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1. Chromosome changes in germ cells

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Studies for structural chromosome changes in germ cells are predominantly carried out in male mammals, mostly in mice. This is due to the difference in germ cell biology between males and females and to the fact that male germ cells are available in abundance while female germ cells are limited in number and difficult to access. Chemically induced structural chromosome changes can be analysed in dividing male germ cells, i.e. in mitotic divisions of differentiating spermatogonia and in meiotic divisions of spermatocytes. The consequences of induced structural chromosome aberrations are detected in the dominant lethal assay and in the heritable translocation test. The dominant lethal assay reflects the induced chromosome damage that after fertilisation is not compatible with embryonic survival. Male germ cells are characterised by their stage-specific response, i.e. different developmental stages of male germ cell development show different sensitivities to chemical mutagens in the dominant lethal assay (Ehling et al., 1972). The heritable translocation test quantifies the induced structural aberrations that allow survival of the embryos to birth, i.e. balanced reciprocal translocations. Upon these data obtained for chemotherapeutic agents or occupational chemicals, a calculation of the genetic risk for exposed human subjects is possible. Carriers of reciprocal translocations in mice and humans are physically normal, however, they show reduced fertility due to the production of imbalanced gametes and the balanced translocations are transmitted to half of their live offspring. Based on heritable translocation data we have estimated that the genetic risk of occupational exposure to 1,3 butadiene is increased by 30% above the spontaneous incidence in the human population (Pacchierotti et al., 1998). Recent data indicate that the chemotherapeutic dose of dacarbazine results in a genetic risk of twice the spontaneous incidence of translocations in the human population. In both cases, the elevated genetic risk is transient, i.e. only spermatids are sensitive to the clastogenic effect of the chemicals. Thus, abstinence for a period of 4-6 months after the end of exposure can prevent the conception of a chromosomally abnormal child. Numerical chromosome changes in germ cells are also of importance for the human population. The Down syndrome (tris 21), the Turner syndrome (XO) and the Klinefelter syndrome (XXY) are the most common human genetic diseases with altered chromosome numbers (aneuploidies). To assess the chemical induction of aneuploidies in germ cells, a molecular cytogenetic assay has been developed, the sperm FISH assay (Adler et al., 1996). It uses fluorescence-labelled chromosome-specific DNA probes hybridised to sperm to detect aneuploidy induction during meiosis in male gametogenesis. A data base for chemicals with aneugenic effects in male germ cells is presently being established.

Reference

Adler *et al*. (1996) *Mutat. Res.,* **372**, 259-268.
Ehling *et al*. (1972) *Mutat. Res.,* **15**, 175-184.
Pacchierotti *et al*. (1998) *Mutat. Res*., **397**, 93-115.

2. Application of comparative genomic hybridyzation for the detection of DNA copy number changes in breast cancer

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In this work the DNA copy number changes were evaluated in 21 breast cancer patients from Kuwait cancer control centre (KCCC) by comparative genomic hybridization (CGH). In CGH, the relative intensities of tumour DNA (detected using green fluorescence) and normal reference DNA (detected with red fluorescence) after hybridisation to normal metaphase chromosomes is used to reveal and map regions of increased or decreased DNA-sequence copy number (Kallioniemi et al., 1992). Amplified loci are visualized as chromosomal regions with predominantly green fluorescence and quantified by digital image analysis at increased green-to-red fluorescence intensity ratio. As no specific probes or previous knowledge of aberrations are required, CGH is especially suitable for identification and mapping of previously unknown DNA copy-number changes that may highlight locations of important genes. In the present study the age of the patients was less than 40 years. This age group of patients comprises 28% of the six age groups investigated. In this particular group of patients the recurrent chromosomal changes were 4p (48%), 6p (43%), 8p (52%), 10p (43%), 12p (57%), 12q (43%), 13q (43%), and 21q (48%). On the other hand, the most frequent losses were 1p (24%), and 22q (19%). These data can be used so that the amplified regions are further investigated.

Reference

Kallioniemi *et al* (1992) Proc.Nat.Acad. Sci. USA **89**, 5321-5325.

3. The genetic basis of adenocarcinoma of the bladder with special emphasis on those arising in 'Clam bladders'

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Since its introduction in 1982 the Clam enterocystoplasty has become widely used for the treatment of detrusor hyperreflexia and instability. There is thought to be a small risk of malignancy in these bladders and in the Cardiff unit alone there have been four such tumours. They were all highly aggressive adenocarcinomas entirely in the native bladder segment. Our aim has been to identify genetic irregularities peculiar to these tumours as reference points.

DNA was extracted from slides of tumours and from biopsy samples taken from the bladder remnant of Clam patients. The DNA was then subjected to an in-situ hybridisation technique called Comparative Genomic Hybridisation (CGH). The methods detect amplifications and/or deletions over all chromosomes compared to controls

Fifteen Clam patients in the 'at risk' latency period have been biopsied. DNA analysis of these biopsy samples from

the bladder remnant have shown that the DNA of the urothelium near the anastamotic line is more genetically unstable compared with DNA of tissue from further away. The tumour DNA (2 Clam cancers and 2 urachal adenocarcinomas) analysis shows several amplifications and deletions particularly on chromosomes 8p and 21q.

The spectre of the possibility of Clam cancer still remains. We need to be able to try and predict which patients are most at risk. If we can identify a sequence of DNA changes leading to tumour formation then we can use them as indicators for the Clam patients. If any of them develop similar DNA changes then they will require closer follow up.

4. Endocrine disruption and the UKEMS

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In the 1970s there was concern that we were on the leading edge of a steep increase in human cancer incidences due to the increasing production of synthetic chemicals, some of which were mutagenic. That concern has waned, albeit mutagens of potential significance to humans can now be detected using the techniques developed to meet that perceived problem. The fact that we still seem to lack a human germ cell mutagen is probably due to a combination of low exposures and the use of inappropriate or insensitive markers of inherited mutations, the latter situation being one that may soon be remedied.

In the 1990s there was a concern that we were on the leading edge of a steep increase in human developmental abnormalities due to increasing production of synthetic chemicals, some of which were hormone mimics. A burst of endocrine disruption assay development or refinement is occurring in this new field, much as happened with mutation assays in the 1970s, but at a much accelerated rate and with access to molecular techniques. Whether or not the hazard presented to humans and wildlife by exposure to environmental hormone mimics is as serious as some fear remains to be determined, but this uncertainty is not slowing the conduct of experimental studies. A curious dilemma is appearing, much as may affect the search for human mutagens in the post-human-genome days ahead. Some chemicals are producing measurable effects that are not associated with any apparent adverse toxicity. This is leading to a debate regarding whether an endocrine disruptor should be defined by its measurable endocrine toxicity, or by its ability to disturb an endocrine function without producing a measurable endocrine deficit. An analogy with mutation would be the heritable production of a protein with an altered amino acid but with unaffected function.

5. Transgenic *in vivo* gene mutation assays – studies on a battery of clastogens with various mechanisms of genotoxicity

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A number of studies suggest that the lacZ transgenic assay (Muta™Mouse) may be insensitive to clastogens i.e. compounds that mainly induce chromosome aberrations and large DNA deletions e.g. mAMSA and camptothecin (Holmstrom and Winters 1992). To investigate this further, we selected 5 known clastogens (bleomycin, camptothecin, ciprofloxacin, mAMSA and oAMSA) with various mechanisms of genotoxic action. Literature surveys indicate these had not been previously tested in the lacZ transgenic model. We determined positive induction of both gene mutations (at the lacZ locus) and clastogenicity (using a flow cytometry peripheral blood micronucleus assay (Litron)). Preliminary studies using male animals (n=5 per group, dosed p.o daily for 5 days) treated with with bleomycin (2.5 mg/kg), camptothecin (0.5 mg/kg), ciprofloxacin (300 mg/kg), mAMSA (3 mg/kg) were conducted. Dose selection was based on positive genotoxicity reported in the literature. oAMSA (3 mg/kg daily) or vehicle (n=10) were used as negative control groups, while ENU (50 mg/kg i.p daily for 5 days) was used as the positive control group. Mutation frequencies (MF) were determined 35 days after the last dose in bone marrow and liver in all treatment groups. Lung samples were also analysed for bleomycin, ciprofloxacin and ENU. Results showed that only ENU induced a significant ($P < 0.05$) increase in MF compared with the vehicle/negative control, whereas ENU and camptothecin induced statistically significant increases in peripheral blood micronucleus frequencies. Bleomycin, ciprofloxacin and m-AMSA, however, were negative for both endpoints. These results mean that no firm conclusions can be drawn from the mutation frequency data at the present time with regard to the sensitivity of transgenic gene mutation assays to clastogens

Reference

Holmstrom and Winters (1992) *Mutagenesis* **7**, 189-193.

6. The safety of genetically modified foods

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Genetically modified foods are the subject of much scientific and public interest. Before their sale is permitted GM Foods undergo a rigorous safety assessment in accordance with the EU Novel Food Regulation 258/97.

In the UK, the Advisory Committee on Novel Foods and Processes (ACNFP) examines all Novel Food Applications (GM Foods are one of the categories of Novel Food) and makes recommendations to the Food Standards Agency for release into the market place. (Prior to 1 April 2000 the ACNFP reported directly to Government Ministers). In order to make its decisions the committee uses the decision tree approach as described in 258/97. For each application a detailed set of data is required relating to source, intended use, likely consumption patterns, nutritional data, toxicological data, details of potential allergenicity, processing route and details relating to the molecular genetics of the product. This will included sequence data, stability of the inserted gene, details of gene expression.

The Committee must keep abreast of a rapidly developing scientific area and can recommend the FSA to commission specific research and has also held open meetings to cover specific issues. It is at the forefront of the Government drive

for transparency and inclusively in the way scientific advice is formulated and handled.

7. The use of molecular techniques in the diagnosis and strain differentiation of *Helicobacter pylori*

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Helicobacter pylori is one of the worlds most common chronic infections. It was first discovered in 1982, and already is known to be the causative agent in chronic superficial/atrophic gastritis, peptic ulcer disease, gastric lymphoma, and more recently gastric carcinoma. The IARC has classified *H pylori* as a group I carcinogen. Histology is considered the gold standard for the diagnosis of *H pylori* in gastric biopsy samples. In 1997, the complete genomic sequence of *H pylori* strain 26695 was published. Since, molecular techniques have increasingly been used in the detection and further evaluation of this bacterium. The polymerase chain reaction (PCR) is cost effective and highly reproducible, plus DNA can be stored for further evaluation.

The aim of the study was to evaluate the sensitivity and specificity of PCR in the detection of *H pylori* compared to histology in gastric biopsy specimens, plus to determine the presence and frequency of the vac-A and cag-A virulence genes and to associated its presence to histopathological findings. 82 consecutive patients attending Neath General Hospital for open access endoscopy were recruited. Four gastric biopsies were obtained. Patients receiving proton pump inhibitors were excluded. Histology was reported by a single pathologist. PCR was performed by the first author. Ethical approval was obtained from the local research ethics committee.

24 patients (29%) were found to have *H pylori* infection on histology. PCR found the same 24 patients positive, together with 2 additional patients. This gave a sensitivity of 100% and a specificity of 92%. The cag-A gene was found in 65% cases. 41% of cag-A positive patients had intestinal metaplasia (precursor to the dysplasia-carcinoma sequence) compared to 11% of cag-A negative patients. The cost per PCR product (consumables only) was £2.92. Thus, the PCR methodology is as sensitive and as cost effective as histology for the detection of *H pylori* infection. Patients with *H pylori* possessing the cag-A gene are more likely to have serious pathology.

8. Quantification of CYP1A1 and CYP1A2 mRNA in human lymphocytes by Taqman PCR: Effects of cryopreservation, mitogen stimulation, long term culture and induction by 3-methylcholanthrene

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Long-term cultures of human lymphocyte have been developed for use in genotoxicity assays, but the expression and inducibility of key xenobiotic metabolising enzymes has not been characterised. In order to investigate this, expression of CYP 1A1 and 1A2 mRNA by freshly isolated human lymphocytes, and phytohaemagglutinin (PHA) stimulated fresh and cryopreserved lymphocytes, were quantified using TaqMan real time PCR. Freshly isolated lymphocytes express low, equivalent levels of both CYP 1A1 and CYP 1A2 mRNA. These levels were increased 420-fold and 4.2-fold respectively in cells cultured with PHA for 7 days. Cryopreserved human lymphocytes were re-cultured in the presence of PHA and were either left untreated, or treated with DMSO or 1μM 3-Methylcholanthrene (3-MC) for 24 hours. The expression of CYP 1A1 and CYP 1A2 was determined at time 0 hours, 7 hours and 24 hours. Expression of CYP1A1 in cryopreserved lymphocytes cultured with PHA for 7 days was a 189-fold greater than in fresh lymphocytes. Expression of CYP1A1 mRNA in cells cultured with 3-MC for an additional 24 hours was induced a further 3.0 fold over untreated levels and 5.8-fold over cells treated with DMSO. CYP 1A2 mRNA levels in lymphocytes re-cultured after cryopreservation were 3 fold higher than in fresh lymphocytes. 3-MC treatment for 24 hours did not induce CYP1A2 mRNA.

9. Cyclophosphamide-induced foetal abnormalities are associated with reduced paternal germ-cell apoptosis: implications for childhood cancer

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Childhood cancer is believed to be often the result of germ-line mutation and evidence from animal studies indicates that this could also be true for a proportion of foetal malformations. It is conceivable that both type of outcome could result from induced abnormalities in the pathways leading to apoptosis. Pre-conceptional, paternal treatment of rats with cyclophosphamide was therefore used to investigate the mechanism of induction of mutations leading to malformed offspring. Adult male rats were administered different doses of cyclophosphamide (along with vehicle controls), 6 days per week for 9 weeks. Some of the animals were then treated with 5-bromodeoxyuridine and testis samples collected for fixation in Carnoy's fluid and subsequent analysis of germ cell proliferation and apoptosis. The remainder were mated to untreated, virgin females resulting in a greater than eleven-fold increase in the proportion of abnormal offspring produced in the 5.1 mg/kg group. Apoptotic cells identified by TUNEL labelling were scored per stage XII/XIII tubular cross-section and the means decreased with increasing dose, significantly at 5.1 mg/kg ($P < 0.05$). No statistically significant effects were found on B-spermatogonial number or proliferation. This cell type is the precursor of that which was most commonly seen to undergo apoptosis in stage XII/XIII. Therefore, a prior reduction in the size of the population of cells that underwent apoptosis is unlikely to be the explanation for the observed reduction in the incidence of apoptotic cells. The inappropriate survival of damaged, germ cells caused by a lowering of the incidence of apoptosis may, therefore, account for the increase in the proportion of foetal malformations. It is, therefore, possible that a similar mechanism could operate to allow cells

bearing mutations predisposing to tumour development to persist in the germ-line.

10. Application of the Restriction Site Mutation (RSM) Assay to detect Benzo[a]pyrene induced liver mutations in the p53 gene of the European Flounder, *Platichthys flesus*

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This research is using the European flounder, *Platichthys flesus*, as a model for the study of environmental carcinogenesis. Studies utilising bottom-dwelling marine flatfish have already indicated significant correlations between the incidence of neoplasia and contamination of the aquatic environment. Benzo[a]pyrene, a highly toxic polycyclic aromatic hydrocarbon, is a component of shipping fuels and is ubiquitous in coastal sites, and is the chemical under scrutiny in this research. Laboratory experiments involve dosing flounder with benzo[a]pyrene. DNA is extracted from the liver tissue and subjected to RSM analysis. Any resultant mutations are then sequenced. Exons 5 to 8 of the tumour suppressor p53 gene are being targeted for analysis as this region is a hotspot for mutations in many other tumour types. Fresh tumour tissue collected from feral fish populations, and archived tumour tissue, will also be examined for mutations. The mutational spectra from these tissues are being compared with those from the laboratory-treated fish. This research will provide experimental evidence to support or not the hypothesis that liver tumours found in feral flounder populations have a chemical aetiology.

