

Fig. 10. Quantification of gene expression profiles in HepG2 cells and/or human hepatocytes treated with benzo[a]pyrene.

from HepG2 cells (Table 4). S9 prepared from HepG2 cells can be particularly useful for detecting carcinogenic heterocyclic amines like IQ and MeIQx because they require acetylation, and this is poor in Chinese hamster cell lines such as CHO. However, even when activated by S9 from HepG2 cells, the lack of penetration of the acetic acid ester metabolites into cells can be a problem. The HepG2 cell system has also been used to elucidate the genotoxic potential of a series of mycotoxins known to be carcinogens *in vivo* but so far (except for aflatoxin B₁) always reported to be negative in all *in vitro* assays studied. For the first time a ranking order could be established for the genotoxic potential of different mycotoxins, such as fumonisin B₁, citrinin and ochratoxin A [18]. Interestingly ochratoxin B, which is structurally related to ochratoxin A but is a non-carcinogen, revealed no genotoxic potential, and aflatoxin B₁ ranked as the most genotoxic. Recently, modulation of gene expression and DNA adduct forma-

tion in HepG2 cells following treatment with different classes of polycyclic aromatic hydrocarbons (PAHs) was assessed. Data indicate that discrimination of high and low potency carcinogenic PAHs by gene expression profiling is feasible [19]. Darroudi also presented data indicating that the anti-oxidants ascorbic acid and β -carotene do give genotoxic responses in HepG2 cells at high concentrations, much as in established rodent cell lines, and therefore the ability of anti-oxidants to become pro-oxidant at high concentrations may be just as likely in HepG2 cells.

The HepG2 cell system proved to be a useful *in vitro* model for detecting environmental and human dietary genotoxicants, anti- and co-genotoxicants [20,21]. Furthermore, this *in vitro* cell system has the potential to discriminate between structurally related carcinogens and non-carcinogens (Table 4) as well as between genotoxic and non-genotoxic carcinogens using gene expression profiling [22]. Consequently, it appears that

Table 4

A comparative study between genotoxicity data *in vitro* using human HepG2 cell system and (non)carcinogenicity data *in vivo*

Chemicals	Carcinogen (<i>in vivo</i>)	HepG2 (<i>in vitro</i>)	CHO cells (<i>in vitro</i>)		Ames test	
			With S9-fraction derived from		HepG2	Rat liver
			HepG2	Rat liver		
2-AAF	+	+	+	-		
4-AAF	-	-	-	-		
B(a)P	+	+	+	+		
Pyrene	-	-	-	-		
CP	+	+	+	+		
DMN	+	+	+	+		
HMPA	+	+	+	-	+	-
Safrole	+	+	+	-	+	-

Abbreviations: CHO, Chinese hamster ovary; 2-AAF, 2-acetylaminofluorene (*in vivo*, carcinogen); structurally related chemical 4-AAF, 4-acetylaminofluorene (*in vivo*, non-carcinogen); B(a)P, benzo(a)pyrene (*in vivo*, carcinogen) structurally related chemical pyrene is non-carcinogen (*in vivo*); CP, cyclophosphamide; DMN, dimethylnitrosamine; HMPA, hexamethylphosphoramide.

Table 5
Induction of micronuclei in AHH-1 and MCL-5 cells by trichloroethylene (University of Swansea data)

Concentration of trichloroethylene (μM)	AHH-1 cells		MCL-5 cells	
	% binucleated (BN) cells	Micronucleated cells/1000 BN	% binucleated (BN) cells	Micronucleated cells/1000 BN
0	55.9	0.90	56.3	1.00
3.8	50.0	1.05	45.1	3.60
9.5	38.7	2.20	35.3	5.10
19.0	37.2	1.60	21.9	5.10
38.0	33.2	1.65	9.3	13.79

the possibility of getting false positive results is low in HepG2 cells, but more experiments on this line of work are required. Furthermore, robust and important endpoints, such as gene mutations, are difficult to study using current protocols in HepG2 and other highly differentiated cells. Method development in this direction will be important if these cells are to be used more widely.

Parry from the University of Swansea presented genotoxicity (mainly *in vitro* micronucleus) data on the MCL-5 cell line, and the human lymphoblastoid AHH-1 cell line from which it was derived. The AHH-1 cell has a high level of expression of CYP1A1 [23]. The MCL-5 cells contain cDNAs for four human CYPs plus microsomal epoxide hydrolase [24]. Both cell lines are heterozygous for *p53*, but undergo a normal repair response and apoptosis following DNA damage. Both cell lines have a modal number of 46 chromosomes although there is some variability and they are probably not euploid. The metabolic competence of these cells avoids the general need for exogenous S9 and, for example, allows for prolonged treatments with pro-mutagens, which could not occur with S9 because of the toxicity induced. The additional CYPs in the MCL-5 cells is seen as an advantage over the AHH-1 for a number of reasons. As can be seen in Table 5, trichloroethylene is more clearly detected as a genotoxin in MCL-5 than in AHH-1 cells. However, as can be seen in Table 6, chloral hydrate is much less active in MCL-5 than in

AHH-1 cells, and would have produced negative results at 50–60% reduction in binucleated cells. The underlying mechanism is unknown and probably not related to CYP expression. It has been the conclusion of the Swansea group that the use of both AHH-1 and MCL-5 provides a more comprehensive screen when testing chemicals of unknown genotoxicity. The cells have been particularly useful at discriminating genotoxic from non-genotoxic chlorinated compounds and studying non-disjunction by synthetic and natural oestrogens. However, very few chemicals have been tested that are neither *in vivo* genotoxins nor DNA-reactive, mutagenic carcinogens. Thus, the susceptibility of these cell lines to false positive results is not currently known. Furthermore, the cells are proprietary, and their routine use for regulatory testing would require appropriate supply and costing arrangements with the supplier.

2.6. New cell systems

White from Health Canada reported on the use of cells derived from the *LacZ* transgenic mouse, MutaTMMouse [25]. FE1 cells isolated from MutaTMMouse lung have a modal chromosome number of 78 (i.e. subtetraploid), but the range is from 62 to 82 chromosomes per cell. The cells appear stable, and have retained important cytogenetic, genetic, biochemical and structural features for 50 generations. The cells can be used to measure gene

Table 6
Induction of micronuclei in AHH-1 and MCL-5 cells by chloral hydrate (University of Swansea data)

Concentration of chloral hydrate (μM)	AHH-1 cells		MCL-5 cells	
	% binucleated (BN) cells	Micronucleated cells/1000 BN	% binucleated (BN) cells	Micronucleated cells/1000 BN
0	58.9	0.85	56.8	1.05
600	48.2	2.25	51.0	0.95
1510	29.7	2.75	40.0	1.25
3020	9.7	6.25	17.9	1.75
6040	2.7	12.39	15.11	2.38

mutations in the *LacZ* transgene (e.g. via the positive selection system described by Gossen and Vijn [26]) or to measure induction of micronuclei in binucleate cells using the cytokinesis block method. They have relatively high levels of inducible CYP1A1 and, with some substrates, can achieve higher glutathione-S-transferase activity than HepG2 cells, several cell lines derived from the Big Blue rat, and several mouse fibroblast lines, and have levels comparable to H4IIEC3 rat hepatoma cells. Good mutant responses have been obtained with several reference mutagens (ethylnitrosourea, ICR-191, benzo[*a*]pyrene), but heterocyclic amines such as PhIP (2-nitro-1-methyl-6-phenylimidazo[4,5-*b*]pyridine) are only mutagenic when a low concentration of Aroclor-1254 induced rat liver S9 is added to the medium, presumably indicating that the FE1 cells do not express the appropriate Phase 2 enzymes and sufficient levels of CYP1A2 to compensate for this deficiency. Several hepatic cell lines have recently been isolated and some have been cultured for 6 months. Whilst the modal chromosome numbers have been closer to diploid, the spontaneous mutant frequency has been very variable from <10 to $>140 \times 10^{-6}$. The metabolic activity of the MutaTMMouse cell lines appears to depend on attachment to a solid surface. Cells forced to grow in

suspension appear to have lost the capacity for metabolic activation. In an effort to miniaturise the assay system, FE1 lung cells have been adapted to grow in small volumes on microcarrier beads coated with Porcine gelatin (Fig. 11). The mutagenic response to benzo[*a*]pyrene for cells attached to microcarrier beads was very similar to that seen in monolayer cultures (Fig. 12). This bead suspension approach has the distinct advantage of being able to screen novel test articles that are only available in minute amounts. As with other systems, very few chemicals have been tested that are neither *in vivo* genotoxins nor DNA-reactive, mutagenic carcinogens. Therefore, the potential for false positive results is not known. Nevertheless, a recent investigation of PAHs did reveal that some *Salmonella* positives that are classified as “inadequate evidence of carcinogenic activity” by IARC (e.g. benzo[*ghi*]perylene) are negative for induction of *LacZ* mutations in the FE1 MutaTMMouse *in vitro* system.

