toxic concentrations. β-Oestradiol gave different responses in two tests and the influence of the sex of the donor is being investigated.

David Tweats (Ware) summarized the available data for detection of aneugens as part of the ongoing L5178Y mouse lymphoma cell gene mutation trial. Compounds such as colchicine needed the longer exposure time of 24 h to show a positive effect. Sofuni's group had probed the mutant colonies for chromosomes 3 and 11 and had found structural chromosome changes, which were often complex. The assay could detect most aneugens and polyploidy inducers. One laboratory using the soft agar method had been unable to detect any effect with vinblastine or colchicine. The inclusion of a 24 h exposure time did not appear to disturb the specificity of the assay.

For many aneugens, particularly spindle poisons, there is a narrow margin of activity between no toxicity and complete toxicity. This means that closely spaced dose intervals may be required and that the effect is not always reproducible.

Silvio Albertini (Basel, Switzerland) presented the results, discussion and conclusions from a collaborative project. This involved four industrial laboratories who tested 59 undisclosed test compounds in-house in both the in vitro micronucleus test (IVMNT) and in vitro chromosome assay (IVCA). Using four cell lines there was an overall concordance between the IVMNT and IVCA and this increased to >90% if aneugens were excluded (generally detected by the IVMNT). A German speaking section of the Environmental Mutagen Society task force has also reviewed the data available on 30 compounds tested in both the IVMNT and IVCA. The main conclusions from the task force were that the IVMNT detects all clastogens, is less prone to false positives and detects aneugens which are not detected in the in vitro chromosome assay. However, the cell cycle has to be considered carefully and appropriate toxicity tests have to be conducted. There will be a 1 year German collaborative trial using V79 cells starting July 1997. Nine laboratories will test 20 compounds using two test methods. David Tweats reviewed the IVMNT using both the database from Japan using CHL cells and data. There was accumulating evidence that the IVMNT and the IVCA produced highly concordant responses. A provisional standard protocol is being developed.

Richard Marshall (Harrogate) described a protocol based on fluorescent in situ hybridization and results for vinblasticine and carbendazim and concluded that measurement of non-disjunction in binucleate human lymphocytes using chromosome-specific centromere probes offers a sensitive method for detection of aneuploidy. Probes had been used for chromosomes 1, 8, 18, 11, X and 17.

Marie-Claude OuldElkim (Vitry-sur-Seine, France) described the procedure for growing CHO-K1 cells without a block and CHO-WBL cells with a block in 6-well plates. Investigators have experienced technical problems in the past, in particular in relation to toxicity and the use of S9 mix, and these were mentioned by James MacKay. Further work was needed to optimize the protocol. The validity of conclusions drawn from comparison of unvalidated data sets was questioned. Proposed OECD guidelines, for both mono- and binucleate cells are being drafted.

Examples of the use of centromeric and whole chromosome probes and some of the practical aspects associated with their use were provided by Gill Stemp (Ware), Richard Marshall...
Meeting report

Aneuploidy can be detected by using centromere-specific probes in interphase cells. Structural and numerical aberrations can be detected in micronuclei. Acridine orange stains cytoplasm as well as the nucleus clearly. For metaphase cells the cytoplasm sometimes needs to be removed and the pH and temperature is important for a successful outcome. There are practical problems associated with the use of centromeric probes to be aware of, such as diffuse signals, stained probes lying on top of one another and the possibility of missing signals because they are lying in a different focal plane within the nucleus.

Dick Shillaker and Jim Parry expressed the view that there is a threshold below which aneugens fail to exert an effect, so it should be possible to specify a no-effect level. Michael Green (Brighton) asked how it was possible to distinguish a no-effect level from an inability to detect an effect.

In vitro studies using cytochalasin B-blocked cells indicate that non-disjunction is more common than chromosome loss. Binucleate cells coupled with the use of probes give the most specific information about the lowest level of effect in vitro. Micronucleus counting with kinetochore staining provides conclusive proof of aneugenic potential in vivo.

Summary
Aneuploidy has important consequences for human health. There is a requirement to detect aneugens that may not be fully satisfied by the current test battery. New cytogenetic techniques look promising.

References

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