11. Investigations into the concept of a threshold mechanism for clastogenicity with topoisomerase inhibitors

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While the use of a threshold concept in risk assessment is widespread, there is very little experimental demonstration for the existence of thresholds for genotoxic compounds other than aneugens[1]. The clastogenicity of drugs that interact with topoisomerases is believed to result from the stabilisation of the topoisomerase enzyme with DNA during the catalytic cycle. This leads to the formation of a stabilised cleavable complex which is directly responsible for an increase in DNA strand breaks. As the induction of DNA damage is by an indirect mechanism the concept of a threshold for clastogenicity may be introduced. To determine whether topoisomerase inhibitors show a genotoxic threshold we have measured micronucleus induction *in vitro* in mouse lymphoma L5178Y cells. Cultures were treated with various concentrations of etoposide, a topoisomerase type II (topo II) inhibitor or doxorubicin, a compound which interacts with both topo II and directly with DNA. The shape of the dose response curves were analysed to assess whether a threshold for clastogenic activty could be determined. In order to discriminate between a NOEL and a practical threshold dose as defined by the minimal dose in which a positive response was elicited, the sample sizes of various concentrations scored immediately above and below the NOEL were increased (from 2000 to 5000 or 10,000 cells/treatment) to improve the sensitivity of the assay. Using statistical modelling, data will be presented to show whether the dose response curves for etoposide (theoretically threshold dependent) may be differentiated from doxorubicin (i.e. theoretically threshold independent because of direct DNA binding).

Reference

Parry *et al* (2000) *Mutat. Res*. **464:** 53-63.

12. Potentiating effects of UV radiation on PAH-induced genotoxicity in *Mytilus edulis*

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Ultraviolet radiation (UVR) is known to alter the toxicity of certain chemical compounds, in particular polycyclic aromatic hydrocarbons (PAHs). In the current study, the potential for environmentally realistic levels of UVR to modify the toxicity of PAHs has been evaluated under different exposure scenarios. Exposures of adult *Mytilus edulis* to (a) B(*a*)P in the absence of UVR; (b) B(*a*)P which had received previous UV photoactivation, or (c) B(*a*)P with simultaneous UV irradiation, were carried out. Analyses of micronuclei (MN) induction were made upon haemolymph samples collected from the adductor muscle of exposed bivalves on days three and six of continuous exposure.

Spontaneous levels of micronuclei induction were within the range of 0-6 per 1000 cells (mean frequency of 1.7 $\pm$ 1.6). Following three days of exposure, the mean frequency of micronuclei was (a) 7.00 $\pm$ 6.05 and 10.5 $\pm$ 4.04 in mussels exposed to 1:g/L B(*a*)P and 10 $\pm$ 0004:g/L B(*a*)P respectively; (b) haemolymph sampled from mussels exposed to previously photoactivated B(*a*)P showed a micronuclei frequency of 13.17 $\pm$ 4.07 and 15.83 $\pm$ 4.9 (1:g/L B(*a*)P and 10:g/L B(*a*)P respectively); and (c) mussels exposed to B(*a*)P with simultaneous UV irradiation, exhibited a mean micronuclei frequency of 16.7 $\pm$ 4.5, in animals which had been exposed to a concentration of 10:g/L B(*a*)P.

These highly significant ($p<0.000$) increases in genotoxicity clearly indicate that there is an interaction of UVR with B(*a*)P in the marine environment. Haemolymph samples following 6 days of exposure, indicated a continuing increase in the induction of micronuclei, only for the lower concentration of B(*a*)P (1:g/L). At the highest concentration of B(*a*)P (10:g/L) this trend was not exhibited. Since MN can only be detected in cells undergoing mitosis, relatively low frequencies of MN observed in these samples may be due to the suppression of mitotic activity, or cell death, rather than reduced genotoxic damage. Observations from this study suggest that the acute phototoxic effects of B(*a*)P and other PAHs should taken into consideration when assessing the impact of physical and chemical agents on aquatic biota.

13. Measurement of cytotoxicity in the L5178Y mouse lymphoma mutation assay

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There are two commonly used measures of the cytotoxicity of a test substance in the L5178Y $TK^{+/-}$ mouse lymphoma assay (MOLY). Day 0 relative survival (RS) measures the cloning efficiency of the cell cultures, relative to the control cultures, immediately after the treatment period whereas relative total growth (RTG) measures the growth of the cell cultures, relative to the control cultures, throughout the expression period and including the post expression cloning efficiency. Recent debate over the assay has raised the question of whether one measurement is preferable and whether using one measurement over the other could qualitatively affect the outcome of an assay. We have reviewed data from 94 studies performed in this laboratory covering a wide range of chemical structures. Of these studies, 16 had given a positive response, and the survival data were examined in more detail. Most (12/16) of the positive studies showed no significant difference between the RS and RTG profiles. Of the remaining studies showing significant and reproducible differences between RS and RTG, all gave lower values for RTG than for RS for any given concentration. Selecting the maximum concentration based on the RTG values, rather than the RS values, would not however, have affected the qualitative outcome of these studies. This targeted review of our database suggests that both measures of cytotoxicity are comparable for the identification of positive materials in the L5178Y assay.

14. Human DNA base excision repair measured with the comet assay reveals inter-individual differences

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DNA repair plays a key role in preventing mutations arising from DNA damage, and differences in DNA repair capacity can help determine individual cancer risk. Inherited DNA repair disorders are associated with elevated cancer risk. But whether DNA repair activity in normal subjects varies significantly is not known, largely because of the lack of a convenient, sensitive assay. We have tested two variants of the comet assay to measure repair of oxidative damage.

1. Lymphocytes were treated with H_2O_2 and incubated; at intervals, samples were analysed for DNA breaks with the standard comet assay. After several hours, breaks were still present, suggesting slow repair, but analysis is complicated since further damage seems to be introduced by exposure to atmospheric oxygen.

2. To avoid this problem, we now measure the repair reaction *in vitro*. A DNA substrate containing 8-oxoguanine is prepared by treating cells with phosensitiser Ro 19-8022 (Hoffmann-La Roche) and visible light. The cells are embedded in agarose, lysed, and then incubated for different times with crude lymphocyte extract. Incisions at damage sites are measured. Extracts from lymphocytes from 5 individuals give reproducibly different time-courses of accumulation of DNA breaks. This new assay is a potentially valuable tool for molecular epidemiological studies.

15. Mutations and inherited disease

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A wide variety of pathological lesions have been reported as a cause of genetic disease in a total of more than 1000 different human genes (see *Human Gene Mutation Database, http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html*). The most common type of mutation in gene coding regions is the single base-pair substitution. Such lesions may either alter the amino acid sequence of a protein (missense) or abolish synthesis of that protein by introducing a stop codon (nonsense). The CpG dinucleotide represents a 'hotspot' for mutation in the human genome with ~25% of all nucleotide substitutions being C·T or G·A transitions at these doublets. This is thought to be due to the propensity of methylated cytosine residues (which occur in CpG dinucleotides) to deaminate to thymine. Some single base-pair substitutions occur in splice sites or promoter regions and cause defective transcriptional activation and mRNA splicing respectively.

Micro-deletions are also fairly common and usually serve to alter the reading frame of the encoded protein. Gross deletions can remove whole exons or even the entire gene. Other types of gene lesion include insertions, duplications and triplet repeat expansions. Such mutations illustrate the principle that mutations may affect any stage of the gene expression pathway from transcription of the gene, through mRNA processing, to translation of the mRNA and the export and stability of the protein product. Whatever the type of lesion, human gene mutations are intrinsically non-random: the likelihood that a given mutation will come to clinical attention is a complex function of gene sequence complexity, the nature of the amino acid substitution, its location within the protein molecule, and its resulting effects on protein structure and function.

16. Characterization of genotoxicity screening tester strains AHH-1 and MCL-5

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AHH-1 and MCL-5 are human B-cell derived lymphoblastoid cell lines frequently used in genotoxicity studies. AHH-1 $TK^{+/-}$ is a cell line that constitutively expresses a high level of the cytochrome CYP1A1. The MCL-5 cell line was developed by transfection of AHH-1 with cDNAs encoding for five different human cytochrome and microsomal epoxide hydrolase carried in plasmids (Crespi et al, 1991). The metabolic components of these cell lines make them a useful screening tool for use in mutagenicity studies.

In the present study the cell lines AHH-1 and MCL-5

were investigated for chromosome stability by Comparative Genomic Hybridization (CGH) and Fluorescence *In Situ* Hybridization (FISH) using whole chromosome probes and telomeric probes.

The combined use of these two techniques allowed us to detect differences in chromosomal stability between the two cell lines, which cannot be attributed to transfection. The two cell lines show a modal chromosome number of 48 and the two extra chromosomes consist of the isochromosomes 3q and 9p. The distal part of the long arm of chromosome 4 is involved in amplification and translocation events although to different degrees in each cell line.

Based on these results we can deduce that although the two cell lines are closely related they have different chromosome stabilities. The AHH-1 strain presents an apparent stability in its karyotype. However MCL-5 cells appear relatively more unstable. This is further supported by our finding that 90% of AHH-1 cells have 48 chromosomes, whereas only 65% of the MCL-5 cells have the same modal chromosome number, which could suggest a lack of stability in the latter. For these reasons we advise that results of aneuploidy studies using the AHH-1 and MCL-5 cell lines should be considered with caution.

17. Detection of aneuploidy in two types of thyroid tumours by application of Comparative Genomic Hybridisation (CGH)

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The quality of cytogenetic analysis of solid tumours has greatly improved over the past decade, but a number of technical difficulties limit the characterisation of solid tumour karyotypes by conventional cytogenetics alone. The advancement of modern high-resolution chromosome banding methods combined with molecular-cytogenetic techniques such as FISH (Fluorescence *In Situ* Hybridisation) have aided the identification of chromosomal aberrations. Among the most recent addition to the FISH technique is CGH (Comparative Genomic Hybridsation), a method that combines the sensitivity of FISH while overcoming many of the limits of the classic cytogenetics, by providing an overview of genetic imbalance within the entire genome.

In this study, two thyroid tumour cell lines, BCPAP and FTC133, derived respectively from a papillary and a follicular carcinoma, have been investigated for their chromosomal stability. The thyroid tumours provide a suitable model for the study of the multi-step process of carcinogenesis. Moreover, for the purpose of our study, the polyploid nature of the cell lines offers an excellent subject for testing the applicability and the sensitivity of the CGH technique in the analysis of polyploid tumour cell lines.

CGH allowed us to detect amplifications and deletions of small chromosomal regions as well as copy number deviation involving whole chromosomes, in both cell lines. These results have also been confirmed by FISH experiments performed in our laboratory. Furthermore, we have been able to highlight some sub-chromosomal regions that might contain genes with an essential role in the cancer progression and we verified the applicability of CGH in the analysis of polyploid samples.

18. Aneuploidy in Barrett's oesophagus

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Barrett's oesophagus is a preneoplastic lesion, in which the normal stratified squamous epithelium of the lower oesophagus becomes replaced by metaplastic columnar epithelium. It develops as a complication of chronic gastroesophageal reflux disease and predisposes the patient to oesophageal adenocarcinoma. Overall a patient with this condition has a cancer risk approximately thirty fold that of the general population.

During the last two decades, a rising incidence of adenocarcinomas of the oesophagus has been observed. In general the prognosis for survival is poor, because most of these patients already have an advanced carcinoma at the time of diagnosis. However, patients with early carcinoma show a favourable long-term survival rate after surgery. Therefore, optimal treatment of Barrett's oesophagus patients requires early detection of malignancy. This has encouraged investigators to study biological parameters for use as prognostic markers, with ploidy, numerical chromosomal abnormalities and proliferation rates being of particular interest. To date, the chosen method of study for numerical abnormalities has been flow cytometry. However, as with many of the findings concerning Barrett's oesophagus the results are conflicting and disagreements as to the findings remain. This study attempts therefore to unravel the confusion into the incidence of aneuploidy at each stage in the progression of Barrett's oesophagus, with the aim of determining whether aneuploidy is an early or late event, and if present how prevalent. In addition, as this a predominately male condition the status of Y chromosome loss has also been studied.

19. An analysis of progression in Barrett's oesophagus using comparative genomic hybridisation

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The technique of Comparative Genomic Hybridization (CGH) has been used to characterise genome wide changes that occur during the progression of Barrett's metaplasia through low-grade dysplasia (LGD) and high-grade dysplasia (HGD) to adenocarcinoma. Biopsies were obtained from 10 patients with LGD, 15 HGD and 5 adenocarcinomas.

CGH allows the global analysis of the whole genome for net loss or gain of chromosome regions in a single hybridization, by competitive binding of test and genomic reference DNA to normal metaphase chromosomes. Hybridization of the DNA samples is visualised by the use of two different fluorochromes. The ratio of fluorescence intensities along each chromosome reflects the relative ratios of test and reference sequences. Thus, CGH produces a map of DNA sequence copy number as a function of chromosomal location.

The data demonstrates that high grade dysplasia is a stage of substantial karyotypic instability with amplifications detected in

all chromosomes other than 15 and the sex chromosomes. Of greatest significance was the amplification seen of 4q, present in 60% of samples. By contrast, adenocarcinomas show less widespread chromosome changes with amplification of chromosome 8q being the common feature. Indicating the presence of multiple copies of the *C-MYC* oncogene in the adenocarcinomas.

Despite ongoing efforts to characterise the molecular changes in Barrett's oesphagus, its pathogenesis remains poorly understood. It is hoped, that this work will lead to a greater understanding of the host/cell factors involved, identifying a clinically useful marker for the progression of this condition, allowing clinicians to stratify patients into high versus low risk groups.

20. Evaluation of DNA replication in Xeroderma pigmentosum variant fibroblasts using the Bromodeoxyuridine (BrdU) Comet assay

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Xeroderma pigmentosum (XP) is an autosomal recessive human disease characterised by extreme sensitivity to sunlight and a very high rate of skin cancer (Sancar 1996; Rasko and Downes 1995). XP has been classified into eight complementation groups, XP-A to XP-G and XP-Variant (XP-V) (Van Steeg and Kraemer 1999; Broughton *et al* 1995). Cells from XP-A to XP-G patients have defects in the process of nucleotide excision repair (NER) (Wood 1996; Seeberg *et al* 1995). In contrast, XP-V patients are NER-proficient but have a defect in 'post-replication repair' (PRR) of UV-damaged DNA (Boyer *et al* 1990; Maher *et al* 1976). The BrdU-Comet assay is a novel technique developed to combine the standard alkaline comet assay with bromodeoxyuridine (BrdUrd) labeling of recently replicated DNA followed by immunolocalisation of the BrdU incorporated using a fluorescein tagged antibody. This assay allows the detection of single strand breaks in the newly replicated DNA of individual cells and hence can be used to assess DNA maturation and post-replication repair in various cell types (McGlynn *et al* 1999).

In this study, the BrdU-Comet assay was used to assess replicative competency in the XP variant skin fibroblast cell line, XP6DU and in control skin fibroblasts, IBR3.7. Cells were exposed to UV-C irradiation, (254nm; 5J/m^2), and incubated in complete medium at 37°C for 1h to recover prior to BrdU pulsing. Pulse-chase experiments were carried out using a short pulse of BrdU which was then chased for a period of 2h by growing the cells in medium supplemented with normal nucleotides.

Prior to irradiation, both the control and XP fibroblast lines demonstrated a gradual reduction of % tail DNA following a 2h chase period, indicative of proficient DNA maturation and repair. A UV-irradiation-induced defect in DNA maturation was seen, however, in the XP variant line, manifested by a marked delay in the reduction of % tail DNA following a 2h chase period.

The BrdU-comet assay has demonstrated that XP-V cells have a greater than normal delay in completing replication of blocked replicons after UV-irradiation.