Hastwell from GlaxoSmithKline reported on the evaluation of a *GADD45a-GFP* reporter assay developed by Gentronix in the UK. In the *GADD45a-GFP* assay, TK6 cells have been transfected with a novel green fluorescent protein reporter based on the human *GADD45A* gene. The tests are performed in microwell plates, which

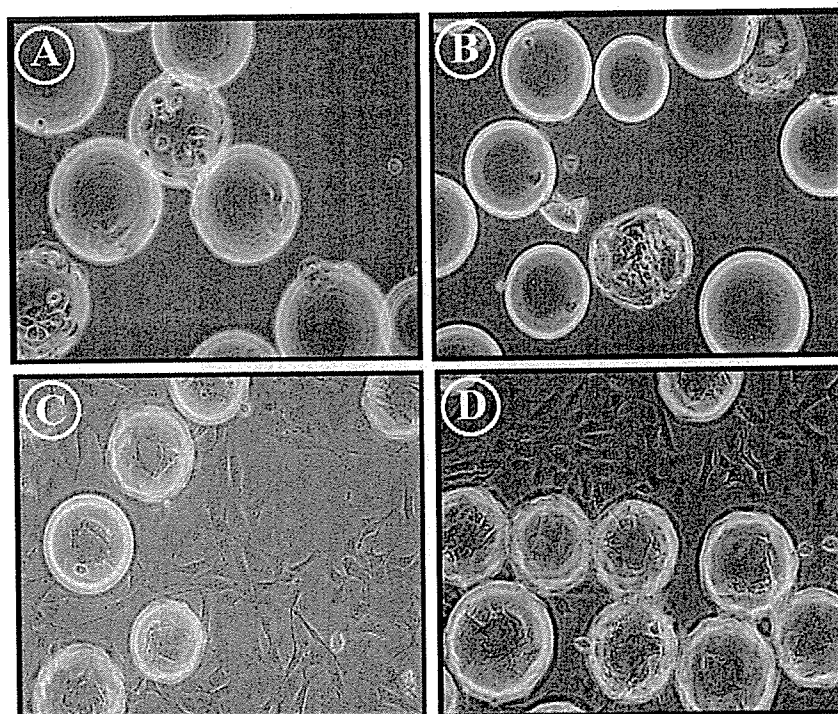


Fig. 11. MutaTMMouse FE1 cells growing on Cytodex[®] microcarrier beads (average diameter 175–190 μ m, dextran coated with Porcine skin gelatin). Upper panels (A and B) show beads and cells after a 2.5 h initial attachment period followed by a 6 h exposure to benzo[*a*]pyrene. The lower panels (C and D) show the culture after 72 h expansion and growth on 100 mm polystyrene culture plates.

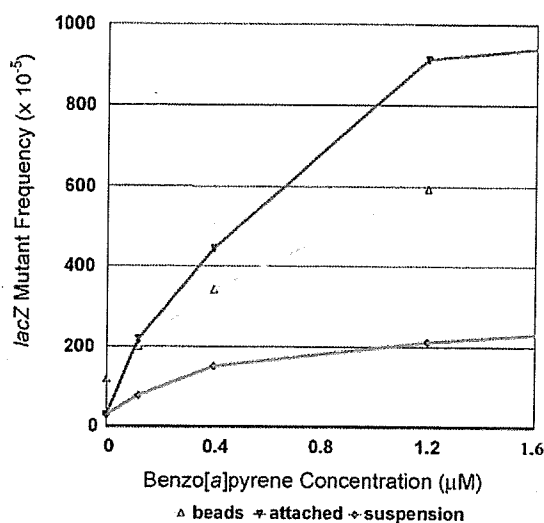


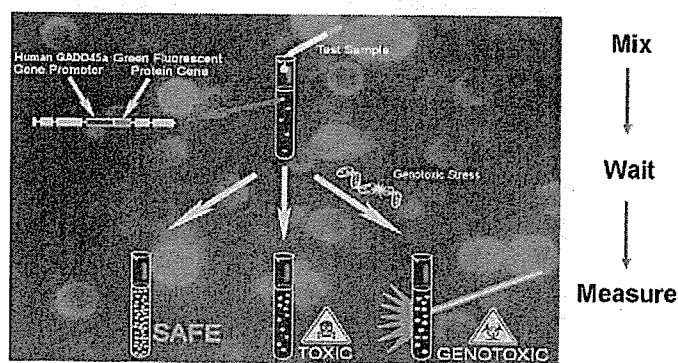
Fig. 12. *LacZ* mutant frequency ($\times 10^{-5}$) induced by benzo[a]pyrene (6 h exposure in serum-free medium followed by 72 h fixation period) in MutaTM Mouse FE1 cells as an attached monolayer, in suspension culture, and attached to Cytodex[®] microcarrier beads.

means that only small quantities of chemical are needed. Generally < 2 mg of a non-toxic chemical, tested up to 10 mM is required. The endpoint of the test is fluorescence, and based on historical data a positive result is concluded when the fluorescence increases 1.5-fold above control levels (Figs. 13 and 14). For the initial evaluation [27], the following sets of chemicals were chosen, and all tested at least four times:

- Thirty-four agents that are genotoxic in the absence of rat liver S9, with known mechanisms of action, that

- were positive in at least one test from the ICH battery [28], namely
 - 10 direct acting genotoxins,
 - 10 aneugens,
 - 7 nucleotide synthesis inhibitors,
 - 4 topoisomerase inhibitors,
 - 3 reactive oxygen species generators.
- Eleven cytotoxic positives, i.e. positive *in vitro* chromosome aberration data associated with cytotoxicity [29].
- Twenty-nine non-genotoxic agents with no positive *in vitro* genotoxicity data.

The 29 non-genotoxic agents and the 11 cytotoxic clastogens all gave negative results in the *GADD45a-GFP* assay, thus giving no false positives. Of the 34 expected genotoxins, 31 gave robust positive responses. Didanosine (a nucleoside analogue), thiabendazole (an aneugen) and methyl viologen (a reactive oxygen inducer) were negative. The evaluation has been extended by looking at marketed pharmaceuticals for which good *in vitro* and *in vivo* data exist (Hastwell et al., manuscript in preparation). From a total of 74 compounds (where rodent carcinogenicity data are available) the sensitivity of the *GADD45a-GFP* assay in relation to rodent carcinogenicity was 81% and the specificity was 94%. This is a much lower false positive rate than experienced with the conventional rodent cell lines used for chromosomal aberration and mutation experiments. Although a large number of chemicals has been evaluated, including a significant proportion of chemicals expected to be negative, none of these



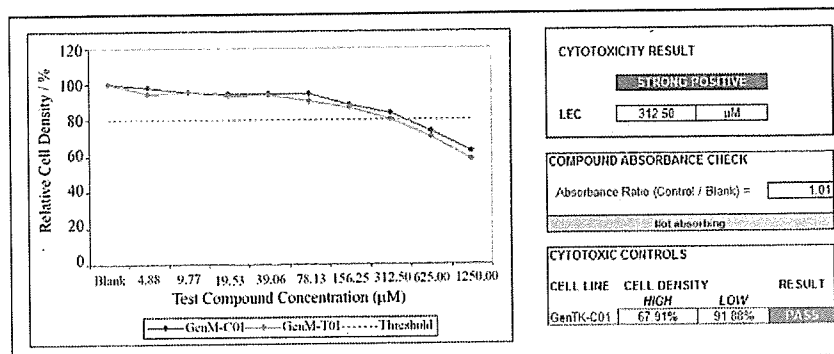
- 4 compounds per plate
- 9 two-fold dilutions
- < 1 mg required to test up to 1000 $\mu\text{g}/\text{ml}$

- Plate set up in 20 minutes
- Automated data collection
- Results in 48 hours

Fig. 13. Schematic of the 96-well plate method for determining genotoxicity in the *GADD45A-GFP* reporter system in TK6 cells (GreenScreen HC).

Cytotoxicity Results

- Positive for cytotoxicity if relative growth drops below 80%.
- Cut off at 30% relative growth.