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21. Mutagenicity Testing Strategies in the USA

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The United States regulatory agencies, Environmental Protection Agency (EPA) and Food and Drug Administration (FDA), have worked for many years to provide mutagenicity testing strategies that are built from a consensus process. For initial testing purposes of new or previously untested chemicals/drugs, most of the testing strategies incorporate a bacterial gene mutation assay, a mammalian cell gene mutation assay (the mouse lymphoma gene mutation assay with colony sizing as the preferred choice), an *in vitro* cytogenetics assay and/or an *in vivo* cytogenetics assay in some combination, depending on the program and legal requirements. Follow-up testing examining possible germ cell heritable effects and/or a more in depth examination of the mutagenic potential of compounds is requested on a case by case basis. The various purposes for mutagenicity testing by the different agencies and their offices result in slight alterations to the initial testing battery of tests. For example, under the EPA=σ Chemical Right to Know program, initial mutagenicity testing of high production volume (HPV) chemicals is reduced to two tests (bacterial gene mutation assay and a cytogenetics assay; the cytogenetics assay is encouraged to be performed *in vitro*) due to the large number of chemicals to be tested and the screening nature of the program. For exposure-based reasons, certain EPA and FDA programs allow a smaller battery of tests (usually two) to be performed when compounds are below certain exposure levels. Mutagenicity tests used in these testing strategies need to be validated, usually through an international consensus process. The mutagenicity tests currently used in the USA mutagenicity testing strategies have this consensus.

22. Genotoxic effects and survival of lymphocytes after *in vitro* exposure to cobalt compounds

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Epidemiological studies have shown that an increased lung cancer risk is associated with occupational exposure to cobalt-containing particles, but the risk varies according to metal speciation. The evaluation of the *in vitro* genotoxic effects and

survival kinetics of human lymphocytes exposed to varying cobalt species may help to explain their *in vivo* carcinogenicity. Therefore, cobalt metal (Co), tungsten carbide (WC), a cobalt-tungsten carbide mixture (WC-Co) and cobalt chloride (CoCl2) were compared for several endpoints at a concentration of 2.0 µg/ ml Co-equivalent: 1) Solubilisation rate of cobalt ions in the culture medium by AAS. After 24h incubation the amount of cobalt ions present was in increasing order: WC-Co < Co < CoCl2. 2) Proportion of apoptotic, necrotic and viable cells by annexin-V staining. Apoptosis was induced, early after onset of treatment (15 min), mainly by WC and by WC-Co and at a later stage also by Co. CoCl2 induced mainly early necrosis. Inhibition of the ceramide-apoptosis pathway by fumonisin and subsequent reduction of apoptosis induction by cobalt compounds is consistent with the production of oxygen free radicals during the solubilisation of cobalt (Lison *et al*., 1995). 3) Inhibition of repair of UV-induced DNA lesions by the alkaline comet assay. Previously, it was demonstrated that cobalt ions and metallic cobalt inhibit the repair of UV- and MMS-induced DNA lesions, respectively (Kasten *et al.*, 1997; De Boeck *et al*., 1998). The present results show that mainly the incision step of nucleotide excision repair is affected. The inhibition is dependent on the compound and the pH of the assay. 4) Induction of chromosome breakage versus loss by combination of the *in vitro* micronucleus test with FISH (pancentromeric probe). This analysis complements the previous *in vitro* genotoxicity studies demonstrating an elevated effect of WC-Co as compared to Co using the alkaline elution and comet assays and the micronucleus test (Anard *et al*., 1997; Van Goethem *et al*., 1997; De Boeck *et al.,* 1998). Cobalt compounds are thus able to induce *in vitro* several types of (geno)toxic damage in human lymphocytes: apoptosis, DNA damage, DNA repair inhibition and chromosome/genome mutations. Their relevance for cancer induction will be dependent on the capacity to cause cell death and subsequent repair proliferation. The WC-Co mixture combines the capacities to induce apoptosis and DNA damage which might contribute to its enhanced carcinogenic potency.

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23. Use of Atlas cDNA expression array technology in rapid screening of genotoxic chemicals

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Gene expression array technology has matured, in the past few years, from an exciting and promising new method into one now in common use. The use of gene expression array technology to elucidate mechanisms of toxicity is becoming widespread and may supersede the Northern blot due to ease, versatility and extremely high throughput. Atlas cDNA expression array technology is described in detail along with the data generated using the carcinogen and oxidative stressor, potassium bromate ($KBrO_3$). This presentation shows the applications and advantages of Atlas cDNA expression array in rapid screening of genotoxic chemicals.

24. The effect of smoking and eating habits on DNA damage in an Indian population as measured in the Comet assay

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This study was undertaken with the aim of assessing DNA damage in the peripheral blood lymphocytes of a normal healthy Indian population using the comet assay. The 62 male volunteers, from Lucknow city aged between 23-57 years, in this study were classified into the smoking, non-smoking, vegetarian and non-vegetarian categories. A significant increase ($p<0.001$) was observed in DNA damage in smokers when compared to non-smokers. When the smokers and non-smokers were separated on the basis of their eating habits into vegetarians and non-vegetarians, a significant increase ($p<0.05$) in DNA damage was still observed in smokers in both groups when compared to the respective non-smokers. Tail moment, the most sensitive parameter measured in the comet assay, was found to increase significantly ($p<0.001$) with increase in the intensity of smoking (smoke pack years) when compared to the non-smokers. When the population was separated on the basis their eating habits, the non-vegetarians showed a significantly higher DNA damage ($p<0.001$) when compared to the vegetarians. A significant ($p<0.001$) age related increase was observed in the DNA damage when compared with the age group of 20-29 yrs. This preliminary study has, for the first time, revealed differences in the extent of DNA damage in the normal Indian population depending on their eating and smoking habits as well as age.

25. The application of cytogenetic and comet assays in marine genotoxicity studies

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The application of cytogenetic methods to marine invertebrate subjects has been hampered by a naturally low mitotic index (due to poikilothermy), lack of *in vitro* cell lines (due to an absence of mitogens and haemopoietic tissues), inadequate karyotypic information (due to too few workers chasing too many organisms) and a failure by chromosomes to band satisfactorily (due to a fundamental difference in chromatin packaging). Nevertheless, some progress has been made over the past 2-3 decades in this research area, particularly by the use of embryo/larval cells, but complicated culturing requirements and seasonal constraints have restricted their use to only a few specialist laboratories worldwide. Given these difficulties, it is not surprising that a considerable amount of effort has been devoted to exploring alternative approaches for the measurement of DNA damage in cells and tissues of aquatic subjects. The comet assay offers considerable advantages over cytogenetic assays since it is relatively cheap and rapid to perform and there is no requirement for cells to be in a dividing state. Furthermore, there is no requirement for any detailed knowledge of the karyotype; an important consideration when dealing with little known species. However, there is still the problem of taking the electrophoresis part of

the method into the field, an important consideration when working in an ecogenotoxicological context at some distance away from the laboratory. Using our recent studies of deep-sea hydrothermal-vent organisms as an example, we report here on a modification of the comet assay that has proved practically useful when working 'on board' a less than stable research ship for weeks at a time.

26. A simple scale for comparison of risks to our lives

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The proposals contained in this talk are the result of development work by the speaker following an initiative of the Royal Statistical Society to provide a simple device, sometimes referred to as a 'riskometer', to enable the general public to make rational decisions where risks to life and limb are concerned.

The risk scale proposed is only intended to be used by an individual who may voluntarily (or involuntarily) expose him/herself to the potential hazard. It is not concerned with industry or society who may by some activity impose a risk on others. It is a logarithmic scale which goes from zero, for a very low risk, to 8, where the activity has 100% certainty of instant death, and in this respect is similar to the Richter scale.

The scale provides a measure of the danger of any activity relative to this maximum value. It takes account of hazards where the consequence may be something less than death and where it is not immediate but deferred. In doing this it makes allowance for the fact that the consequence may be mitigated before it takes its toll; for instance continuing improvements in effective treatment for cancer may reasonably be expected.

Distinctions are also made between one-off risks and those due to chronic or habitual exposure, the scale number for the latter being determined by integration over the remaining expected lifetime.

Some crudely calculated risk numbers illustrate the use of the scale for a wide variety of risky activities, and these show how seemingly incomparable activities may be simply compared; for instance, a 35 year old man smoking 40 cigarettes a day for the rest of his life is taking a comparable risk to playing a single game of Russian roulette.

27. Genotoxic activity *in vivo* of four spices in the *Drosophila* wing spot test

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The wing Somatic Mutation And Recombination Test (SMART) in *Drosophila melanogaster* were used to evaluate the genotoxicity effect of four spices, Cinnamon, Ginger, red and black pepper. The study was performed by a standard cross (ST), with a normal bioactivation, and high bioactivation cross (HB) which is characterised by a high cytochrome P-450 dependent bioactivation capacity. The third instar larvae, transheterozygous for two recessive mutations of wing trichomes, multiple wing hairs (mwh) and flare (flr3), were treated by chronic feeding with the test compound mixed to a standard dry. The wings from the resulting flies were scored, under 400X magnification, for the presence of clones of cells possessing malformed wing-hairs. Such clones appeared as mwh-flr3 twin spots and single spots of either mwh or flr3 phenotype. The result show that ginger increase significantly (<0.05) the small (1 or 2 cells) single spot, both in ST and HB, compared to the other three spices. For the large ($>$ 2 cells) single and twin spot, the results were not significant.

28. Mutagenicity of 2-Amino-1-methyl-6-phenylimidazo [4,5-*b*]pyridine (PhIP) in the germ cells of male *lacZ* transgenic mice (Muta™Mouse)

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The genotoxic carcinogen PhIP, has been investigated for its mutagenicity in germ cells at the lacZ locus of male transgenic mice (Muta™Mouse). In an initial study male mice were administered 20 mg/kg p.o for 4 days and sacrificed 7 days later. High molecular weight testicular DNA was extracted, and the lambda vectors containing the lacZ reporter gene were recovered by in vitro packaging. Bacteriophage plaques were analysed after infecting *E. coli.* C (*lacZ*$^-$ *galE*$^-$) under a positive selection system in the presence of phenyl-β-galactoside. Suspected mutants were confirmed by re-plating with *E. coli.* C (*lacZ*$^-$) and X-gal. The mutation frequency (MF) was calculated for the PhIP- treated, and vehicle control groups. Initial analysis of the data revealed that PhIP caused a 2.5-fold increase in MF compared to the vehicle control, which is marginally positive under these experimental conditions. We investigated further these initial findings in a second study, undertaken with a similar treatment regime, but extended to included a longer manifestation period of 25 days (c.f 7 days) to allow for germ cell maturation. The results obtained from this second study showed that PhIP caused only a 1.3-fold increase in MF above background. Our experiments suggest that on sub acute exposure PhIP is equivocal as a germ cell mutagen.

29. Consultation on revised COM guidelines on strategy for testing of chemicals for mutagenicity

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The Committee on Mutagenicity of Chemicals in Food, Consumer Products and the Environment (COM) is an independent expert advisory committee whose members are appointed by the Chief Medical Officer (CMO) for England. Its remit is to advise those government departments/agencies with regulatory responsibility for chemicals in various sectors on all aspects of the mutagenicity of chemicals. Their existing guidelines were published in 1989 and need updating. The COM issued a consultative document in February 2000 on a strategy for testing chemicals for mutagenicity. It is expected

that this will be influential in development of testing strategies by the regulatory agencies over the next few years.

In outline the revised draft guidelines are similar to the existing guidelines. Three stages are recommended. Stage 1 involves screening for mutagenic activity in vitro and comprises 3 test systems. The main change is that this now includes screening for aneugenicity. The 3 tests proposed are a bacterial assay for gene mutation, a test for clastogenicity and aneugenicity (in vitro micronucleus test or metaphase analysis) and a mammalian cell mutation assay. The later test is not recommended for those substances where there will be little or no human exposure. Stage 2 consists of in vivo testing in somatic cells. The main purpose is to ascertain whether activity seen in vitro can be expressed in vivo. However one in vivo test is recommended for those compounds negative in vitro but for which exposure is expected to be high or moderate and sustained. It is recognised that the initial test will in most instances be a bone marrow (or peripheral blood) assay. A second test is needed before conclusions can be drawn regarding absence of in vivo activity. A range of assays are given for consideration, but the appropriate assay needs to be identified on a case by case basis. Stage 3 comprises in vivo testing in germ cells. It is recognised that this stage is necessary only when it is important to consider the potential of somatic cell mutagens to affect germ cells.

30. Evaluation of FISH in the assessment of genetic risk in occupational exposure to ionizing radiation and ultrasound: a case report

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For several decades, chromosome aberration assay (CA) has been the recommended method for cytogenetic risk estimation following clastogenic exposure to humans. The method is based on the analysis of unstable genome damage, which decreases significantly 6 months after cessation of exposure. However, the introduction of FISH enabled detection of stabile genome damage accumulated over a lifetime. In order to evaluate the significance of FISH in the assessment of genome damage, we followed up a subject exposed occupationally to 192 Ir and ultrasound who showed a significant increase in chromosome aberrations. The annual dose measured by physical dosimetry was below 50 mSv. In a period of 12 months the results of two blood samplings showed a significant increase in bicentric chromosomes, acentric fragments and chromatid breaks. The frequency of translocations measured by FISH (0.02) was significantly elevated over published control (0.003 translocations per cell); p = 0.018). After cessation of exposure to Ir exposure, blood samples were taken twice. The results of CA approached the control values while the frequency of translocations was still significantly increased. We conclude that FISH is a potent and superior method for the estimation of genome risk after exposure to clastogens, which would have been overlooked in this described case had we used the chromosome aberration assay.

31. Is the *in vitro* micronucleus a suitable alternative to *in vitro* metaphase analysis using cultured cells?

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The *in vitro* micronucleus assay has many advantages over other *in vitro* assays. The main advantage being that it is a multi-endpoint assay that can be used to evaluate many different genotoxic endpoints such as mitotic delay, apoptosis, chromosome breakage and chromosome loss. It has the potential to detect both clastogens and aneugens, however it is unable to detect gene mutations. It is technically less demanding and less time consuming than metaphase analysis, has high statistical power and has the potential for automation using image analysis.

Compound X is an intermediate in the synthesis of a new antiviral dug, and has been assessed for genotoxicity in a wide range of *in vitro* and *in vivo* assays. In the majority of *in vitro* assays (Ames, MLA, L5178Y comet, cytogenetics using CHL cells) it has produced a positive result. However, in the *in vitro* micronucleus using L5178Y cells it has produced a negative result, leading us to question the validity of the *in vitro* micronucleus test. Both the *in vivo* mouse micronucleus assay and *in vivo* mouse comet assays targeting bone marrow and liver were negative after exposure to compound X. However an *in vivo* 'site of contact assay' (eg comet induction in rodent stomach) has yet to be carried out on this compound.

Further investigations into the genotoxicity of compound X were carried out to elucidate the type of DNA damage being caused, and to explain the negative data sets. Two additional *in vitro* studies that have not been previously carried out using compound X were performed; a cytogenetics study using human peripheral lymphocytes and an *in vitro* micronucleus also using human peripheral lymphocytes. The cytogenetics assay gave a positive result whilst the *in vitro* micronucleus was negative. In addition fluorescence *in situ* hybridisation has been carried out on metaphases prepared from mutants isolated from the L5178Y mouse lymphoma assay.

In conclusion compound X is highly genotoxic in a majority of in *vitro* assays. However this positive response is not expressed in the *in vitro* micronucleus using either L5178Y mouse lymphoma cells or human peripheral lymphocytes. The current draft COM guidelines suggest that *in vitro* metaphase analysis and the *in vitro* micronucleus assay can be regarded as equivalent tests for detecting chromosome damage. This data set does not support this statement. The negative *in vitro* micronucleus data also calls into question the relevance of the *in vivo* micronucleus test in terms of safety assessment?