Genotoxicity Results

- Positive for genotoxicity if relative fluorescence induction exceeds 1.5 fold.

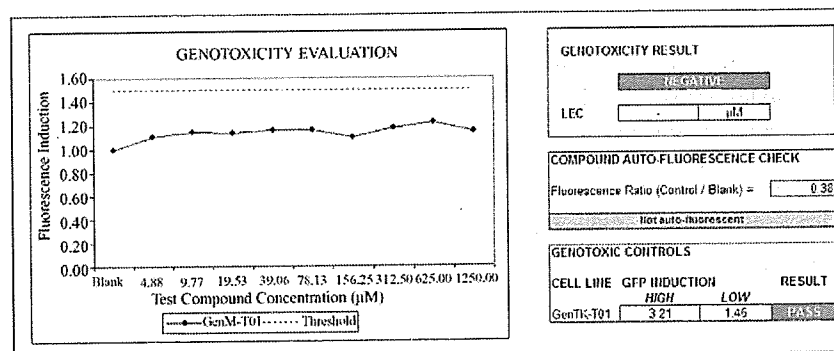


Fig. 14. Criteria for cytotoxicity, genotoxic response and graphical output from the GreenScreen HC assay system.

compounds required metabolic activation. S9 is fluorescent and absorbing and therefore could interfere with the current assay. Cyclophosphamide has been tested in a modified microwell plate. After a 24-h treatment in the presence of S9, significant fluorescence was observed in treated cultures. In principle, therefore, the *GADD45a-GFP* assay in TK6 cells may be adaptable to testing in the presence of S9. A modification of the assay using flow cytometry at 24 h after the start of treatment (4 h treatment, 20 h recovery period) has successfully detected responses with cyclophosphamide, 7,12-dimethylbenz[*a*]anthracene and benzo[*a*]pyrene in the presence of S9, but a better option may be to use HepG2 cells with the same target (*GADD45A*) and reporter (green fluorescent protein) genes. This system will also be proprietary when finally developed.

2.7. 3D skin models

False positive results in mammalian cell genotoxicity tests cause particular problems for the cosmetics industry because, according to the 7th Amendment to the EU Cosmetics directive, from 2009 onwards in the EU it will not be possible to follow up these findings with *in vivo* genotoxicity tests. As an alternative, 3D models of human skin are being evaluated for the possibility of measuring genotoxic endpoints.

Aardema from Procter and Gamble presented data on development of a MN assay in the EpidermTM 3D human skin model [30]. Normal human-derived epidermal keratinocytes are grown on a membrane placed at an air-liquid (medium) interface inside a 9 mm culture insert. The cells differentiate and within 3 weeks tissue closely resembling human epidermis develops (Fig. 15). Methods were developed to reproducibly isolate individual cells from EpidermTM cultures so as to prepare high quality slides for MN analysis. Gentle trypsinisation produces around 300,000 cells per tissue, and by using cytochalasin B to collect binucleate cells, around 40–50% of collected cells were found to be dividing. The background MN frequency has been found to be low (mean 0.05%, range 0–2/1000 cells) and clear induction of MN has been seen after treatment with mitomycin C, vinblastine sulfate, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and methyl methanesulfonate (e.g. Fig. 16). More importantly, the rodent skin non-carcinogens trichloroethylene, 2-ethyl-1,3-hexanediol, 4-nitrophenol and 1,2-epoxydecane were negative. Some metabolic characterisation has been performed, and EpidermTM expresses numerous xenobiotic metabolism related genes observed in normal human skin. Further studies are in progress including experiments with chemicals requiring metabolic activation.

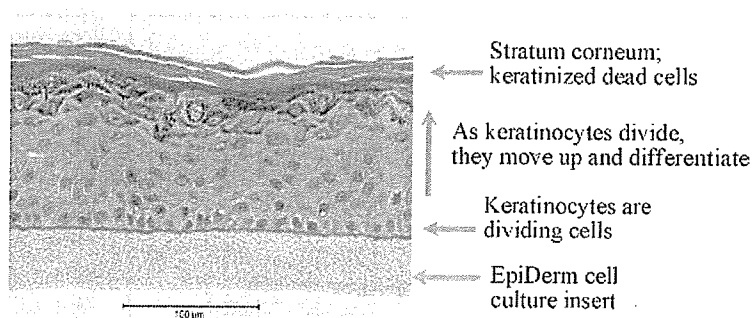


Fig. 15. Histology of EpiDerm™ after *in vitro* culture of normal human keratinocytes.

Meunier from L'Oréal presented data from a similar 3D skin model, Episkin™. DNA damage was assessed by induction of comets in isolated keratinocytes. The induction of DNA damage by UV-A light was clearly enhanced by the photogenotoxic fluoroquinolone lomefloxacin (Fig. 17). 4-Nitroquinoline-*N*-oxide also induced comets in cells of Episkin™. However, no data were presented for chemicals that are accepted as neither genotoxic *in vivo* nor DNA-reactive, mutagenic carcinogens.

3. Discussion and recommendations

Discussion of the issues and presentations took place in four break-out groups, and then in a final plenary session. There was general agreement that the false positive rate with the current mammalian cell systems, in particular with the rodent cell lines, is not acceptable. There are several actions that need to be taken in order to improve the situation, and the consensus recommendations are summarised in the following paragraphs. Some of the actions are for the shorter term, i.e. to reduce the risk of false positives with the existing systems. Other actions are medium or long term requiring the development and evaluation of modified or new systems.

3.1. Interpretation of positive results as relevant or irrelevant

Until mammalian cell tests with higher specificity are identified or developed, it is necessary to obtain evidence on the relevance for humans of positive results in genotoxicity tests, in particular in cultured mammalian cells. It was acknowledged that the level of understanding of the mechanisms that might lead to false positive results is poor amongst many scientists involved in the safety of chemicals and drugs, and that education is needed. It is understood that several reviews of some of the accepted threshold and non-relevant mechanisms of genotoxicity, and approaches to obtain evidence, are in preparation. However, a detailed "trouble-shooting" manual on approaches to investigate whether a positive result is relevant could be very helpful. In addition to avoiding cells with unstable karyotypes and excessive cytotoxicity (see below) some of the approaches that can be taken are:

- Look for (lack of) suspicious activity in microarrays and quantitative structure activity databases.
- Determine whether reactive oxygen species were generated by reaction of the test chemical with culture medium.

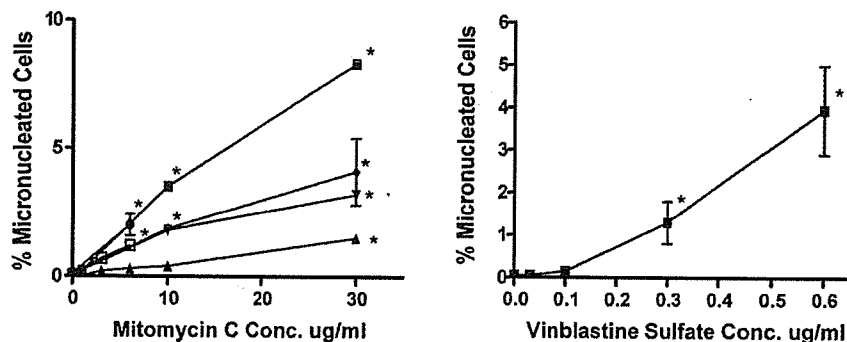


Fig. 16. Induction of micronuclei in EpiDerm™ by mitomycin C and vinblastine sulfate.

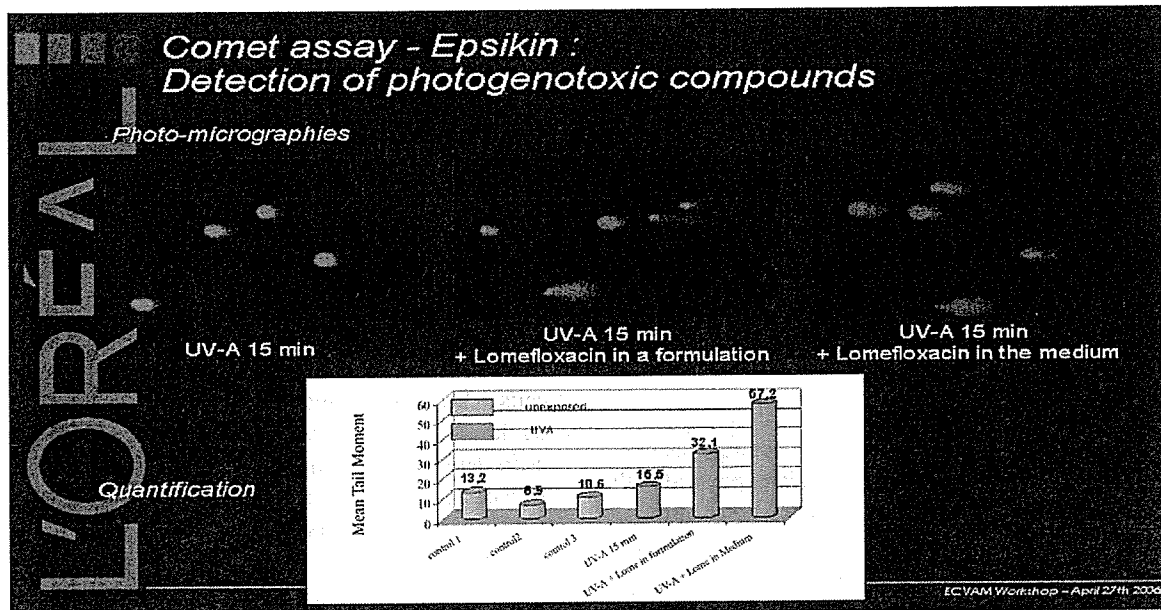


Fig. 17. Induction of comets by lomefloxacin + UV in Epsikin. For the photosensitization experiments with lomefloxacin, the Epsikin® epidermises were treated either by adding a 50 μM lomefloxacin solution in ethanol to the Epsikin® culture medium “underneath the skin” (mimicking the systemic flow) or by topical application of 1 mg/cm^2 of a 4% lomefloxacin cream (kindly provided by L’Oreal Applied Research Laboratories at Chevilly LaRue, France). In those cases the Epsikin® was treated for 1 h with lomefloxacin, rinsed three times with PBS and then irradiated 15 min with UVA light. The comet assay was performed immediately after the irradiation. All the unexposed controls (topically treated or culture-media treated epidermises, untreated epidermis) were included in the experiment as described in the figure.

- Check for degradation products in culture medium.
- Investigate possible confounding effects of apoptosis (e.g. by using CTLL-2 *bcl2* cells or Annexin-V analysis) and necrosis.
- Check for agonist and antagonist effects on kinases.
- Check for loss of cellular homeostasis (e.g. high osmolality, low pH).
- Check for metabolic poisoning and inhibition of DNA synthesis.
- Check for possible exposure to UV light.
- Check for nucleotide pool imbalances.
- Check for metabolic overload (e.g. glutathione depletion).
- Determine absence of DNA adducts under genotoxic conditions, preferably using radiolabeled chemical rather than ^{32}P -postlabeling.

In order that practising scientists and regulatory reviewers may become more familiar with the likely causes of genotoxic responses that are not relevant for humans, we encourage journals to publish data on coded compounds (from industrial in-house databases) that will help exemplify non-DNA or non-relevant mechanisms of genotoxicity. However, in such cases, as much information as possible should be provided on each test agent,

and an independent review of the classification of positive and negative calls will probably be needed.

In addition to the above suggestions, it was acknowledged that 3D tissue models, such as those presented for skin, could provide valuable information on the relevance of *in vitro* positive results, and the further development of such models is to be encouraged. However, given that relatively few human or animal skin carcinogens (UV light, PAHs) exist, these models may be of limited value as replacements for whole animals.

3.2. Cell culture conditions and techniques

It was agreed that “good housekeeping” of cell cultures is a requirement for reliable and reproducible results. Practitioners should avoid working with high passage cells and should look at chromosomal content, karyotype and other characteristics such as metabolic capability and response to reference genotoxins for evidence of genetic drift. There are some suggestions that cell density and culture size can have an impact on the response of the cultures to chemical insult and this needs to be investigated. The ECVAM task force on good cell culture practice (GCCP) has previously published recommendations on GCCP [8]. These recommendations need to be reviewed in light of the current workshop to

see if they need to be up-dated and/or adapted to *in vitro* genotoxicity testing.

There was concern at the possibility of reactive oxygen species being formed by reaction between the culture medium and test chemical. More data are needed before advice can be given on whether certain cell/media systems are less likely to produce artefactual results through oxidative stress than others, and Halliwell is encouraged to continue his investigations in the hope that such recommendations can be made.

3.3. Biotransformation

The xenobiotic-metabolising system comprises several hundred enzymes, which are usually expressed with high selectivity in varying tissues, cell types and ontogenetic stages, and in rodents also often with high sex specificity. Some enzymes are involved in the biotransformation of many genotoxicants, others are important only for a small number of compounds, and some reaction types involve a higher risk of formation of reactive metabolites than others. Various enzymes are only present at significant levels after induction by specific endogenous or xenobiotic factors. Thus, no cell type *in vivo* reflects the full biotransformation capacity of the organism. Even hepatocytes, which are heavily involved in biotransformation, only express a limited selection of xenobiotic-metabolising enzymes. For example, there are now strong indications that the hepatocarcinogenicity of PAHs in rodents is due to bioactivation by CYP1B1 in extrahepatic tissues, and that hepatic CYP1A1 – which normally is used for “promiscuous” activation of PAHs *in vitro* – acts as a major PAH-detoxifying enzyme *in vivo*. Moreover, the expression of numerous enzymes ceases, or is drastically decreased, in cells in culture. In part, this simply reflects the propensity of the organism to avoid expression of risk-borne enzymes in proliferating cells. This is true even for hepatic cell lines (e.g. HepG2) that have retained much biotransformation activity in comparison to fibroblastoid or lymphocytic cell lines. Classical S9 is a rich source of selected CYPs, but otherwise the spectrum of enzymes present in active form is very low.

The risks resulting from the formation of reactive intermediates is reduced *in vivo* by the presence of detoxifying systems, which are often extremely efficient (but overlap with toxifying systems). Two processes of detoxification can be distinguished:

(i) Metabolic “sequestration” of a promutagen into pathways that avoid the formation of the ultimate mutagen.

(ii) Inactivation of an active metabolite after its formation.

These processes differ in the enzyme systems involved. Sequestration commonly occurs by CYPs, reductases/dehydrogenases, UGTs and SULTs. The individual members of these enzyme classes are often expressed with high selectivity in certain cell types of physiological stages. Inactivation of active (electrophilic) metabolites is normally conducted by epoxide hydrolases or GSTs. These enzymes – although not every single form – are widely expressed in many tissues and cells. Thus, microsomal epoxide hydrolase and substantial levels of GST activity towards some substrates have been detected in all mammalian cell lines studied [31]. However, high-efficiency (high V_{max}/K_m) enzymes may be most important for sequestration and inactivation, especially in systems with high-efficiency, rather than promiscuous, activation. Some proximate and ultimate genotoxicants equilibrate *in vivo*. In this case, efficient protection may even occur through enzymes located at sites different from the site of activation. However, when this equilibration is limited, the appropriate localisation of the detoxifying system may be critical. An example is aflatoxin B₁, which is a potent hepatocarcinogen in the rat but only weakly active in the mouse. Constitutive expression of Gst a5, an enzyme that efficiently inactivates aflatoxin B₁ 8,9-oxide in mouse but not rat liver, appears to be an important mechanism underlying this difference. Although a rat Gst a5 is constitutively expressed in various extrahepatic tissues, this localisation appears to be inefficient for toxification. However, after hepatic induction of Gst a5 by certain chemicals, the rat becomes resistant towards the hepatocarcinogenicity of aflatoxin B₁ [32]. It is not possible to mimic such complex, varying pharmacokinetic processes in a simple *in vitro* screening model.

Detoxification may occur to the parent compound or downstream to its metabolites. The capacity of *in vitro* systems to detoxify is usually modest, often limited to the nanomolar to low micromolar concentration range over the entire exposure period. Thus, no significant competition between toxifying and detoxifying activities would occur unless the maximum concentration of parent compound tested was rigorously restricted to a very low range, and the relevant enzymes were present. The situation is very different for metabolites. Their concentrations are low, implying high-affinity (or more precisely high-efficiency) detoxification reactions can potentially occur.