32. The concept of pseudo-thresholds for mutagenicity

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We are frequently told that unlike other toxicological endpoints, there is no threshold for mutagenicity and that any exposure to a mutagenic agent is potentially harmful. Although this concept may be true in theory, I suggest that it may be misleading and counterproductive in practice. The mutagens that we screen exist against a background of components of

our environment, which cannot be avoided and are capable of causing genetic damage. Furthermore our bodies produce a wide variety of endogenous DNA damaging agents, including reactive oxygen species, nitric oxide, alkylating agents and products of lipid oxidation. Our ability to respond to this onslaught of damage is itself under the influence of the environment, and indeed some protective mechanisms are inducible. Thus induced mutation must be considered against the level of background mutation. If background mutation were an unchangeable constant, then there might be a case for considering very much smaller induced effects as important, but background mutation is variable and is itself influenced by the environment. This has two implications: Firstly it is difficult or impossible to judge very low dose effects accurately against a variable background mutation rate. Secondly, strategies to modify the 'background' mutation rate may be the most relevant to reducing the overall human mutation burden. The corollary of these considerations is that although there may indeed be no true threshold for a mutagen, there may be a pseudo-threshold, where other factors will have a far greater influence on the overall mutation rate, and where it would be far simpler and more cost effective to modify these. If we recognise the reality of pseudo-thresholds and can generate a useful working definition, it may help us focus resources on where they will have the greatest impact on health and it may help us to avoid basing choices between products on an exaggerated emphasis on genotoxicity.

33. Evaluation of DNA damage induced by three genotoxins with different modes of action in the haemocytes of the marine mussel, *Mytilus edulis*

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Induction of DNA damage was evaluated at the molecular and cellular levels using the comet and micronucleus (MN) assays, from haemocytes collected from the bivalve mollusc, *Mytilus edulis*, at intervals over a period of time. Three genotoxins, with different modes of action, Ethyl methane sulphonate (EMS) (a direct acting alkylating agent), Hydrogen peroxide (H_2O_2) (which produces free radicals and possibly triggers the activation of nucleases) and the beta emitting radionuclide tritium, (in the form of tritiated water) were used in this study.

The comet assay detected the maximum DNA damage after 1 hour for all three genotoxins. After 24hrs the H_2O_2 exposed mussels showed no significant difference in comparison to the controls, although the EMS and tritium exposed mussels still showed evidence of DNA damage. After 48 hours there was no difference between the tritium exposed mussels and the controls, however the EMS exposed mussels still showed a significant difference in the number of normal haeomcytes compared to controls. On the other hand, the induction of MN was only observed following 24 hours in all of the genotoxic exposed mussels with a slight increase at 48 hours.

The studies suggested that the induction of MN is generally observed following 24 hours exposure irrespective of the mode of action of the genotoxin used over a range of concentrations. In comparison, the comet assay showed an increased sensitivity for detection of DNA damage induced by various genotoxins. The H_2O_2 produced short-term effects, followed by tritium and finally EMS, which produced a continuous level of DNA damage.

34. Japanese mutagenicity testing strategy

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In Japan, there are different genotoxicology guidelines and strategies for chemicals used in different fields Pharmaceuticals and agricultural chemicals, for example, are subjected to risk assessment and an approval system, while industrial chemicals are subjected to hazard identification and a classification system. This presentation focuses on industrial chemicals. The Ministry of International Trade and Industry (MITI) and the Ministry of Health and Welfare (MHW) adopted the Chemical Substances Control Law promulgated in 1973, amended in 1986, and revised in 1997 to protect humans from exposure to hazardous substances through the environment that have the properties of persistence and bioaccumulation, and are potentially toxic. If newly produced or imported chemicals ($>$1 ton/year) are neither degradable nor bioaccumulated, then screening toxicity tests are required. The screening tests consists of the 28-day repeated-dose toxicity test, the reverse mutation assay in bacteria (corresponding to OECD guideline 471), and the chromosomal aberration (structural and numerical) test in cultured mammalian cells (corresponding to OECD guideline 473). The law requires evaluation of the toxic and genotoxic potential of chemical substances semi-quantitatively by application of a ranking system. The 'no observed effect level' (NOEL, mg/kg body weight/day) for toxicity, 'the number of induced revertants per a given dose (mg/plate)' (Mutagenic activity, revertants/mg) for the reverse mutation assay in bacteria, and 'the estimated dose at which chromosomal aberrations are observable in 20% of metaphases' (D20, mg/ml) for the chromosomal aberration test in cultured mammalian cells. Chemicals are evaluated first by these values and then the effects are characterized more precisely by experts. Then each chemical is classified as a 'designated' or 'no restriction' chemical. The designated chemicals are monitored by post-marketing surveillance and, if necessary, are subjected to full-scale toxicity tests, including an in vivo genotoxicity assay (*e.g.*, mouse bone marrow micronucleus assay).

35. Determining biological damage in marine invertebrates resulting from environmental contaminants

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The consequences of environmental pollution for wildlife inhabiting contaminated sites are complex. In many cases of accidental contaminant release into the environment there is an obvious acute effect where ecological damage is easily identified. However, the long-term effects due to prolonged

exposure to complex mixtures of low levels of contaminants are very difficult to detect and measure. Despite this, there is an increasing realisation that environmental monitoring practices need to ensure the protection of the environment as a whole. This will require an approach that can first quantify the biological damage and then secondly, link this damage with the health of the species under assessment.

This project is investigating the level of biological damage in marine invertebrates of the north-east Irish Sea. The level of damage will be related to three potentially contributing factors - organic chemicals, metals and radionuclides - through the course of the study. Recent work has now assessed the feasibility of applying genotoxic techniques, derived from human monitoring of radiation exposure, to marine invertebrates. We describe the techniques and their application in quantifying biological damage as the first part of an ecological risk assessment for non-human species.

36. Cationic thiols enhance the clastogenicity of bleomycin in lymphocytes

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The anticancer drug bleomycin (BLM) is a radiomimetic clastogen, resembling ionizing radiation in the S-phase independence of its action and the induction of chromosome-type aberrations in G_0. Bound to DNA noncovalently as Fe^{+2}-BLM, it is activated by the addition of oxygen, followed by a one-electron reduction. Activated BLM, a ferric hydroperoxide, abstracts a hydrogen from the 4′ position of deoxyribose. Addition of oxygen to the resultant free radical forms a peroxyl radical whose decomposition leads to a strand break. The same molecule of BLM can then be reactivated in situ, cleaving the complementary strand to leave a double-strand break.

The radioprotective aminothils (2-(3-aminopropylamino) ethanethiol (WR-1065) and cysteamine (CSM) exhibit antimutagenic effects against BLM in some assays, especially under hypoxic conditions. However, these compounds enhance, rather than diminish, the radiomimetic clastogenicity of BLM in G_0 human lymphocytes. The cytokinesis-block micronucleus assay was used to determine whether related compounds similarly potentiate BLM. Amines that lack a thiol group [spermine; spermidine; putrescine; 2-(3-aminopropylamino) ethanol] potentiate the clastogenicity of BLM more weakly than do the aminothiols, and they do so only at high doses. Their action may stem from conformational alterations that make the 4′ position more accessible to BLM attack. Thiols lacking an amino group [dithioerythritol; 2-mercaptoethanol] weakly potentiate BLM. Thus, the strong potentiating effect of the aminothiols is ascribable to the combination of the amino and thiol groups rather than to either alone. The thiol may provide electrons for BLM activation or for the reduction of inactive Fe^{+3}-BLM. The amino groups target this redox mechanism to the site of BLM action on DNA. Thus, cationic thiols, which bind to DNA, are effective potentiators, whereas thiols lacking an amino group are not. The charge (Z) can explain the greater potency of WR-1065 ($Z = +2$) than CSM ($Z = +1$) and the relative ineffectiveness of related compounds that lack a net positive charge at physiological pH. Unlike WR-1065 and CSM, cysteine ($Z - 0$) and N-acetylcysteine ($Z = -1$) potentiate the clastogenicity of BLM in G_0 lymphocytes only very weakly.

37. Dietary genotoxins - always a bad thing?

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It is known that diet plays an important role in the development of certain cancers and compounds such as heterocyclic amines (HA) have been identified as dietary genotoxins. Mutagenesis is a complex process and the overall biological effects of dietary genotoxins interacting with each other and with environmental or endogenous genotoxins within cells of target tissues are poorly understood. In this study, the HA, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*] pyridine (PhIP), and free radicals generated during normal cellular metabolism are being used as examples of dietary and endogenous genotoxins, respectively. HA, found in well-cooked meat, are activated predominantly in the liver to produce reactive species which can form DNA-adducts. HA have been shown to induce tumours within target tissues in rodent models. *In vitro*, these HA-DNA adducts have been shown to be rapidly removed by cellular DNA repair although the repair mechanisms involved are not clearly defined. Although oxidative DNA damage caused by free radicals generated from cellular metabolism, is constantly repaired by the cell, such damage is thought to play a role in mutagenesis. The aim of this project is to determine *in vitro* whether a dietary genotoxin, PhIP, has a beneficial, neutral or deleterious effect on DNA oxidative damage caused by cellular free radicals, perhaps by inducing DNA repair.

Studies on short-term primary cultures of human hepatocytes and human colorectal cells are being supplemented with experiments on the human cell lines Hep G2 (hepatocyte) and HT29 (colorectal). Assays which have been adapted to measure oxidative damage are the Comet assay, which monitors global DNA repair in single cells, and ligation-mediated PCR, which measures DNA damage within a cell population at a specific selected gene, p53. Induction of transcription of repair enzymes has been analysed by RT-PCR, and DNA repair activity is measured by the incorporation of radiolabelled nucleotides into an oxidatively damaged DNA template using cell extracts. DNA-PhIP adduct formation is monitored using the accelerated mass spectrometer at CSL.

In initial studies using a human stress cDNA array to detect mRNA, no significant induction of DNA repair enzymes was detected in Hep G2 cells after incubation with PhIP (20 μM for 16h). Therefore real-time RT-PCR using an ABI 7700 Sequence Detection System has now been set up to study the induction of DNA repair enzymes after PhIP treatment. To date, no effect of PhIP treatment has been detected on actual repair activity in HepG2 cells using the cell-free assay. Initial experiments using the Comet assay suggest that HT29 and Hep G2 cells show a similar dose-response to oxidative damage. All these techniques will now be extended to study both cell types.

The information gained from this study will, it is hoped, give an insight into whether threshold levels for HA can realistically be established.

38. The development of the Polymerase Inhibition (PI) assay, a high-throughput screen for genotoxic chemicals

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We have developed a high-throughput screen for the detection of genotoxic chemicals based on the polymerase chain reaction (PCR) (1). The assay relies upon the fact that chemically induced DNA damage is often a hindrance to the DNA polymerases used in PCR. Hence, DNA damaging agents can be identified by a corresponding drop in PCR efficiency post treatment. We present here data showing the optimisation of the assay using UV light and highlight the parameters identified as important in maximising the sensitivity of the assay. In addition, we present data on the characterisation of the polymerase inhibiting effect of known DNA damaging agents as examples. These examples include alkylating agents (ENU and MNU), bulky heterocyclic amines (N-OH-PhIP) and ROS generators (H_2O_2). The success of the PI assay in identifying the DNA damage induced by such chemical genotoxins holds promise that this methodology may be suitable for high-throughput screening of putative genotoxins. The high-throughput status of this methodology stems from the fact that the assay is performed in microplates with an immobilised DNA target, which is damaged and subsequently amplified.

The main drawback of the methodology, at present, is that it is not capable of analysing chemicals which require metabolic activation. The use of S9 mix or commercially available microsomal preparations of P450 enzymes, results in the destruction of the immobilised DNA target by contaminating nuclease enzymes. However, the introduction of purified P450 enzyme cocktails should overcome this hurdle.

Reference

Jenkins *et al* (2000). *Molecular Carcinogenesis*, **27**, 289-297

39. Determination of highest concentration for analysis in the human lymphocyte *in vitro* cytogenetics assay

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Primary human lymphocytes are a commonly used cell type for regulatory *in vitro* cytogenetics (IVC) assays. Regulatory guidelines recommend that toxicity in these cultures is determined by measurement of the depression in mitotic index (MI) produced by a test substance relative to the concurrent solvent control. The highest concentration used for metaphase analysis should produce a depression in MI of at least 50%. Examination of a large number of data sets within this laboratory showed that depression in MI alone was the criterion for dose selection in only 6% of the cases. For the majority of test substances cytotoxicity, as manifested by changes in chromosome morphology (*e.g.* fuzzy or indistinct chromatids) was the main criterion for choice of highest concentration. In some cases this coincided with an appropriate reduction in MI. In most cases, 50% MI depression was not achieved but analysis of higher concentrations was not possible due to the observed cytotoxic effects on the chromosome morphology. Examination of MI values for positive control cultures in these experiments showed a range of 30-100% of concurrent solvent controls, demonstrating the variability of MI as an indicator of toxicity over repeated experiments. We recommend that the highest concentration analysed in these assays be chosen based on an assessment of both chromosome morphology and MI depression.

40. Practical interpretation of the revised OECD guidelines for genotoxicity testing: the recommendations of the Industrial Genotoxicity Group

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Revised guidelines for the core genotoxicity assays were published by the OECD in 1998. As with all regulatory guidelines there are areas where legitimate alternatives are allowed. However, there are also points which are open to a number of interpretations. The Industrial Genotoxicity Group (affiliated to UKEMS) convened a panel of experienced genetic toxicologists to review the guidelines and, where alternatives or uncertainty existed, to make recommendations on best scientific practice to aid in consistent interpretation of the guidelines. The review covered the five core assays: bacterial mutation; *in vitro* cytogenetics, mammalian cell gene mutation (with particular emphasis on the L5178Y $TK^{+/-}$ assay); erythrocyte micronucleus; *in vivo* UDS.

General points covered by the review included: presentation and use of historical control data; definition of maximum dose level; evidence of absorption/tissue exposure in *in vivo* assays; justification for use of a single sex in *in vivo* assays; strategy for repeat assays in *in vitro* assays; testing of insoluble materials.

Assay specific recommendations included the following topics.

Bacterial mutation assay: strategy for anti-bacterial compounds; choice of strains.

In vitro cytogenetics assay: measurement of polyploidy; test design; cell cycle measurement.

Mammalian cell gene mutation assay: colony size data for L5178Y; design of repeat test.

Erythrocyte micronucleus test: choice of dose route.

Liver UDS assay: study design.

The details of the panel's recommendations will be given in this presentation to enable them to be discussed within the genetic toxicology community.

41. From epidemiology, through risk assessment to standards design: formulations of the new WHO Guidelines for recreational waters

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Compliance of recreational waters with current EU standards costs Wales alone some £1.3 billion. This cost has been incurred in an attempt to achieve the Guideline values specified in Directive 76/160/EEC. This is required to gain the status

of a European Blue Flag beach. However, present standards have no clear public health foundation and compliance cannot be justified in 'health gain' terms. This was recognised by the European Commission in 1994 when a new proposal was published for amendments to Directive 76/160/EEC. This proposal was heavily criticised by the UK House of Lords Select Committee Enquiry in 1995 which, again, could find no clear public health foundation for the proposed standards.

The UK has completed a series of epidemiological investigations which provide the basis of 'health based' standards design for recreational waters. The data from these epidemiological investigations has been linked to a new 'disease burden' approach to facilitate quantification of both present health impacts and the 'health gain' attributable to remediation schemes. This offers potential for 'benefits assessment' to set against the very high costs of compliance. The science underpinning this approach has recently been used in the design of new WHO Draft Guidelines, now in consultation draft stage. These Guidelines are now the subject of much debate but offer a useful way forward limiting adverse health impact of coastal recreational exposures.

42. Evaluation of steroid hormones for micronuclei induction in human cell lines

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In contrast to the mechanisms of carcinogenesis produced by chemical and viral agents the hormone related cancers have a different mechanisms in which hormones, both endogenous and exogenous, by driving the cell proliferation; increase the opportunity for random genetic errors or mutations. There has been considerable discussion on the cancer hazard of synthetic hormones such as those used in some countries to promote animal growth. Currently the available data on the possible genotoxicity of the compounds is confused and often misinterpreted.