The purported lack of enzymic detoxification *in vitro* is in contrast to observations that various chemicals,

when tested at high concentrations, are positive in direct genotoxicity assays, but negative in the presence of S9. Although the underlying mechanism(s) for the lack of genotoxicity in the presence of S9 have not been elucidated in most cases, it has been demonstrated for some cases that heat-inactivated S9 was also protective (H.R. Glatt, unpublished results). This suggests that physical trapping (e.g. of lipophilic compounds in microsomes) or chemical trapping (e.g. reaction of electrophiles with nucleophilic sites), rather than enzymatic activities, produced the effect. Therefore, it is difficult to assess the *in vivo* significance of this observation.

Currently the impact of metabolic differences between *in vitro* and *in vivo* test systems on the false positive rate in *in vitro* genotoxicity tests is not known. However, it is clear that variation of the metabolising system can have dramatic effects on the results of *in vitro* tests as well as for animal studies, where genetic knockout or inhibition of an individual enzyme can eliminate the ability of a carcinogen to induce tumours. In an ideal world the same metabolic modulation should have parallel consequences *in vivo* and *in vitro*.

A review of the important *in vivo* genotoxins and DNA-reactive mutagenic carcinogens is needed to determine whether metabolic differences between *in vitro* and *in vivo* test systems are, in fact, contributing to the high false positive rate, and to better define the relevant metabolic systems to include in *in vitro* tests. Perhaps we will have to include a much larger variety or different set of enzyme systems than have been traditionally used in *in vitro* tests to predict better what happens in animals. Genetic engineering may be used to help address this goal, but this approach will be very time- and cost-intensive. In addition, to achieve the proper balance of all of the relevant enzymes in a given engineered cell line to appropriately detect all *in vivo* genotoxins and DNA-reactive, mutagenic carcinogens is likely to be an insurmountable task. Moreover, such an approach would not reflect the *in vivo* situation, where different enzymes are often compartmentalised in different cells. Alternatively, a panel of individual cell lines, each with a small number of expressed enzymes, could be used. Almost infinite possibilities for permutations of various enzymes exist with such an approach, generating ideal tools for research on mechanisms. However, the selection of a cell battery for broad scale genotoxicity screening would require some arbitrary, pragmatic decisions, and this arbitrary character, as well as large deviations from the balance of enzymes *in vivo*, would remain obvious. This is in contrast to alternative metabolising systems (S9, conventional “metabolically competent” cell lines) where the situation may be similar but less obvious and

less open for remedies by systematic, hypothesis-driven research in a concrete situation.

From the point of view of genetic engineering, one may either start with a cell line that has retained some residual xenobiotic-metabolising activities (such as HepG2), or from a relatively “clean” cell line (V79 or a human equivalent, if available). The former model has the advantage that less engineering is required to achieve broad xenobiotic-metabolising capacities. The latter model is favoured when used as an analytical tool to specify critical host factors, as background activities are minimised. Further aspects that should be taken into account in the selection of the basic system(s) are:

- (a) Genetic engineering intrinsically involves numerous culture passages and clonal selections; therefore, long-term stability of critical properties (obviously including biotransformation activities) is pivotal.
- (b) The cell line selected should be suitable for efficient analysis of important and robust endpoints, such as gene mutations.

3.4. Top concentration for testing

Current OECD guidelines for genotoxicity testing in mammalian cells require that the top concentration with soluble and non-toxic substances should be 10 mM or 5000 µg/ml, whichever is the lower. There was some discussion about whether this may be appropriate for complex mixtures and technical grade (impure) industrial chemicals for example, where the objective is not only to test the genotoxicity of the main ingredient. However, given that mutagenic impurities are only detected in an Ames test carried out to 5 mg/plate (when spiked at a level of about 5% [33]), it was questioned whether detection of impurities is a sufficiently important role for genotoxicity testing to justify pushing to such high concentrations. However, the K_m for many biochemical reactions, whether involved in metabolic activation/inactivation, general cellular defence/balance, cellular transport or cellular turnover is less than 100 µM [34,35,36]. It is probable that low K_m reactions primarily determine the bioactivation pathway *in vivo*. These kinetic characteristics suggest that the high concentrations currently required for *in vitro* testing may not be informative for human risk assessment. The 10 mM and 5000 µg/ml requirements are seemingly based on a small number of carcinogens that needed high concentrations before giving positive responses in mammalian cell tests *in vitro*, sometimes using inappropriate metabolic conditions. It is not known whether the carcinogens that require these high concentrations for detection *in vitro*

are “important”, how robust the *in vitro* mammalian cell findings are, or whether these chemicals are positive in other test systems (e.g. the Ames test) currently used in a standard battery. The fact that the published data on these chemicals are probably quite old could mean that under current chromosomal aberration and gene mutation protocols they could be detected at lower concentrations. It also has to be considered that simple detection of a carcinogen at high *in vitro* concentrations that are not relevant *in vivo* does not mean there is a mechanistic correlation between the *in vitro* genotoxicity and the *in vivo* carcinogenicity.

Further to the above considerations on the K_m of important biochemical processes, general considerations on *in vivo* exposure to (toxic) compounds have been put forward. In this context one can consider knowledge about intentional high dose or long-term exposure to pharmaceuticals as worst case examples. It is clear that even high dose pharmaceuticals such as antibiotics (e.g. penicillins, fluoroquinolones) or pain relief agents such as acetaminophen (also known as paracetamol) seldomly yield systemic or tissue levels $>10 \mu\text{M}$ [37]. Thus, taking chronic intake, possible accumulation and overdosing scenarios into account, a lowering of the current maximum *in vitro* concentration, perhaps by 10-fold or more, may be justified, at least for certain types of chemicals (such as pharmaceuticals) from scientific and consumer protection viewpoints.

The participants therefore agreed that a new review of existing data is needed to determine whether such high concentrations as 10 mM or 5000 $\mu\text{g}/\text{ml}$ are needed to detect *in vivo* genotoxins and DNA-reactive, mutagenic carcinogens. The following actions are recommended:

- An expert panel should be assembled to determine which *in vivo* genotoxins and DNA-reactive, mutagenic carcinogens need to be detected in *in vitro* mammalian cell tests. This subset of chemicals should be determined from both published and industry (confidential) data. A first suggestion of an important data set would be the IARC groups 1, 2A and 2B carcinogens, omitting those, such as hormones and immunosuppressants, which are acknowledged to be of a non-genotoxic mode of action. In view of the on-going initiative of the Health and Environmental Sciences Institute of the International Life Sciences Institute (ILSI-HESI) with regard to false positives in *in vitro* mammalian cell genotoxicity tests, it was suggested this action could be best achieved in conjunction with ILSI-HESI, and the outcome of their workshop (held in June 2006) will be reported elsewhere.
- The role of metabolism in the activity of the above *in vivo* genotoxins and DNA-reactive, mutagenic carcinogens needs to be reviewed in order to define the appropriate metabolic systems to include in *in vitro* genotoxicity assays.
- The published and industry data should be reviewed to determine whether concentrations as high as 10 mM or 5000 $\mu\text{g}/\text{ml}$ are needed to detect this important subset of chemicals, or whether a lower level could be justified.
- If high concentrations are needed in the mammalian cell tests, data from other tests such as the Ames test should be reviewed to see if the chemical(s) would be detected in other parts of the standard battery.
- If high concentrations are needed, and genotoxicity was not detected in other parts of the standard battery, an opinion should be formed as to whether a more modern protocol or modified metabolic conditions would be likely to detect genotoxic effects at lower concentrations. If necessary, new testing should be initiated.
- If high concentrations are needed (with appropriate metabolic conditions) a scientific effort should be mounted to elucidate whether the mechanism(s) of genotoxicity for these chemicals would trigger responses in other toxic endpoints.
- A thorough evaluation of various human exposure scenarios should be made to determine an upper limit of *in vitro* testing from the viewpoint of human consumer protection.

It is evident that the concentration of a pro-genotoxicant required for a positive test result can vary dramatically depending on the activating system used. General improvements in the activation systems, or in a chemical class-dependent manner (with corresponding positive control compounds), might be an important pre-requisite for a reduction in the top concentration. For the time being, the participants of the workshop concluded that it is prudent to challenge the current recommended upper concentration of *in vitro* testing (10 mM or 5000 $\mu\text{g}/\text{ml}$), and that a lower level appears to have scientific merit.

3.5. Measures and extent of cytotoxicity

Many different measures of cytotoxicity are used in mammalian cell tests, in particular the chromosomal aberration test. Reductions in cell count, confluency, mitotic index and population doubling are all widely used and equally accepted, but it is unlikely that all these measures would select the same top concentra-

tion for testing. Other indicators of toxicity such as ATP levels, mitochondrial function, LDH-leakage may also be appropriate. Greenwood et al. [11] showed that several non-DNA-reactive chemicals and metabolic poisons would not have given positive chromosomal aberration results if the 50% cytotoxic concentration had been chosen based on a reduction in population doubling rather than a reduction in cell count, and that, using this measure, no important DNA-reactive genotoxins would have been missed. These findings have not been independently verified and there are no other publications comparing different measures of cytotoxicity in relation to genotoxicity.

The participants therefore agreed there is a need for a thorough comparison of different measures of cytotoxicity in case some measures may select concentrations for testing that allow the detection of all important *in vivo* genotoxins and DNA-reactive, mutagenic carcinogens but lowers the risk of false positives. In particular:

- A collaborative trial is needed on a selected set of chemicals. This could be the same subset of chemicals selected for evaluation of top concentration (above). Additional sets of chemicals, such as the non-DNA-reactive chemicals of the Greenwood et al. [11] publication, and agreed non-genotoxins also need to be included. If possible, this trial should be co-ordinated with any initiatives coming from the ILSI-HESI workshop (June 2006) which will be reported elsewhere.
- Multiple endpoints of toxicity need to be compared at the same time in each participating laboratory.
- Dose–response relationships for cytotoxicity and genotoxicity should be determined, and include the current required levels (i.e. at least 50% toxicity for chromosomal aberrations, at least 60% toxicity for the micronucleus assay and at least 80% toxicity in the mouse lymphoma assay).
- Human cells such as lymphocytes should be included as well as the rodent (and any other, e.g. TK6, HepG2) cell lines, and measures of cytotoxicity for lymphocytes other than mitotic index need to be identified.
- Robust endpoints of genotoxicity that are not sensitive to interference by cytotoxicity (e.g. gene mutations and DNA adducts) should be identified and included in the trials.

Although the only indication came from the data of Elhajouji, there was a belief amongst many participants that a majority of false positive results in chromosomal aberration and mouse lymphoma tests probably occur in the prolonged, continuous treatments in the absence

of exogenous metabolic activation. Several factors such as extent of exposure, prolonged cytotoxicity, and lack of detoxification by S9 may be involved, but the exact reasons are not known. However, it was noted that the 50% and 80% toxicity requirements for the chromosomal aberration and mouse lymphoma assays were originally based on short (e.g. 3–6 h) treatments, and the need for these levels of toxicity (or even the appropriateness of the current measures) has not been independently justified. Therefore, this collaborative trial must include chemicals that are only positive after prolonged (e.g. 20–24 h) treatments.

3.6. Criteria for and evaluation of new mammalian cell test systems

Certain characteristics of the commonly used rodent cell lines (CHO, CHL, V79, L5178Y, etc.) such as their *p53* status, karyotypic instability, DNA repair deficiencies, etc. are recognised as possibly contributing to the high rate of false positives. The need for exogenous metabolism with the cell systems is also expected to contribute to the false positive rate. If these cell types are to be replaced in the future, any new systems should ideally:

- Be early passage.
- Be karyotypically stable and, if possible, normal.
- Be *p53* proficient.
- Be DNA repair proficient.
- Preferably consist of human cells.
- Be metabolically competent (at least Phase 1 and Phase 2 capacity should be defined), if necessary through genetic engineering. Expert advice is needed on what are the essential Phase 1 and Phase 2 enzymes that should be functional in any new system for the biotransformation of *in vivo* genotoxins and DNA-reactive, mutagenic carcinogens. Several different cell lines with different enzyme profiles may cover a reasonable fraction of the complex biotransformation machinery present *in vivo*. However, the selective expression of a limited number of enzymes in a cell line may direct the biotransformation into pathways that are different from the major pathways occurring in animals and humans, where many different enzymes compete and interact with each other.
- Be able to detect the majority of genetic endpoints relevant to human somatic and inherited disease.
- Show improved specificity without reducing the ability to detect *in vivo* genotoxins and DNA-reactive, mutagenic carcinogens.

The participants were aware that this wish list is far too ambitious to be realised in a single test system, at least within a reasonable time. Therefore, several systems may be required, which in combination will facilitate the distinction between correct and false positive results.

Some of the data presented at the workshop indicated that the human lymphocyte cell system might produce a lower level of false positives than the common rodent cell lines. If the use of the clastogenicity endpoint *in vitro* is to be continued, further work is needed to establish whether human lymphocytes do offer a lower false positive rate, and also to find alternative methods (other than mitotic index) for measuring cytotoxicity.

Although many of the new systems presented at the Workshop show promise, and fulfil some of the criteria given above, none fulfils all of the criteria. In many cases (e.g. MCL-5, HepG2, transgenic cell lines) it is the lack of data on specificity that is the problem. In the case of the *GADD45a-GFP* assay it is mainly the lack of data with compounds requiring metabolic activation. The new 3D skin models are also at an early stage of development. It was therefore agreed that a collaborative research program is needed to evaluate new mammalian cell-based methods and models for genotoxicity. In addition to the cell systems discussed at the workshop, other cells that have retained some xeno-metabolic activities (e.g. HepaRG [38] and AR42J-B13 rat pancreatic stem cells [39,40]) or cell lines genetically engineered to express appropriate Phase 1 and Phase 2 metabolism should be considered.

This collaborative research program will be a major exercise, but if it is not undertaken we will still be faced with an unacceptable level of false positives and the consequential unnecessary follow-up *in vivo* testing, for decades to come. A steering committee, consisting of genotoxicity, metabolism and chemistry experts from academia, industry and the regulatory agencies should be established to draft a plan for this program. The participants did not reach any conclusion as to how this trial should be organised, but it is hoped that ECVAM may be able to contribute, to liaise with other related activities from organisations such as ILSI-HESI and to begin to identify sources of funding and support.

4. Conclusions

The workshop participants agreed that some of the commonly used cells for genotoxicity testing (in particular some of the rodent cell lines) have produced an unacceptably high level of false positive results when compared with known *in vivo* genotoxins and DNA-reactive, mutagenic carcinogens. In most of the rodent

cell lines used, deficiencies in metabolism, *p53* function and DNA repair capability almost certainly contribute to this high false positive rate. Better guidance on the likely mechanisms resulting in positive results that are not relevant for humans, and on how to obtain evidence for those mechanisms, is needed both for practitioners and regulatory reviewers.

Testing up to high concentrations and high levels of cytotoxicity as is currently required in mammalian cell genotoxicity tests are also likely to contribute to the high frequency of false positive results, and may not be justified. A thorough review of published and industry data to determine whether such levels are required for the detection of *in vivo* genotoxins and DNA-reactive, mutagenic carcinogens is urgently needed. Suggestions to lower the current upper limit (perhaps by 10-fold or more) may be justified in terms of metabolic and cellular processes, and human tissue exposures. This needs urgent but careful evaluation.

Various measures of cytotoxicity are currently allowable under OECD guidelines, but there is little comparative data on whether different measures would select different concentrations for test. A detailed comparison of multiple measures of cytotoxicity, in relation to endpoints such as clastogenicity, is needed. Also, genotoxicity endpoints that are not intrinsically linked with processes leading to cytotoxicity need to be developed.

There was agreement amongst the workshop participants that cell systems preferably of human origin, which are *p53* and DNA-repair proficient, and have defined Phase 1 and Phase 2 metabolism, covering a broad set of enzyme forms, and used within the context of appropriately set limits of concentration and cytotoxicity, offer the best hope for reduced false positives in the future. Whilst there is some evidence that human lymphocytes are less susceptible to false positives than the current rodent cell lines, other cell systems based on HepG2, TK6 and MCL-5 cells, and 3D skin models based on primary human keratinocytes also show some promise. However, much effort will be required to introduce a broader spectrum of metabolic capabilities into these or other target cells. Other human cell lines such as HepaRG have not been used for genotoxicity investigations and should be studied. A collaborative research programme is needed to identify and evaluate new cell systems with appropriate sensitivity but improved specificity.

Perhaps most importantly, the participants in this workshop felt that it is time for the scientific community to give careful consideration to the carcinogens and *in vivo* genotoxins we expect any new assay, or modified existing assay, to detect. Rodent bioassays have flaws in terms of detecting human carcinogens, and this is com-

pounded by trying to use genotoxicity assays to detect as many rodent carcinogens as possible. This has led to the continued expansion of genotoxicity test protocols (e.g. addition of new treatment and sampling regimens, new strains of bacteria, etc.), and the combination of these tests in batteries. The consensus of the group was that there is a need to refocus on the detection on human carcinogens and *in vivo* genotoxins that are DNA reactive. Without this change of focus, the development and validation of any new methods or assays will likely not lead to an overall improvement.