In this study cytokinesis blocked micronucleus assay is used to evaluate six steroid hormones, i.e. 17-$ Estradiol, Progesterone, Testosterone, Zearanol, Trenbolone and Megesterole acetate, for their ability to induce micronuclei in the primary human fibroblast cell line Wills1 and human lymphoblastoid cell line MCl-5. Apoptosis and Necrosis is also measured using Cytokinesis blocked micronucleus technique as inclusion of these parameters is important for the accurate description of mechanisms of action and measurement of cellular sensitivity to a chemical. The CBMN assay has been used for assessing the induction of both structural and numerical aberrations by chemical agents. Clastogenic and aneugenic responses may be distinguished by the visualisation of kinetochore proteins by immunofluorescent labelling.

43. A high throughput genotoxicity screen using eukaryotic cells: reagent free and metabolically competent

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Current genotoxicity assays monitor either mutagenicity or induction of DNA repair in prokaryotes including *Salmonella*, *Escherichia* and *Vibrio*. These bacterial cells are not particularly robust, and they differ from (eukaryotic) mammalian cells in such factors as uptake, metabolism, chromosome structure and DNA repair processes. Tests conducted *in vitro* generally require the use of an exogenous source of metabolic activation. Such tests can be a misleading source of information on the mutagenic and carcinogenic potency of a substance in mammals. We are developing a genotoxicity test using yeast cells, which is both robust and eukaryotic. Repair activity is linked to synthesis of the Green Fluorescent Protein (GFP), which is very stable and can be estimated non-invasively. Expression of a mammalian cytochrome P450 in yeast leads to *in vivo* metabolic activation, allowing detection of the promutagens aflatoxin B1 and 2-amino anthracene.

44. Detection of DNA damage by the Comet assay in MCL-5 cells exposed to extracts of urban air particulate matter

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The Comet assay can provide an important indication of the genotoxic potential of airborne particulates. The DNA damaging activities associated with inhaleable particulate matter (PM10) collected from Faisalabad (an industrial city of Pakistan) have been determined using the Comet assay. Air samples from seven different locations of the city were collected on fibreglass filters using a high-flow air sampler. To study the induction of comets by airborne particulate matter, MCL-5 cells were exposed for 30 minutes at 37^{B}C to both inorganic (50% nitric acid) and organic (methylene chloride) extracts of the air particulates. These cells were then subjected to alkaline single cell gel electrophoresis under optimised conditions. Out of 7 organic extracts, 3 induced significant DNA damage to MCL-5 cells; comets with mean tail length greater than 50µm were observed. Inorganic extracts of the same samples were also positive. Longer exposure (18 hours) of MCL-5 cells to air particulate samples resulted in higher cytotoxicity and longer mean tail length of comets. To determine the effect of DNA repair inhibitors on comets, additional studies were set up to perform the Comet assay with or without repair inhibitor compounds (HU/araC). Relatively higher number of comets with greater tail lengths were induced in the presence of repair inhibitors than in their absence. The results of the present study indicate that there is a need to monitor airborne particulates on a regular basis for their potential to damage the genetic material of living organisms.

45. An investigation of damaged mitochondrial DNA in smoking related Warthins Tumour

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The epithelial cells of Warthins tumour are characterised by numerous prominent and often pleomorphic mitochondria. In conjunction with mitochondrial structural abnormalities, previous studies have shown an alteration in respiration, respiration control and oxidative phosphorylation. Recent surveys show a strong correlation (96%) between the development of Warthins tumour and cigarette smoking. Therefore we hypothesised that the mitochondrial proliferation could be a compensatory mechanism for a reduction in ATP synthesis due to a high percentage of non-functional mitochondria caused by increased oxidative damage. Increased oxidative stress in the mitochondria would lead to an increase in mitochondrial DNA (mtDNA) damage including deletions which can be measured quantitatively by the polymerase chain reaction (PCR). We observed the presence of mtDNA deletions in archival tumour and normal parotid samples. One of the deletions was quantified and a significantly high level was observed in all tumour samples. We also developed 2-colour fluorescent in-situ hybridisation (FISH) to detect mtDNA deletions in paraffin wax-embedded sections allowing the technique to be used on archival material.

46. The Mammalian Gene Mutation Database: a major new resource for molecular biologists

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The Mammalian Gene Mutation Database (MGMD) has been constructed to provide a comprehensive collection of data on somatic mutations detected in mammalian cells following exposure to chemical and physical agents. The database is comprised of published base pair substitutions, deletions, duplications, insertions and more complex rearrangements in *sup*F, *HPRT, APRT, gpt, DHFR, Lac*I and *Lac*Z. The database is accessible through the Internet (http://lisntweb.swan.ac.uk/cmgt/index.htm) where the user is able to search for specific mutation data via a user-friendly multiple choice search page. The relevant mutation data is then displayed online and may be saved or copied directly into the user's database, spreadsheet or word processing package. Each mutation entry in MGMD includes an unique identifier, full mutagen/dose details, species, tissue origin, cell and gene details, a standard unambiguous mutation description, sequence change, amino acid change (if applicable), mutation frequency description, the reference abstract source and access to the genes cDNA sequence. The mutation spectra information in the database will be used to study causal relationships that exist between the mutations induced in specific genes by environmental mutagens and the induction of human genetic disease. These analyses will attempt to answer questions such as which parameters influence the induction of a gene's mutational spectra? How do laboratory induced mutation profiles compare with those found in tumours? Why do specific mutation hotspots exist in target genes? While the database has been developed for research purposes it is hoped that it will acquire a broader utility among researchers in the field of genetic toxicology.

47. Use of biomarkers in studies of personal exposure and risk from air pollution

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Biomarkers of exposure, susceptibility, and genetic damage from air pollution were studied in the US, Japan, and the Czech Republic. Personal exposures to airborne particulate polycyclic aromatic hydrocarbons (PAH) were measured using personal exposure monitors that collected fine particles (#1.7Φm). A wide range of personal exposure concentrations were measured over several orders of magnitude. Exposure biomarkers included urinary PAH metabolites, cotinine, and trace metals in blood. Biomarkers of genetic damage measured in blood included DNA adducts, protein adducts, chromosomal aberrations, and sister chromatid exchanges. Mutagenicity in urine was measured in several Salmonella tester strains. The influence of metabolic susceptibility (GSTM1 and NAT2 genotypes) on the association between personal air exposures and biomarkers of exposure, dose, and genetic damage were also evaluated. At environmental exposures to fine particles ($<$1- 140Φg/m^3), the personal exposures to carcinogenic PAH ranged from 2 to 26 *n*g/m^3 and these measures were highly correlated ($r = 0.79$, $p < 0.0001$). Personal exposures to carcinogenic PAH were significantly correlated with total urinary PAH metabolites ($r = 0.48$, $p = 0.0002$) and two of the trace element levels in blood, selenium ($r = 0.55$, $p < 0.0001$) and lead ($r = 0.39$, $p = 0.003$). The urinary PAH metabolites and trace element levels in blood provided potential tracers for apportioning exposure to the specific combustion sources. The selenium was associated with high sulfur coal and lead with vehicles. The PAH profiles in each geographic region were consistent with the PAH profiles of combustion sources in each region. DNA adduct levels were significantly correlated with PAH exposure for a sub-group of the nonsmokers ($r = 0.36$, $p<0.050$) and both the correlation and significance were increased in the GSTM1 genotype group ($r=0.59$, $p<0.005$). There was also a significant increase in correlation between personal PAH exposure and urinary PAH metabolites for NAT2 slow acetylators ($r=0.58$, $p=0.001$) compared to NAT2 rapid and GSTM1 genotypes. Variations in metabolic genotypes accounted for some of the inter-individual variability observed in these biomarker measurements. At higher PAH occupational exposures in coke oven workers, the exposure-DNA adduct relationship became nonlinear and stratifying the subjects by genotype and smoking status did not alter the lack of an exposure-response relationship.

48. The Polymerase Stop Assay for the determination of DNA adduct frequency, single strand breaks and double strand breaks on the human p53 gene exposed to increasing doses of various chemical mutagens

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The Polymerase Stop Assay (PSPCR) is a quantitative PCR based assay that provides evidence of DNA damage induced by various chemical and physical mutagens (McCarthy *et al.*1996). The unrivalled ability of the polymerase chain reaction to exponentially amplify a given target region of DNA is

successfully exploited by the polymerase stop PCR reaction. Generally, PCR is manipulated to allow preferential amplification of rare mutated target sequences. In contrast, the Polymerase Stop assay relies on a loss of amplification efficiency due to DNA damage, which directly inhibits the exponential nature and therefore full potential of the PCR. DNA polymerases undergoing the process of DNA synthesis are less likely to circumvent large bulky adducts, polymerisation is also halted at single strand and double strand breaks, the resulting loss of template for subsequent cycles of amplification greatly inhibits the amplification procedure resulting in a quantitative loss of PCR product. With stringent practical application the assay is sensitive enough to detect damage on DNA regions of 300bp, but is robust enough to be equally accurate at assessing the damage incurred on regions over 14kb in length. The Polymerase Stop assay was applied to various regions within the human p53 tumour suppressor gene treated with increasing doses of specific chemical mutagens.

Reference

McCarthy *et al* (1996). Mutat. Res. **363**: 57-66.

49. PAHs in the aquatic environment: genotoxicity and phototoxicity

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Polycyclic aromatic hydrocarbons (PAH) are ubiquitous, persistent organic contaminants of marine ecosystems. PAHs enter the marine environment from a range of sources including the combustion of fossil fuels and industrial effluent. A considerable amount of indirect evidence suggests that such contamination can account for a number of adverse effects in exposed aquatic organisms, including carcinogenicity. While the direct measurement of PAH, by chemical analysis, can provide detailed information regarding spatial and temporal trends in contaminant distribution, it provides little indication of the biological effects of such compounds. We are currently employing a suite of biomarkers in an attempt to determine whether current levels of PAH contamination in UK waters pose a carcinogenic risk to exposed aquatic organisms. Model organisms include the marine flatfish dab (*Limanda limanda*) and flounder (*Platichthys flesus*). Studies involve environmental monitoring utilising biomarkers (e.g. DNA adducts) of genotoxin exposure and surveys for cancer prevalence, along with laboratory studies investigating mechanistic links between PAH exposure and cancer induction. Furthermore, certain PAHs are known to possess phototoxic properties, which can increase their toxicity to aquatic organisms by several orders of magnitude when exposures occur in the presence of ultra-violet (UV) light. Experiments are described which highlight the potential impact of PAH phototoxicity and its relevance to determining the impact of PAHs in aquatic ecosystems.

50. ^{32}P-postlabelling analysis of DNA adducts and EROD induction as biomarkers of genotoxin exposure in Dab (*Limanda limanda*) from British coastal waters

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Dab (*Limanda limanda*) were sampled from a number of polluted and unpolluted areas in British coastal waters. The ^{32}P-postlabelling assay was used to analyse the level of aromatic/hydrophobic DNA adducts in pooled samples of liver tissue. The mean levels of DNA adducts detected from areas known to receive anthropogenic pollutants ranged from 4 to 33.8 adducts per 10^8 nucleotides, with all sites containing samples displaying DNA adduct profiles consisting of diagonal radioactive zones. In contrast, no DNA adducts were detectable in samples from an unpolluted reference site. The ranking of polluted sites based on DNA adduct levels did not correspond with the ranking of sites based on sediment associated polycyclic aromatic hydrocarbon levels, highlighting the problem of linking the presence of contamination with detectable biological responses. No correlation was found in this study between EROD activity and the level of DNA adducts.

51. Evaluation of mitomycin C-induced DNA damage and repair in RT4 bladder cancer cells using the alkaline comet assay

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The alkaline comet assay is a rapid, sensitive and relatively inexpensive test for the detection of a variety of DNA lesions. Modifications of the standard protocol have been proposed for the simple and sensitive detection of crosslinking agents (Olive *et al*., 1992, Olive and Banath, 1995, Pfuhler and Wolf, 1996, Miyamae *et al*., 1997, Merk and Speit, 1999). Since crosslinking agents connect DNA with DNA or DNA with proteins they inhibit spontaneous and mutagen induced DNA migration. In this study the comet assay was used to evaluate the DNA crosslinks induced by mitomycin C (MMC) by determining the reduction of DNA migration following 5 Gy gamma irradiation. The effect of MMC alone in the comet assay was also investigated.

MMC was observed to reduce gamma ray induced DNA migration in the comet assay in a dose dependent manner. MMC treatment alone produced positive trends in several parameters measured using Komet 4.0 (Kinetic Imaging Ltd). The most notable parameter was comet optical intensity where an increase of greater than four fold was observed over the dose range used. Repair of cross-links following MMC treatment was followed over a two hour period with cells being exposed to 5Gy gamma radiation just prior to cell lysis. An increase in DNA migration was observed with increasing MMC repair time, as the radiation induced breaks were released to form comet tails.

These results indicate that DNA crosslink damage and repair can be measured in RT4 cells following 5Gy gamma irradiation performed immediately prior to cell lysis. It also indicates that it may be possible to measure DNA damage and repair induced by crosslinking agents directly with the comet assay, using a

novel parameter, without the use of a strand breaking agent such as gamma irradiation. These findings merit further investigation.

Reference

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Olive and Banath (1995) Exp. Cell Res. **221**: 19-26
Olive *et al.* (1992) Exp. Cell Res. **198**: 259-267
Pfuhler and Wolf (1996) Environ. Mol. Mutagen **27** :196 – 201

52. EU Technical Guidance Document for Mutagenicity

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In 1995 the European Competent Authorities for regulation of chemicals (new and existing substances) adopted a technical guidance document (TGD) in order to harmonise criteria for risk assessment of chemicals. Concerning the strategy for mutagenicity testing, some main characteristics are as follows:

- 1 step-wise approach, triggered by (a) the extend of exposure (or tonnage as surrogate for exposure), or (b) the need for clarification of positive findings.
- 2 basic testing refers to a bacterial gene mutation test and an in vitro chromosomal aberration test.
- 3 further testing for substances with negative findings consists of a mammalian cell gene mutation test and, in case of high exposure, an in vivo test (in general, a bone marrow micronucleus test).
- positive in vitro findings need clarification which, in general, is done by conduct of an in vivo micronucleus test and, if this is negative, an UDS test in vivo.

In early 2000 a revision of the TGD was initiated including a modification of the genotoxicity testing strategy. Up to now discussion focuses on the following aspects:

(1) alternatives for the in vitro chromosomal aberration test: can it be replaced by the in vitro micronucleus test or by the mouse-lymphoma assay?
(2) local genotoxicity: are new methodologies, such as the in vivo comet assay or gene mutation tests with transgenic mice, useful for investigation of local (non-systemic) genotoxic effects?
(3) bacterial mutagens: is immediate and extensive in vivo testing needed if mammalian cell culture assays were negative?
(4) genotoxic carcinogens: can more guidance be given for definition, identification and risk estimation (including the threshold problem)?

From the viewpoint of the German Competent Authority main goals of the revision process are to allow for more flexibility in the choice of test systems, to establish the need for investigation of local genotoxicity (esp. at the site of contact) and to re-consider the impact of genotoxic potentials for the risk characterisation of carcinogens.

53. Genotoxins and the initiation of sporadic breast cancer

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Breast cancer is the most frequent malignancy occurring in women worldwide, accounting for 20% of all female cancers. Aetiological risk factors that could account for the majority of cases, which are post-menopausal and/or sporadic, remain obscure and genetic predisposition probably only accounts for some 5-10% of breast cancer cases. In simple terms, the breast consists of 70-90%, by weight, of adipose tissue, in which are dispersed the functional elements that are lined with epithelial cells from which breast cancers commonly arise. Numerous fat-soluble compounds, identified in the human diet and in the environment, have been shown to induce mammary cancer in rodents. Extracts of lipid obtained following collagenase digestion of elective reduction mammoplasty tissues from healthy UK-resident women (18-45 years) revealed activity in short-term genotoxicity assays in approximately 40% of cases (Martin *et al.*, 1996). There was a tendency for the most active lipid extracts to be those obtained from donors whose human mammary epithelial cells (HMECs) also contained the most pre-existing DNA single-strand breaks (SSBs) (Martin *et al.*, 1997). Active lipid extracts induced the morphological transformation of mammalian cells *in vitro*: heterocyclic aromatic amines were not present at detectable levels (Martin *et al.*, 1998). In an attempt to increase cohort size, extracts of UK breast milk were examined and some 40% were again found to be active in short-term genotoxicity assays (Martin *et al.*, 1999). Viable cells, a large percentage of which were epithelial, were recovered from breast milk and examined for the presence of SSBs and for the ability of the donor's own milk extracts to induce SSBs (Martin *et al.*, 2000a). Again, donors whose untreated cells contained the most SSBs tended to yield genotoxic breast milk extracts. Breast milk cells are also known to be able to activate rodent mammary carcinogens to DNA-damaging species (Martin *et al.*, 2000b). Together these studies provide compelling evidence of the *in vivo* exposure of normal HMECs to as-yet-unidentified genotoxic agents several years prior to the peak in the occurrence of sporadic breast cancer. Work is in progress to try and characterise these agents and to determine their possible role in the aetiology of breast cancer.