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Early Pregnancy Failure Induced by Dibutyltin Dichloride in Mice

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ABSTRACT: In this study, we examined the adverse effects of dibutyltin on initiation and maintenance of pregnancy after maternal administration during early pregnancy in mice. Following successful mating, female ICR mice were given dibutyltin dichloride (DBTCl) at 0, 7.6, 15.2, or 30.4 mg/kg bw/day by gastric intubation on days 0–3 or days 4–7 of pregnancy. Female mice were sacrificed on day 18 of pregnancy, and the pregnancy outcome was determined. After administration of DBTCl on days 0–3, the rate of non-pregnant females and the incidence of preimplantation embryonic loss were significantly increased at 30.4 mg/kg bw/day. The incidences of postimplantation embryonic loss in females given DBTCl on days 0–3 at 15.2 mg/kg and higher and on days 4–7 at 7.6 mg/kg bw/day and higher were increased. No increase in the incidence of fetuses with external malformations was observed after the administration of DBTCl on days 0–3 or days 4–7. A decline in the serum progesterone levels was detected in mice given DBTCl at 30.4 mg/kg bw/day on days 0–3 or days 4–7 of pregnancy. The data show that DBTCl adversely affects the initiation and maintenance of pregnancy when administered during early pregnancy in mice and suggest that the decline in serum progesterone levels is responsible for pregnancy failure. © 2007 Wiley Periodicals, Inc. *Environ Toxicol* 22: 44–52, 2007.

Keywords: dibutyltin dichloride; organotin; pregnancy failure; early embryonic loss; progesterone

INTRODUCTION

Organotin compounds are chemicals widely used in agriculture and industry. Disubstituted organotin compounds are commercially the most important derivatives, being used as heat and light stabilizers for polyvinyl chloride (PVC) plastics to prevent degradation of the polymer during the melting and forming of the resin into its final products, as catalysts in the production of polyurethane foams, and as vulcanizing agents for silicone rubbers (Piver, 1973; WHO, 1980). Wide-spread use of organotin compounds has caused increasing amounts to be released into environment.

The most important route of entry of organotin compounds as nonpesticides into the environment is through the leaching of organotin-stabilized PVC by water (Quevauviller et al., 1991), and its use in antifouling agents resulting in the entry of organotin into the aquatic environment (Maguire, 1991). The identification of dibutyltin (DBT) and tributyltin (TBT) in aquatic marine organisms (Sasaki et al., 1988; Lau, 1991) and marine products (Suzuki et al., 1992) has been reported. TBT is degraded spontaneously and biochemically via a debutylation pathway to DBT in the environment (Seligman et al., 1988; Stewart and de Mora, 1990). Food chain bioaccumulation of butyltin in oysters (Waldock and Thain, 1983), mud crabs (Evans and Laughlin, 1984), marine mussels (Laughlin et al., 1986), Chinook salmon (Short and Thrower, 1986), and dolphin, tuna, and shark (Kannan et al., 1996) has been reported. These findings indicate that butyltins accumulate in the

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food chain and are bioconcentrated, and that humans can be exposed to butyltins via food.

Organotin possesses toxic effects on reproduction and development in experimental animals (Ema and Hirose, 2006). We previously reported that dibutyltin dichloride (DBTCl) by gavage throughout the period of organogenesis resulted in a significant increase in the incidence of fetal malformations in rats (Ema et al., 1991) and that rat embryos were highly susceptible to the teratogenic effects of DBTCl when administered on day 7 and day 8 of pregnancy (Ema et al., 1992). Tetrabutyltin (TeBT) is metabolized to TBT, DBT, and monobutyltin (MBT) derivatives (Fish et al., 1976; Kimmel et al., 1977). The TBT compound is metabolized to DBT and MBT derivatives and DBT is metabolized to MBT derivatives (Iwai et al., 1981). The developmental toxicity studies on butyltins suggest that the teratogenicity of DBT is different from those of TeBT, TBT, and MBT in its mode of action, because the susceptible period for teratogenicity and types of malformations induced by DBT are different from those induced by TeBT, TBT, and MBT (Ema et al., 1995, 1996). Tributyltin chloride (TBTCl) (Harazono et al., 1996, 1998ab) and DBTCl (Ema and Harazono, 2000ab) during early pregnancy produced pregnancy failure in rats. In rats, the predominant adverse effects on reproduction and development of TBTCl and DBTCl on days 0–3 of pregnancy were a decrease in the pregnancy rate and an increase in the incidence of preimplantation embryonic loss, and TBTCl and DBTCl on days 4–7 of pregnancy mainly caused postimplantation embryonic loss (Harazono et al., 1998b; Ema and Harazono, 2000ab). The doses of DBTCl that caused early embryonic loss were lower than those of TBTCl (Ema and Harazono, 2000b). Thus, the possibility exists that DBTCl and/or metabolites participate in the induction of early embryonic loss due to TBTCl.

The reproductive and developmental effects of organotin compounds, including DBT, were extensively investigated in rats (Ema and Hirose, 2006). We are unaware of any studies in which the adverse effects of DBT on initiation and maintenance of pregnancy have been assessed in mice. Studies in mice would be of great value in evaluating the reproductive and developmental toxicity of DBT. The present study was therefore conducted to determine the adverse effects on the initiation and maintenance of pregnancy of maternal exposure to DBTCl during early pregnancy in mice.

MATERIALS AND METHODS

Animal Husbandry and Maintenance

Male and female Crlj:CD1(ICR) mice at 8 weeks of age were purchased from Atsugi Breeding Center, Charles River Japan, (Yokohama, Japan). The mice were acclimat-

ized to the laboratory for 11 days prior to the start of the experiment. Male and female mice found to be in good health were selected for use. Female mice were caged with male mice and checked the following morning for signs of successful mating by examining vaginal plugs. The day when vaginal plugs were detected was considered to be day 0 of pregnancy. Successfully mated females were distributed into eight groups of 12 mice each and housed individually. Animals were reared on a γ -irradiated basal diet (CRF-1; Oriental Yeast, Tokyo, Japan) and filtered tap water *ad libitum*, and maintained in an air-conditioned room at $22^{\circ}\text{C} \pm 3^{\circ}\text{C}$, with a relative humidity of $50\% \pm 20\%$, under a controlled 12 h light/dark cycle, and ventilation with 10–15 air changes/hour. This study was performed in 2005 at the Safety Research Institute for Chemical Compounds. (Sapporo, Japan) in compliance with the "Law for the Humane Treatment and Management of Animals" (Ministry of the Environment, Japan, 1973), "Standards Relating to the Care and Management, etc. of Experimental Animals" (Prime Minister's Office, Japan, 1980) and "Guidance for Animal Care and Use of the Safety Research Institute for Chemical Compounds, Co."

Chemicals and Dosing

DBTCl was purchased from Tokyo Kasei Kogyo (Tokyo, Japan). The DBTCl used in this study was 99.5% pure, and it was kept in a dark and cool place. DBTCl was dissolved in olive oil (Wako Pure Chemical Industries, Osaka, Japan). The female mice were dosed once daily by gastric intubation with DBTCl at a dose of 7.6, 15.2, or 30.4 mg/kg bw (25, 50 or 100 $\mu\text{mol}/\text{kg}$ bw) on days 0–3 of pregnancy or on days 4–7 of pregnancy. The dosage levels were determined based on the results of our previous studies, in which increases in the incidence of pre- and postimplantation embryonic loss were caused in female rats gavaged with DBTCl at 7.6 mg/kg bw/day and higher on days 0–3 and days 4–7 of pregnancy, respectively (Ema and Harazono, 2000ab) and our dose-finding study in which no adverse effects on embryonic survival at 15.2 mg/kg bw/day and lower, increased embryonic loss at 30.4 mg/kg bw/day, and one death and three pregnancy failure in four females at 60.8 mg/kg bw/day were found in mice gavaged with DBTCl on days 0–3 of pregnancy. The volume of each dose was adjusted to 5 mL/kg of body weight based on the daily body weight. The control mice received olive oil only on days 0–3 or days 4–7 of pregnancy. All DBTCl solutions were prepared fresh daily.