Reference

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54. Susceptibility of primary cultures of human prostate epithelial cells to chemical carcinogens as detected using the Comet assay

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Epidemiological studies suggest that dietary and/or environmental factors play a role in the aetiology of prostate cancer. However, there is little data on the susceptibility of human prostate epithelial cells (PECs) to known carcinogens. Two sources of prostate tissue, from a large cohort of men, are transurethral resection of the prostate (TURP) and radical retropubic prostatectomy. These procedures are designed to

alleviate the effects of benign prostatic hyperplasia (BPH), a risk factor in the aetiology of prostate cancer, or to remove malignant tissue. Following removal, TURPs or tumour-adjacent tissue fragments were placed in buffered saline (4°C) and transported to our laboratory. After mincing in PFMR-4a medium (37°C), homogenates were seeded onto collagen-coated petri dishes. Epithelial cells were allowed to adhere and non-adherent material was removed by washing with the medium after 12 hr. The abilities of adherent PECs to activate the chemical carcinogens 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]pyridine (PhIP), its N-OH metabolite and benzo[*a*]pyrene (B[*a*]P) were examined using the alkaline single cell-gel electrophoresis ('Comet') assay as described previously (Martin *et al.*, 1999). PECs, removed by scraping, were concentrated by centrifugation and were treated with PhIP, N-OH PhIP or B[*a*]P for 30 min at 37ºC. DNA damage was quantified as comet tail length (CTL) (μm). Treatment of PECs from different individuals (n=4) with varying concentrations of PhIP (2.0, 20.0 and 200.0 μM), N-OH PhIP (0.01, 0.1 and 1.0 μM) and B[*a*]P (0.9, 9.0 and 90.0 μM) induced comet formation. Control median CTLs (n=4) ranged from 7.5 – 10.0 μm and 13.5 – 18.0 μm in the absence or presence of HU/ara-C respectively. After exposure to increasing concentrations of PhIP (n=4) or N-OH PhIP (n=2) median CTLs ranged from 25.5 – 40.5 μm, 52.5 – 78.5 μm and 71.5 – 126.0 μm and 45.5 – 71.0 μm, 69.5 – 134.0 μm and 96.0 – 188.5 μm, respectively. After exposure to increasing concentrations of B[*a*]P (n=2) median CTLs ranged from 22.0 –114.0 μm, 28.0 – 138.0 μm and 75.0 – 184.0 μm, respectively. However, PECs (n=2) exposed to PhIP, N-OH PhIP and B[*a*]P were less susceptible to the genotoxic effects of these compounds after 48 hr in culture. After 96 hr in culture no comet-forming activity was observed. The results of this pilot study suggest that activating enzymes present in PECs are maintained at high levels *in vivo*, but decline in culture possibly due to the absence of inducers. However, we have shown that primary cultures of PECs can activate chemical carcinogens and future development(s) may provide a robust model system for the study of the aetiology of prostate cancer.

Reference

Martin *et al.* (1999) *Mutat. Res.* **445,** 21-43.

55. Development of a methodology to map oxidative DNA damage at nucleotide resolution in human cells

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Reactive Oxygen Species (ROS) can be produced during cellular metabolism, infections by microorganisms like *Helicobacter* p*ylori* or after exposure to factors like ionizing radiations and chemicals. ROS can induce damage to different cell components which includes the DNA. Many organisms including mammals have developed enzymes, antioxidant molecules to protect the cells against ROS. However, ROS can escape destruction and then generate a wide range of DNA damage (base damage, strand breaks, abasic sites). If not removed by various repair mechanisms (Base excision and Nucleotide excision repair), those DNA damages can lead to mutation, cancer or cell death.

Oxidative DNA damage can be induced at preferential sites depending on factors like the sequence context, protein binding, chromatin structure.... Those hotspots or fingerprints can also depend on the ROS induced thus can be used as a biomarker.

The fingerprinting of ROS-induced DNA damage can make use of ligation-mediated polymerase chain reaction (LMPCR). This technique allows the detection of very low frequencies of DNA damage while starting with few copies of the DNA target. However, it is a very demanding approach that requires ligatable ends at the damage sites.

The 3′end labelling technique is an alternative technique to detect oxidative DNA damages at nucleotide resolution. This process is simpler than LMPCR, allows the detection of damages without PCR steps and does not require ligatable ends. However, a high copy number of the DNA sequence is required which thus limits its use with mammalian cells.

The aim of the project is to develop and validate a methodology for the detailed mapping of oxidative DNA damage at the level of the nucleotide in specific target sequences of human DNA. We present here the development selected which combines LMPCR and the 3′end labelling technique.

56. Screening compounds for genotoxicity and cytotoxicity: an SOS bioluminescence *Salmonella typhimurium* test to measure genotoxicity kinetics

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Vitotox® assay is a SOS-bioluminescence *Salmonella typhimurium* test to measure genotoxicity, toxicity and mutagenicity. The assay is based on recombinant bacteria that contains the bacterial luciferase genes luxCDABE of *Vibrio fischeri* under transcriptional control of the mutated recN promoter, which is controlled by the bacterial SOS-system. After incubation of the cells with a genotoxic product, the recN promoter will become derepressed, resulting in expression of the lux operon. Expression results in light production in function of the genotoxicity. A similar recombinant bacterial strain with a constitutively expressed lux operon is used simultaneously for the detection of possible cytotoxicity of the same compound. This is the unique feature in Vitotox test that the user in fact performs simultaneously two tests that has always before been done separately. The simultaneous test systems reduces time and costs needed for the testing of the certain compound.

We claim that so far, Vitotox test is the only existing genotoxicity test that can be placed in secondary screening, or even in HTS screening phase. This is the main potential of the Vitotox test. It takes only micrograms of test compound, it can be automated, and high enough throughput can be achieved for testing of very many compounds in reasonable time frame. The almost perfect correlation with the results obtained by Ames test quarantines to the user, that when he has to do Ames test for legal reasons, he already well knows that the compound will past that test. Therefore, with using Vitotox test, the number of Ames test done can be clearly reduced from the present number.

The unique nature and patent of Vitotox test gives us a

good possibility to create a large product folio based on this technology. The same technology could be used for development of several similar test kits for different purposes. With the HTS format Vitotox assay, that is at the moment under development, it will be possible to test over 800 compounds during one working day. This kit would make it possible to do genotoxicity testing already even in early screening phase of drug discovery.

57. *Helicobacter pylori*, oxidative damage and gastric carcinogenesis

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Helicobacter pylori, is a class 1 carcinogen whose presence is associated with cancer of the mid or distal stomach. One proposed mechanism for *H. pylori's* carcinogenic effect is the release of reactive oxygen species from the inflammatory cells sent to eradicate it from the gastric mucosa.

The most important reactive oxygen species in biological systems are the oxygen intermediates such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and the hydroxyl radical ($^{\cdot}OH$). The most ubiquitous oxidative DNA base modification is 8-hydroxy-guanine, and can produce GC: TA transversions. One study has shown that the levels of 8-hydroxy-guanine in the DNA from gastric tissue infected with *H.pylori* are elevated when compared to those of uninfected individuals.

To investigate mutations *in vitro* to those seen *in vivo,* a transformed gastric cell line was dosed with varying concentrations of H_2O_2, to mimic oxidative damage. Exons 5 to 8 of the tumour suppressor gene p53, were analysed using the Restriction Site Mutation (RSM) assay. Resistant bands representative of mutations were only found in exon 7. These RSM results were then compared with mutations from the IARC database and from patient biopsy samples (normal, gastritis, intestinal metaplasia, dysplasia and carcinoma).

58. The immunological consequences of UVB-induced DNA damage

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Exposure to UVB has genotoxic effects but also results in suppression of some cell-mediated immune responses to antigens encountered shortly after the irradiation. The latter change has critical implications for the control of skin tumours and various infectious diseases. It is likely that a cutaneous chromophore is involved as the initiating step in the complex series of changes resulting in immunomodulation. Three approaches have provided evidence that DNA can act as one important initiator of this process. Firstly, mouse models have been developed in which suppression of contact hyper-sensitivity (CH) occurs when the sensitiser is applied to the skin after the UV exposure, followed by challenge several days later. If the cutaneous thymine dimers, induced by UV, are repaired with T4 endonuclease V or photolyase plus light, then the suppression in CH does not occur. The effect is not confined to the skin as some dendritic cells which accumulate in lymph nodes as a result of UV exposure display DNA damage: antigen presentation to T lymphocytes or the local cytokine milieu may be changed as a result. Secondly, various strains of nucleotide repair deficient mice (XPA, XPC, TTD, CSB) have been generated recently. Here the minimal dose of UVB necessary to produce erythema (MED), the minimal dose necessary to suppress CH (MID) and the tendency to develop skin cancers have been compared. Perhaps surprisingly, these three parameters do not correlate; for example, the XPA knockout mice have a low MED and and a low MID in comparison with the parent strain, while the CSB knockout mice have a low MED but a high MID. Thirdly, UV-induced DNA damage has been shown to lead directly to changes in the production of various immunological mediators by keratinocytes, such as the induction of interleukin-10, the suppression of interferon-γ stimulated ICAM-1 expression, and the up-regulation of interleukin-6.

59. Genetic changes in colorectal cancer

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Colorectal cancer, which is an excellent example of the complex multistage process of carcinogenesis, is the second most common cause of cancer deaths in the United Kingdom and much of the industrialised world. Most colorectal cancers are thought to develop from adenomas (benign tumours sometimes referred to as polyps) in what is often called the adenoma carcinoma sequence. Adenomas are derived from the normal colonic epithelium. Approximately 10-20% of colorectal cancers are familial and the rest are sporadic with diet believed to be an important factor in the development of the sporadic form of the disease. The two best characterised forms of familial bowel cancer are familial adenomatous polyposis (FAP) and hereditary nonpolyposis colorectal cancer (HNPCC). FAP results from the inheritance of a mutation in a single allele of a tumour suppressor gene (the adenomatous polyposis coli (APC) gene). Importantly, mutations in the APC are also thought to initiate the development of tumours in the more common sporadic forms of the disease. HNPCC results from the inheritance of mutations in DNA mismatch repair genes which leads to genomic instability and an increase in the mutation frequency which ultimately leads to mutations in important growth control genes such as the APC tumour suppressor gene. Thus tumorigenesis in HNPCC patients is also thought to be initiated by mutations in the APC tumour suppressor gene. Mismatch repair genes are also mutated in a subset of sporadic colorectal tumours. Mutations in both allele of APC are thought to be required for the development of adenomas.

The progression of premalignant adenomas to carcinomas can take many years and requires further tumour suppressor genes and proto-oncogenes to become mutated and/or deregulated. Other gene(s) believed to be important in colorectal carcinogenesis include the p53 tumour suppressor gene, transforming growth factor β receptor and its signalling pathway and ras and bcl-2 family of oncogenes. Recently,

cyclooxygenase 2, which is involved in arachidonic acid metabolism and prostaglandin production has been reported to be upregulated during colorectal carcinogenesis. Some of these genes are currently being investigated as targets for both the prevention and treatment of bowel cancer.

60. Pesticides effects on humans: a molecular epidemiological study

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Epidemiological evidence suggests that 60-80% of all human cancers may be the results of lifestyle and environmental factors.

Contamination of our environment with chemicals is a major concern considering that 1000 new synthetic chemicals are being introduced each year.

Of particular importance are the potentially hazardous effects associated with the widespread application of pesticides. These compounds are widely used in agriculture, and present a large proportion of chemicals to which man is environmentally exposed. The acute toxic effects of pesticides are well established, with 100000 non-fatal cases of human poisoning reported each year.

Genotoxic effects are considered among the most serious of the possible side effects of agricultural chemicals. Such effects are of special concern because of the generally irreversible nature of the processes and the long latency associated with their manifestation. Many pesticides have been tested for mutagenicity by a variety of *in vitro* and *in vivo* assays and mutagenic compounds have been found among all major categories of pesticides. A limited number of 'field' studies have also been done aiming at obtaining epidemiological evidence for the presumptive genetic risk at the somatic level for the human population. Some studies have shown an increased risk of lung cancer in pesticides exposed agricultural workers. The impact of pesticides on the health of agricultural workers is however still largely unknown.

In this project which we coordinate and is financed by E.U. with the participation of Greece, Spain, Poland and Hungary, the effects of pesticides in populations exposed in greenhouses was examined.

With the use of comet assay (Piperakis *et al* 1998,1999) we measured:

1) DNA damage; 2) antioxidant protection; 3) DNA repair efficiency; and 4) protection by antioxidant agents (vitamin-C). Health, lifestyle, diet, age etc of the examined population was recorded by a detailed questionnaire and taken into account. Preliminary results show a difference in the response of the Greek exposed population, if compared to the controls.

Reference

Piperakis *et al* (1998) *Carcinogen*. **19,** 695.
Piperakis *et al* (1999) *Methods Enzymol*. **300**, 184.

61. GeneTEX, a new HTS genotoxicity assay for drug discovery

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With the introduction of combinatorial chemistry and High Throughput Screening (HTS), new leads can be rapidly identified, with achieved throughput of primary screening of up to hundreds thousands compounds a week . The classical strategy in drug development involves a second step of removal of molecules with toxicological properties, including genotoxic properties. With an average positive compounds in the first positive screening of 5-10%, present methods used induce an important bottleneck: they can't screen thousands of compounds a week.

Here, we present a new, patented, test which fulfils the requirements of HTS.

This test detects genotoxic compounds on the basis that DNA damage induced by such molecules are recognised by specific human DNA repair proteins. The test is able to detect and discriminate alkylating agents, clastogens and adduct-forming molecules. Genomic DNA bound on microplates wells is incubated with the compounds to be tested, with or without metabolic activation. In the following step, a human cell-free extract is added and the DNA repair proteins bind to the damaged DNA. Then, specific antibodies are added, recognising the different proteins bound on the damaged DNA, depending on the type of damage. A secondary enzyme-labelled antibody is added and the final detection uses chemiluminescence.

The achieved thresholds of sensitivity are comparable and often lower than those obtained with classical, non HTS, tests such as Ames test or SOS chromotest. For example, the thresholds obtained with cisplatin, N-acetylaminofluorene, N-methyl-N-nitrosourea, bleomycin, representing the 4 main classes of genotoxins are 0.2µm (3ng), 130µM (1.6µg), 0.6mM (3µg) and 70nM (3ng) respectively. The required amounts of compounds fit with the few mg synthesised with combinatorial chemistry.

The entire test takes less than 3 hours to be completed and can work on existing robotic workstations.

A variation of the test consists in the use of cultured cells to screen genotoxic compounds. In this case, cells are incubated with the compounds, which provides data on cell penetration.

62. Evaluations of carcinogenic risk to humans: use of data on mechanisms of carcinogenesis

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The *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans* contain reviews and evaluations of published scientific literature on chemicals, complex mixtures, occupational exposures, physical and biological agents, lifestyle practices, and other agents and circumstances that may present a carcinogenic hazard to human beings. Re-evaluations are conducted from time to time as new data are published. Overall evaluations indicate the strength of the total available evidence that an agent or an exposure circumstance can increase the risk of human cancer, and are based principally on epidemiological studies of cancer risk in exposed humans and on bioassays for carcinogenicity in experimental animals. Other relevant data are also taken into account, including data on mechanisms of carcinogenesis, and such data can raise or lower the overall evaluations (Table I). The use of data on genetic and related effects in carcinogenic hazard evaluation has recently been reviewed (McGregor *et al.*, 1999).

Table I. 860 Overall evaluations of carcinogenicity, *IARC Monographs* Volumes 1-77 (1972-2000)

Group	Definition	Cancer data alone	Plus other data	Total
1	Carcinogenic to humans	75	3↑	78
2A	Probably carcinogenic	25	38↑	63
2B	Possibly carcinogenic	229 + (1↓)	5↑	235
3	Not classifiable	477 + (1↓)	5↓	483
4	Probably not carcinogenic	1	0	1

↑, Overall evaluation raised from a lower 'default' level; ↓, overall evaluation lowered from previous level.

Forty-six IARC evaluations (5.3 per cent) have been revised upwards by considerations of mechanisms-based evidence. In all but one of these, bioassay data in animals were strengthened by supporting evidence that a DNA-reactive (genotoxic) mechanism of action operates in animals and is likely to operate in humans. More than half the agents currently classified as *probably carcinogenic to humans* (Group 2A) are in this category. In contrast, the criteria for a Group 1 classification are very strict, as there must be strong evidence in exposed humans that the agent acts through a relevant mechanism of carcinogenicity. Only three of the 78 agents in Group 1 have been so classified using mechanistic evidence. Two of these, ethylene oxide and neutron radiation, act by DNA-reactive mechanisms; the third, 2,3,7,8-tetrachlorodibenzo-*para*-dioxin, operates by a non-genotoxic, receptor-based mechanism.

A comparatively few evaluations have been revised downward on the basis of newer findings. Two of these revisions, for acrylonitrile (2A→2B) and for dimethylformamide (2B→3), resulted from newer, more powerful epidemiologic studies which did not validate previous limited evidence for increased cancer risk in exposed humans. Several other evaluations have now also been revised downward, from Group 2B to Group 3, on the basis of evidence that carcinogenesis by these compounds in experimental animals occurs by non-DNA reactive mechanisms that do not operate in humans under any known conditions of human exposure to the agents in question. Such mechanisms include:

- urolithiasis and deposition in the urinary bladder of irritant phosphate-containing urinary precipitates, for bladder cancer induced in rats by melamine and by saccharin salts;
- α_{2u}-globulin nephropathy, for renal cell carcinoma induced in rats by *d*-limonene;
- peroxisome proliferation, for hepatocellular neoplasms induced in rats and mice by di(2-ethylhexyl) phthalate; and
- the non-DNA reactive mechanism by which the non-estrogenic compound atrazine induces mammary tumours in female rats of one strain.

These evaluations were preceded by the publication of proceedings of IARC scientific symposia at which all aspects of the relevant carcinogenic mechanisms (except that for atrazine) were discussed. In these symposia a consensus was reached regarding the use of data concerning these mechanisms in carcinogenic hazard identification (IARC, 1995; Capen *et al.,* 1999). Criteria have also been developed for using data on mechanisms by which chemicals give rise to follicular neoplasms of the thyroid. These will be applied in *IARC onographs* Volume 79 (October 2000).

IARC Monographs working groups have had little difficulty in applying data on DNA-reactive mechanisms to raise overall evaluations. However, there has been no consensus on precisely what such data should include, beyond general acceptance of the predictive value of certain kinds of data, together with a preference for experimental methods and endpoints that are widely used (McGregor *et al*., 1999). These endpoints include gene mutation in bacteria and mammalian cells and chromosome abnormalities *in vitro* and *in vivo*. There are known (Group 1) and probable (Group 2A) human carcinogens that cause chromosome abnormalities in mammalian cells by non-DNA reactive mechanisms, but have no effect in bacteria (*e.g.*, DNA topoisomerase II inhibitors: IARC, 2000). Such examples indicate that overly prescriptive guidelines for what mechanistic data may be acceptable or necessary must be avoided.

It has been more difficult to achieve consensus regarding use of data on other mechanisms of carcinogenicity, for either raising or lowering overall evaluations. To date, IARC evaluations of only five 'rodent carcinogens' have been revised downward on the basis of mechanistic evidence. Each of these compounds is carcinogenic at only a single organ site in experimental animals, and is active by a single non-DNA reactive carcinogenic mechanism. The more difficult problem of non-genotoxic substances that cause tumours in rodents at multiple sites and by more than one mechanism remains to be addressed.

Reference

Capen *et al.* (1999) IARC Scientific Publications No. 147.
IARC (1995) IARC Technical Report No. 24.
IARC (2000) IARC Monographs Volume 76.
McGregor *et al.* (1999) IARC Scientific Publications No. 146.

63. Cytogenetic analysis of E6 human thyroid tumour cells

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The K1E6 human thyroid epithelial cells are a subclone isolated from K1 cells - a well differentiated papillary cancer cell line. They contain the HPV16E6 human papilloma virus protein, which confers loss of p53 function. These cells belong to a set of cell lines which are iso-genic and iso-phenotypic both with and without functional p53.

Using both fluorescence *in situ* hybridisation (FISH) and Giemsa staining we have analysed the chromosomal composition of the E61A (early passage) and E61D (late passage) cell lines. There was an increase in chromosome number from a near diploid median of 41-51 in the early passage cultures, to a near tetraploid median of 87-97 in the late passage cultures.

It was evident that the chromosomal composition of the late passage cultures was far more unstable than that of the early passage cultures. In the E61A cell line 7 of the 24 human chromosomes showed some translocations and there was one insertion. While in the E61D cell line 14 chromosomes showed some translocations, while there were 2 insertions. Both of the cell lines contained 3 stable derived chromosomes previously seen in the iso-genic $p53^{+}$ and$p53^{-}$ cell lines. These marker chromosomes were t(8:1), t(17:18) and ins(9:1:9).

Reference

Parry *et al* (1998) *Chromosoma* **107**:491-497.
Wyllie *et al* (1999) *British Journal of Cancer* **79** (7-8): 1111-1120.

64. *In vitro* DNA repair activitiy as a tool for the rapid screening of genotoxins

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To easily screen genotoxic compounds, we have developed two microplate assays based on the reconstitution of the excision repair reaction *in vitro*. Damaged-plasmid or genomic DNA from treated cells was adsorbed in sensitized microplate wells, then cell extracts were added to perform the repair reaction used to test the presence of base damage either during the recognition ('4D TEST')or the repair synthesis ('3D TEST') stage. In the Damaged DNA Detection assay ('3D TEST'), the yield of incorporation of biotinylated deoxynucleotides during the repair synthesis step was measured in an ELISA-like reaction with chemiluminescence detection (Salles and Provot 1999). The '3D TEST' allows us not only to screen genotoxic compounds but also protective agents such as antioxidants and excision repair inhibitors. In the '4D TEST' excision repair proteins from cell extracts were immunodetected with antibodies that recognise XP (xeroderma pigmentosum) proteins. An ELISA reaction using a secondary antibody allowed to quantify the recognition reaction (Li *et al* 1998). Taking into account the potential automation of the '4D TEST', weset up an assay with the purified *E. coli* Fpg repair protein in order to detect oxidants or antioxidants (Sattler *et al* 2000). '3D' and '4D TESTS' can be used in chemical screening procedures.

Reference

Li *et al* (1998) *J. Mol. Biol*., **281,** 211-218.
Salles and Provot (1999) *In vitro* chemiluminescence assay to measure excision repair in cell extracts, in 'DNA repair protocols' vol. 113, D. Henderson and J. Walker (eds) Humana Press Inc., pp. 393-401
Sattler *et al* (2000), *Arch. Biochem. Biophys*., **376**, 26-33.

65. Things are never as simple as we think

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It is natural to seek quick and simple solutions to problems. This is no less true in toxicology than in other human endeavours. Unfortunately, complex problems rarely yield to simple solutions. The following examples serve to illustrate where we have pursued simple solutions, with limited success; these may serve as lessons as we move into new areas of toxicology research.

Although the earliest concern of genetic toxicology was for mutations induced in human germ cells, attention soon turned to the potential of genetic toxicity tests to detect chemicals that posed a threat of cancer. Reports that approximately 90% of rodent carcinogens were detected in bacterial mutation tests led to high hopes for a simple method to identify carcinogens. Effort was then directed toward finding other tests that would complement the bacterial tests. Years of effort were mounted to determine the optimal combination of tests and protocols to detect carcinogens. 25 years later, genetic toxicity tests have settled into an integral role in safety assessment but they have not provided a simple method to discriminate between carcinogens and noncarcinogens.

Later, a relatively simple approach to identify chemicals that induce mutations in mammalian germ cells seemed apparent and was published in 1996. Consistent with all available data was a scheme by which only chemicals positive in the male dominant lethal test were subjected to the male mouse morphological specific locus test. Within 4 years an exception was found. Bleomycin was negative in the male mouse dominant lethal test but, surprisingly, it induced specific locus mutations in spermatogonia. This simple solution was helpful but flawed.

When a high incidence of malformed frogs was discovered in ponds in Minnesota, we volunteered to cooperate with state and federal agencies, thinking that the toxicology and chemistry resources of the NTP would contribute to the quick identification of the environmental factor(s) resulting in the malformations. Four years later the causal factor(s) is not known but it appears that multiple factors, both natural and manmade, may interact to result in malformations. The answer was not as simple as we had hoped.

Currently the 'endocrine disrupter' issue is one of the most scientifically and politically active areas of toxicology. With very limited knowledge of the existence or magnitude of the environmental or human health threat, bold conclusions and simple solutions are being presented. Although there is reason for concern, understanding the possible toxic effects of hormonally active chemicals will require extensive research and testing.

Biology is complicated and it should not be assumed that toxicology is 'easier' than other fields of biological research. In seeking answers to the practical questions of toxicology, it is important to maintain the highest scientific standards in designing and conducting experiments as well as in analysing and interpreting data. Simple solutions to toxicology problems are alluring but, they seldom serve science or public health.

66. Genetic stability of DNA repair-deficient human cell lines

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During cancer progression *in vitro* and *in vivo* many genetical and morphological changes occur in cells. These changes are being investigated in two human cell lines that are DNA repair-deficient, Xeroderma Pigmentosum (XP) and Trichothiodystrophy (TTD), and in a control, a normal human cell line (1BR). Micronucleus assays (MN) and kinetochore staining were performed in order to investigate any chromosomal mutations occurring at the different stages.

All the experiments were performed in primary, pre-crisis and immortalised cultures as received from the MRC Cell Mutation Unit in Brighton.

All three immortalised cell lines had a much higher growth rate in comparison to the primary cell lines. Also they have lost the ability to proliferate after confluency and grow beyond a single cell suspension. They have a rounded morphology compared to the elongated morphology of the primary cell lines that reach confluency and stop growing. Primary cells

also divide for only a limited number of generations and then division ceases (these cells are said to be senesced).

Pre-crisis cells are very difficult to culture as they die after 1-2 days from the start of the culture. Some of the TTD cells have escaped crisis and become immortalised in culture by forming multi-layered foci within the flask, that can be seen by the naked eye. XP cells die after a period of 2 days. However, the pre-crisis control cell line (1BR) proliferates and MN assay and kinetochore staining was performed. The doubling times of all the cultures were estimated in order to perform the MN assay, these were varied between the cell lines with the immortalised cells having the fastest doubling times.

From the experiments it was shown that the binucleate frequencies (BNC) of the immortalised control cell line (1BR), was higher than both the XP and TTD in contrast with the other categories where the control cell line had the lower percentages. XP and TTD frequencies were comparable to each other in this category. Also, the percentages of the micronuclei scored were low under these conditions in all the cell lines tested.

67. Telomere length regulation in mouse DNA repair mutants and human cancer

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Telomeres are essential functional elements of eukaryotic chromosomes responsible for chromosome stability maintenance. In yeast, telomeres play a role in cellular responses to DNA damaging agents such as ionizing radiation (IR), a potent inducer of DNA double strand breaks (DSB). DSB repair proteins including Ku, are permanently present at yeast telomeres and they relocate to the site of DSB following exposure to IR. We are interested in telomere maintenance in mammalian DNA repair mutants. The *scid* (severe combined immunodeficiency) mouse is a DNA-PKcs mutant. DNA-PK is a large enzyme complex consisting of the catalytic subunit, DNA-PKcs, and a heterodimer protein, Ku. The major roles of DNA-PK involve DSB repair and V(D)J recombination. We analyzed telomere length in both *scid* mice and established cell lines. Our results clearly show that *scid* mice have 1.5-2 times longer telomeres than corresponding control mice. This difference is even more pronounced in established *scid* cell lines in comparison with control cell lines. Therefore, the *scid* mouse is the first mammalian mutant shown to have abnormally long telomeres. In spite of long telomeres chromosomes from *scid* mice have a high incidence of chromosome fusions indicative of the loss of telomere function. We observed similar telomere defects in Ku86 deficient mice. To investigate if DNA-PK is directly involved in telomere length regulation we used *scid* cells transfected with the normal copy of the human *DNA-PKcs* gene. Telomere length analysis in transfectants revealed reversion to the normal telomere length suggesting that DNA-PKcs may be directly involved in telomere length regulation. In addition to these effects on telomere length, DNA-PKcs also affects chromatin structure and chromosome organization. To investigate further the role of telomeres in mammalian DSB repair we used mouse lymphoma cells that have functional DNA-PK but show deficient DSB repair. These cells show severely shortened telomeres in comparison with parental cells. In addition we are investigating telomere length in radiosensitive breast cancer cases and preliminary results show an inverse correlation between telomere length and chromosomal radiosensitivity. Taken together, these results indicate that, similarly to yeast, mammalian telomeres may be involved in cellular response to DNA damage.

68. Molecular analysis of genotoxin-induced mutations in mouse p53 gene by the Restriction Site Mutation (RSM) assay

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The RSM assay is a sensitive genotype mutation detection method. It has the potential to detect mutations at any of the selected restriction site in a sequenced gene of an organism.

In this study, the RSM assay was employed to study mouse p53 gene mutations induced by various chemicals. 9 restriction sites of five p53 gene regions (namely exon4, exon5, intron6, exon7 and intron8) were analysed and mutation frequencies were determined at each of the restriction sites studied. The effectiveness of the RSM assay was confirmed by detecting a total of 192 mutated sites in the p53 gene: 28 in CD-1 mice (liver, kidney, lung) treated *in vivo* with cyclophosphamide, 13 in CD-1 mice testes treated *in vivo* with 1-ethyl-1-nitrosourea (ENU), 8 in Muta^{TM}mouse testes treated *in vivo* with ENU, 109 in mouse lymphoma L5178Y tk+/- cell lines treated *in vitro* with 4-Nitroquinoline 1-oxide, and 35 spontaneous mutated sites (*in vivo* and *in vitro*). Here we summarise the mutation data accumulated and highlight the trends discovered which are relevant to mutagenesis in general. Rare spontaneous germline mutations were for the first time detected by using the RSM assay. This further emphasises the sensitivity of the RSM assay. Various specific mutation characteristics were revealed in this study. These included: (i) mutation occurred at different rates in transcribed and non-transcribed strand (ii) neighbour base influence (iii) intron regions were generally more mutable than exon regions (iv) tissue specificity of mutation spectra. The results gave strong evidence that the RSM assay is a powerful tool to study the mechanisms of mutagenesis and carcinogenesis *in vivo* and *in vitro*.

69. DNA adducts, abasic sites and DNA repair

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DNA adducts result from endogenous and exogenous exposure to electrophilic chemicals and/or metabolites and are thought to be causally involved in the formation of mutations and cancer. It is clear, however, that not all DNA adducts are equally efficient in causing mutations. Some adducts, such as N^2,3-ethenoguanine and O^4-methylthymine, are highly efficient promutagenic adducts, while other lesions such as N-7-alkylguanine adducts do not cause active miscoding during DNA replication. The proposed mechanism for the weak mutagenicity of N-7-alkylguanine adducts is through the formation of abasic sites that result from glycosylase activity and/or chemical depurination. We have recently developed a

highly sensitive slotblot assay for abasic sites and applied this assay to investigate the relationships between DNA alkylation, the presence of abasic sites and DNA repair. Studies conducted with MMS have demonstrated that exposure to 0.5, but not 0.15 mM MMS for 24 hours results in increased numbers of abasic sites (0, 0.15, and 0.5 mM had 3.9'0.7, 3.9'0.5, and 8.0'1.7 per 10^6 nucleotides, respectively). The same DNA had 23 and 197 7MG /10^6 nucleotides, respectively for 0.15 and 0.5 mM MMS. Thus, it was clear that very high numbers of 7MG were needed to cause increased numbers of abasic sites. In a related experiment, rats were exposed by inhalation to 500 ppm propylene oxide for 20 days (6 hr/day) and the number of N-7-hydroxypropyl-guanine (7HPG) adducts and abasic sites were measured in target and non-target tissues for carcinogenesis. The nasal respiratory mucosa had 133 7HPG/ 10^6 nucleotides, while lung, liver and testis had 15, 7 and 3 7HPG/10^6 nucleotides, respectively. Abasic sites were not increased in any of the tissues from PO exposed versus control rats. This was unexpected, as the number of 7HPG adducts in nasal mucosa was similar to that measured in cells exposed to 0.5 mM MMS. It is possible that DNA repair was induced during the 20 days of exposure, minimising any increase in abasic sites. Current investigations are examining the role of single versus multiple exposure regimens on the formation of DNA adducts, abasic sites and the induction of base excision repair pathways. A thorough understanding of the relationship between the formation of N-7-alkyguanine adducts, the multiple steps involved in repair of these DNA lesions, and whether or not the repair is balanced or unbalanced is necessary for the accurate assessment of genetic risk.

70. Mutagenicity Testing Strategies for Pharmaceuticals

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The two ICH guidelines for genotoxicity testing of pharmaceuticals, issued in 1995 and 1997, have been adopted as national and pan-national guidelines in the USA; Japan and the European Union. These supersede any pre-existing national guidelines in the three regions for pharmaceuticals. They are regarded as formal regulatory guidelines and thus any departure for the guidance given must be strongly justified ie they have strong regulatory weight. To help minimise any future divergence in requirements within the three regions, the ICH Steering Committee has drafted a 'maintenance process' in which existing ICH guidelines can be updated in the light of compelling new scientific evidence. As genetic toxicity testing is a dynamic field, the two ICH genotoxicity guidelines are candidates for maintenance. In preliminary (unofficial) meetings, several topics have been identified for consideration, including up-grading the status of the in vitro micronucleus test to be equivalent to accepted tests for chromosome damage; providing more options for in vivo tests to investigate compounds positive in vivo; including alternative technical procedures for carrying out in vivo micronucleus tests etc. However, the ICH Steering Committee is yet to agree to a formal maintenance process for these guidelines.

Unlike the ICH guidelines, which are specific to pharmaceuticals, the UK COM guidelines encompass a much broader group of compounds to which man may be exposed. They give advice on the assessment of mutagenicity for chemicals of interest to UK Government Departments. However, these guidelines are intended to cover pharmaceuticals. Indeed the COM 1989 guidelines were adopted largely unchanged by the EU pharmaceutical regulatory Committee for Proprietary Medicinal Products, until they were replaced by the ICH guidelines in the later 1990′s. The proposed revision of the COM guidelines, although congruent with ICH guidelines in many respects, do differ in some details. The most important of these differences is the definition of a 4 test battery to define absence of genotoxicity rather than the 3 tests defined in the ICH guidelines. The in vitro micronucleus test in mammalian cells is also given as one of the options. This is controversial as there is no universally accepted protocol for this assay.

Many of the differences between the ICH and COM guidelines are due to differences in the time that they have been drafted and thus a maintenance process for ICH guidelines is likely to bring the two in closer harmony. In the interim, the ICH guidelines are expected to be followed to register pharmaceuticals in the three regions, including the UK.

71. The *in vivo* gut micronucleus test detects clastogens and aneugens administered orally by gavage

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A general testing battery for pharmaceuticals generally includes a bacterial gene mutation assay, an *in vitro* chromosomal aberration or a gene mutation test on mammalian cells, and an *in vivo* test for chromosome/genome mutations. The aim of this study was to determine the sensitivity of the *in vivo* mouse gut micronucleus assay for direct genotoxins as compared to the *in vivo* bone marrow micronucleus assay (which can also detect indirect genotoxins) to detect clastogens and/or aneugens administered orally by gavage.

Both bone marrow cells and gut cells of the same animals were analysed. The reference substances tested in this study were colchicine (COL), carbendazim (CAR), tubulazole (TUB), and griseofulvin (GRI), all known aneugens, and 1,2-dimethylhydrazine (DMH) which is a colon carcinogen with clastogenic activity (both intraperitoneally and orally as shown by Wargovich *et al.*, 1983; and by Goldberg *et al.*, 1983). COL was slightly genotoxic and strongly toxic in the gut, and genotoxic in the bone marrow, DMH was very genotoxic and toxic in the gut, and not genotoxic in the bone marrow, CAR was slightly genotoxic in the gut and not genotoxic in the bone marrow, GRI was slightly genotoxic and toxic in the gut, but not at all in the bone marrow, and TUB was strongly toxic and genotoxic in both tissues. Thus for all substances tested the *in vivo* gut micronucleus test was as sensitive or even more sensitive than the *in vivo* bone marrow micronucleus assay. The results indicate that in the case of absence of proof of bone marrow exposure (like for three of the five substances tested), another tissue should be considered. The gut micronucleus test may be a good alternative to the bone marrow micronucleus test for compounds which do not target the haematopoietic system. Furthermore, for compounds intended to be administered orally to man, the cells of the

gastrointestinal tract are the first to come in contact with the compounds.

In conclusion, this study shows that the micronucleus test on gut cells is able to detect clastogens and aneugens given orally by gavage of which some of them were not detected in the bone marrow micronucleus test. Sensitivity and specificity of the gut micronucleus test for the detection of colon carcinogens will be further explored.

Reference

Goldberg *et al.* (1983), *Mut. Res*., **109**:91-98.
Wargovich *et al*. (1983), *JNCI*, **71**(1): 133-137.

72. The Bromodeoxyuridine Comet: measuring DNA maturation in biopsies

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The single cell gel electrophoresis (Comet) assay is a simple method of measuring DNA strand breaks in individual cells. We have combined this with bromodeoxyuridine (BrdUrd) labelling of DNA and immunolocalisation of BrdUrd to assess DNA replicative integrity on a single cell basis. Using this modified assay, single strand breaks in recently-replicated DNA can be detected, as can their disappearance as the newly-replicated DNA matures (McGlynn *et al* 1999). These discontinuities may be caused during semiconservative replication or exacerbated by the arrest of replicative polymerases at UV- or chemical-induced lesions. The validity of this assay has been demonstrated using several cell lines of known replicative status (SVM84, xeroderma pigmentosum variant, etc).

We have also applied the BrdUrd Comet assay to single cells derived from small endoscopic biopsies of human colonic mucosa. Misreplication is known to be an important factor in the development of many colonic carcinomas. The BrdUrd Comet assay provides a simple, accurate and automatable technique for the assessment of replicative integrity in the small amounts of material available from mucosal endoscopic biopsies. It should therefore be possible to use this assay to screen populations to establish correlations between known colon cancer risk factors and colon replicative integrity.

Reference

McGlynn *et al* (1999) *Cancer Res*., **59**, 5912-5916

73. Enhanced sensitivity of the Comet assay towards benzo[*a*]pyrene

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The alkaline single-cell gel electrophoresis ('Comet') assay was developed to assess DNA damage in individual cells in the form of single-strand breaks (SSBs), as well as alkali-labile sites, incomplete excision repair events and DNA interstrand cross-links. Cytosine arabinoside (ara-C) allows recognition and excision of such lesions but inhibits DNA resynthesis and subsequent religation, resulting in accumulation of SSBs. Addition of hydroxyurea (HU) has a synergistic affect on this inhibition. In a standard protocol in our laboratory, a metabolically-competent human cell line, MCL-5 cells ($\approx$1 x 10^5 cells/ slide) was incubated with increasing concentrations of benzo[*a*]pyrene (B[*a*]P) (0.0036, 0.036 and 0.36 mM) in the presence or absence of HU (10 mM) and ara-C (1.8 mM) at 37°C in 5% CO_2 for 30 minutes. Comet-forming activity was quantified by measuring comet tail length (CTL) and was observed only in the presence of HU/ara-C (Martin *et al*, 1999). In a typical experiment, B[*a*]P induced median CTLs of 22.0, 24.5 and 30.0 μm, respectively, compared to the control value of 18.0 μm. In attempts to improve sensitivity, experiments involving longer exposure of cells in 25 cm^3 flasks (7.0 x 10^5 cells/ml in 10 mls), in the presence or absence of HU/ara-C were performed. Longitudinal experiments with cells in the presence of HU/ara-C alone revealed no appreciable increase in median CTL or cytotoxicity (assessed by trypan blue exclusion) up to 4 hours incubation (median CTL 9.5 μm compared with a control value of 7.5 μm in the absence of HU/ara-C). After 6 hours, some increases were observed (23.5 μm) as compared to the control value (11.0 μm), increasing greatly by 18 hours (49.5 μm) with cell viability falling below 75%. Low concentrations of B[*a*]P (0.0072-0.72 μM) gave a dose response for comet formation with 4 hours incubation in the presence of HU/ara-C, without inducing appreciable cytotoxicity. For example, at 0.72 μM, median CTL of 32.0 μm was significantly greater than the control value of 6.5 μm ($P < 0.0001$, Mann-Whitney test). At a concentration of 0.036 mM similar levels of DNA adduct formation (measured by ^{32}P-postlabelling analysis) were seen in the presence or absence of HU/ara-C. In one experiment, the potent comet-forming activity of *anti*-7,8-diol 9,10-epoxide of B[*a*]P under similar conditions was also demonstrated. A concentration of 0.00072 μM compared to 0.72 μM B[*a*]P in the same experiment gave median CTLs 2.1 and 2.2-fold greater, respectively than the control ($P < 0.0001$). These experiments point to a requirement for DNA repair inhibitors in order to observe comet-forming activity by B[*a*]P due to the efficiency of repair processes in MCL-5 cells. When HU and ara-C are incorporated into the Comet assay, an extremely sensitive assay for genotoxicity can result.

Reference

Martin *et al* (1999) *Mutat. Res.,* **445,** 21-43

74. Mutations of K-ras and p53 genes in colorectal carcinomas

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Colorectal cancer is the second commonest malignancy in the western world. Nearly 20000 deaths are attributed to it annually in the UK. Accumulation of various genetic events is associated with the stepwise transformation of normal colonic mucosa into carcinoma. These events include mutational activation of the proto-oncogene K-ras and mutation/deletion of the p53 tumour suppressor gene. Up to 50% of sporadic colorectal

tumours are found to contain K-ras mutations. The majority of them seem to be missense mutations at codons 12 and 13. Mutations of p53 appear in 70% of tumours. K-ras mutations are thought to occur at an early stage in the adenoma-carcinoma sequence, whereas p53 mutations manifest later prior to metastasis. In our study, twenty anonymous sporadic colorectal tumour samples obtained at operation were analysed for mutations of K-ras and p53. Twelve adenomas (polyps) obtained from routine colonoscopy were also analysed. Genomic DNA extracted from these tumours was amplified with PCR. Aliquots of the PCR product were digested with the restriction enzyme Bst NI for K-ras mutation detection; direct sequencing was employed for mutation detection of the p53 gene.

Our early results reveal 3 out of 20 tumours (15%) had a mutation of K-ras and only 1 of 12 adenomas (8%). Results of direct sequencing and p53 analysis are awaited. We hope our results will help point us towards genetic factors involved in the early changes of carcinogenesis. Subsequently a highly sensitive mutation detection system, the Restriction Site Mutation assay, is used to carry our work forward in the detection of the rarer mutations involved in early colorectal carcinogenesis.

75. Extended-term lymphocyte use in genotoxicity testing – the cause of aneugen resistance identified

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The use of extended-term lymphocytes (ETLs) for genotoxicity testing offers several advantages over conventionally used cell types (O'Donovan *et al.* 1995). In contrast to the use of 'standard' lymphocytes in genetox assays, which require fresh blood samples per assay, up to 40 assays may be run from an ETL culture created from a single donor blood sample. This not only represents a more practical approach for testing but also reduces the inter/intra donor variation characteristic of lymphocyte use (Elhajouji *et al*. 1994). Also, as a frozen bulk culture ETLs have the handling ease of rodent cell lines but offer the advantage of being a karyotypically stable cell type, essential for non-disjunction aneuploidy detection.

As part of the validation effort for the *in vitro* micronucleus (MN) test, we have been conducting studies to assess the suitability of ETLs for use with this assay. However, these studies have shown, that despite producing the normal genotoxic response to clastogens (*i.e*. induction of MN), the ETL cultures tested were relatively resistant to the genotoxic effects of aneugens (*i.e*. no MN were induced). Kinetochore staining revealed that the background level of chromosome loss was less than a quarter of that seen in with conventional lymphocytes. Having aneugen resistance and low levels of spontaneous chromosome loss would suggest the ETLs to have increased cell division fidelity compared to conventional lymphocytes.

Various hypotheses to elucidate the cause of this phenomenon were examined *e.g*. was cyropreservation or extended culture time responsible? Eventually it was the medium pH that was found to determine aneugen sensitivity. The ETL protocol employed medium at pH 7.0, changing to medium at pH. To 7.8 (as used for conventional lymphocyte culture) conferred aneugen sensitivity on cultures previously demonstrated to be aneugen resistant at pH7.0.

Reference

O'Donovan *et al*. (1995) *Mutagenesis*, **10**(3): 189-201.
Elhajouji *et al* (1994) *Mutagenesis,* **9**(4):.307-313.

76. Genetic polymorphism in CYP1A1 gene: association with ethnic origin and cancer susceptibility

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Individual variation in susceptibilities to cancer is partially due to genetic variability in the activation and detoxification of environmental pro-carcinogens. The CYP1A1 gene plays a major role in the metabolism of various carcinogens present in the environment. To date, four polymorphisms have been described in the human CYP1A1 gene. These polymorphisms have been found to be associated with a higher risk for several cancers including lung, colon, breast and for Parkinson disease in specific ethnic origins. Moreover, CYP1A1 genetic polymorphisms show large differences in frequencies in the different populations especially between Asian and African-American. In addition, individuals with particular CYP1A1 genotypes were found to have synergistically increased cancer susceptibility when combined with other particular genotypes (e.g. GSTM1). We present our analyses of case-control studies over the past ten years. The analyses show that the CYP1A1 gene is a promising genetic biomarker for susceptibility to cancer in specific ethnic groups, particularly in the case of lung cancer.

77. CYP1A1 polymorphism as a predicator of susceptibility to oral cancer risk

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Oral cancer is a tobacco-related disease with a high incidence in many parts of the world. Moreover, the survival rates are still very low with this type of malignancy. Thus, predication is a very effective strategy to reduce death caused by oral cancer. The information of potential sensitivity of individual susceptibility to oral cancer should be very valuable in indicating high-risk individuals, allowing them to avoid the intake of carcinogenic substances (such as tobacco). CYP1A1 is one of the key enzymes in the metabolism of various chemical carcinogens from environment. It plays an important role in the activation of mutagens such as benzo(a)pyrene and other aromatic hydrocarbon found in cigarette smoke. We have been undertaking analyses of the published literature on the role of CYP1A1 in oral cancer susceptibility. Our research suggests that individuals with particular CYP1A1 alleles are associated with higher risks for oral cancer, in particular, at a low dose levels of cigarette smoking. Thus, individual differences in the CYP1A1 polymorphisms can be regarded as a key factor in the assessment of oral cancer susceptibility.