Observations

All mice were observed for clinical signs of toxicity twice a day during the administration period and daily during the nonadministration period. Females showing a moribund condition were euthanized under ether anesthesia. Maternal

TABLE I. Maternal findings in mice given DBTCl by gastric intubation on days 0–3 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of females showing clinical signs				
Dead	0	1	0	0
Moribund condition (euthanized)	0	1	1	1
Vaginal discharge	0	1	0	0
Jaundice	0	2	7*	10*
Decreased locomotor activity	0	2	1	1
Hypothermia	0	1	1	1
Soil of perigenital fur	0	0	1	0
Initial body weight (g) ^a	27.4 ± 2.0	27.2 ± 2.1	27.2 ± 2.4	27.2 ± 2.1
Body weight gain (g) ^a				
Days 0–4	1.7 ± 1.1	0.6 ± 1.2	1.2 ± 1.6	0.3 ± 0.9*
Days 4–8	2.9 ± 1.5	2.5 ± 2.6	2.1 ± 2.0	1.6 ± 1.5
Days 8–18	20.1 ± 9.1	21.3 ± 12.4	13.6 ± 12.2	8.6 ± 12.2
Adjusted weight gain ^b	8.9 ± 3.4	9.9 ± 3.8	7.9 ± 4.8	5.3 ± 5.0
Food consumption (g) ^a				
Days 0–4	18.2 ± 1.8	15.0 ± 1.9*	16.7 ± 3.2	14.8 ± 2.3*
Days 4–8	22.9 ± 4.9	22.0 ± 2.7	21.7 ± 3.5	20.9 ± 3.5
Days 8–18	71.7 ± 10.1	71.0 ± 12.5	64.6 ± 13.3	57.8 ± 13.4*

^a Values are given as mean ± SD.

^b Adjusted weight gain refers to body weight gain excluding the uterus.

* Significantly different from the control, $P < 0.05$.

body weight was recorded daily, and food consumption was recorded on days 0, 4, 8, 12, and 18 of pregnancy. The females were euthanized by exsanguination under ether anesthesia on day 18 of pregnancy. The uterus was weighed and the number of corpora lutea was recorded. The numbers of implantations, live and dead fetuses, and of resorptions were counted. The uteri were placed in 10% ammonium sulfide for confirmation of the dam's pregnancy status (Salewski, 1964). The live fetuses removed from the uterus were sexed, weighed, and inspected for external malformations and malformations within the oral cavity. The placental weight was also measured.

Analysis of Serum Steroids Hormone Levels

Blood samples were collected from the abdominal aorta under ether anesthesia on day 4 or day 8 of pregnancy, 24 h after the last administration of DBTCl at 0 or 30.4 mg/kg bw/day on days 0–3 or days 4–7 of pregnancy. The serum was separated and stored at -80°C for later assay of steroid hormones. Serum progesterone and 17β -estradiol were measured by Teizo Medical (Kawasaki, Japan) using the liquid chromatography-electrospray ionization Tandem Mass Spectrometry (LC-MS/MS, Applied Biosystems/MDS SCIEX). The detection limits of serum progesterone and 17β -estradiol were 10.0 and 0.25 pg/mL, respectively. The intra- and interassay coefficients of variation for 17β -estradiol were below 6.4% and 8.9%, respectively. The intra- and interas-

say coefficients of variation for progesterone were below 9.0% and 7.9%, respectively.

Statistical Analysis

The statistical analysis of fetuses was carried out using the litter as the experimental unit. Maternal body weight, body weight gain, adjusted weight gain, food consumption, numbers of corpora lutea, implantations, embryonic/fetal loss and live fetuses, fetal weight, and placental weight were analyzed for statistical significance as follows. Bartlett's test of homogeneity of variance was used to determine if the groups had equivalent variances at the 5% level of significance. If the variances were equivalent, the groups were compared by one-way analysis of variance. If significant differences were found, Dunnett's multiple comparison test was performed. If the groups were not equivalent, the Kruskal-Wallis test was used to assess the overall effects. Whenever significant differences were noted, pair-wise comparisons were made using the Mann-Whitney U test. The incidences of pre- and postimplantation embryonic loss and fetuses with external malformations were analyzed using Wilcoxon's rank sum test. The incidence of clinical signs in dams, pregnancy, nonpregnancy, and litters with fetal malformations, and the sex ratio of live fetuses were analyzed using Fisher's exact test. The levels of serum progesterone and 17β -estradiol were analyzed by Student's t -test. The 0.05 level of probability was used as the criterion for significance.

TABLE II. Reproductive and developmental findings in mice given DBTCl by gastric intubation on days 0-3 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of nonpregnant females	1	3	4	7*
No. of pregnant females	11	9	8	5*
No. of implantations per female ^{a,b}	9.5 ± 5.1	9.8 ± 7.1	8.3 ± 7.0	5.4 ± 6.7
Pre-implantation loss per female (%) ^{a,b}	9.7	29.7 ^c	34.0	58.3*
No. of pregnant females surviving until scheduled sacrifice	11	8	7	4
No. of litters totally resorbed	0	0	1	1
No. of corpora lutea per litter ^{a,d}	10.5 ± 4.3	13.1 ± 4.9	12.4 ± 4.4	13.3 ± 1.3
No. of implantations per litter ^{a,d}	10.4 ± 4.3	12.6 ± 4.9	12.3 ± 4.4	13.3 ± 1.3
Pre-implantation loss per litter (%) ^{d,e}	1.5	3.3	1.1	0
No. of post-implantation loss per litter ^{a,d}	1.0 ± 1.0	1.1 ± 1.5	4.1 ± 3.2	4.0 ± 5.4
Post-implantation loss per litter (%) ^{d,f}	10.1	14.1	41.3*	32.2
No. of live fetuses per litter ^{a,d}	9.4 ± 4.2	11.5 ± 5.3	8.1 ± 5.0	9.3 ± 6.2
Sex ratio of live fetuses (male / female)	50/53	47/45	30/27	21/16
Body weight of live fetuses (g) ^a				
Male	1.54 ± 0.19	1.30 ± 0.12*	1.14 ± 0.22*	1.12 ± 0.10*
Female	1.42 ± 0.15	1.28 ± 0.20	1.08 ± 0.26*	1.01 ± 0.11*
External examinations of fetuses				
No. of fetuses (litters) examined	103 (11)	92 (8)	57 (6)	37 (3)
No. of fetuses (litters) with anomalies	1 (1)	0	1 (1)	0
Cleft palate	1	0	1	0
Kinked tail	0	0	1	0
Placental weight (mg) ^a	125 ± 56	116 ± 15	120 ± 17	119 ± 16

^a Values are given as mean ± SD.^b Values obtained from females successfully mated.^c Value obtained from 11 females, because corpora lutea were indistinguishable in one female.^d Values obtained from pregnant females surviving until scheduled sacrifice.^e [(No. of corpora lutea—no. of implantations)/no. of corpora lutea] × 100.^f (No. of resorptions and dead fetuses/no. of implantations) × 100.* Significantly different from the control, *P* < 0.05.

RESULTS

Administration of DBTCl on Days 0-3 of Pregnancy

Table I shows the maternal findings in mice given DBTCl on days 0-3 of pregnancy. One death was observed at 7.6 mg/kg bw/day, and one female each showed a moribund condition at 7.6, 15.2, and 30.4 mg/kg bw/day, and was euthanized. The female mice in the DBTCl-treated groups showed vagina discharge, jaundice, decreased locomotor activity, hypothermia and/or soiled perigenital fur, and the incidence of females showing jaundice was significantly increased at 15.2 mg/kg bw/day and higher. A significantly decreased body weight gain on days 0-4 was noted at 30.4 mg/kg bw/day. Food consumption on days 0-4, days 4-8, and days 8-18 in the DBTCl-treated groups were reduced, and significantly decreased food consumptions on days 0-4 at 7.6 and 30.4 mg/kg bw/day and on days 8-18 at 30.4 mg/kg bw/day were observed.

The reproductive and developmental findings in mice given DBTCl on days 0-3 of pregnancy are shown in

Table II. The total absence of any implantation site, i.e., nonpregnancy, was found in one, three, four, and seven of the 12 females in the control, 7.6, 15.2, and 30.4 mg/kg bw/day groups, respectively. In the successfully mated females, the pregnancy rate was significantly decreased, and the incidence of preimplantation embryonic loss per females was significantly increased at 30.4 mg/kg bw/day. In the pregnant females that survived until the scheduled sacrifice, the number of corpora lutea per litter, implantations per litter, live fetuses per litter, the incidence of litters totally resorbed and of preimplantation loss per litter, and the sex ratio of live fetuses were not significantly different between the control and DBTCl-treated groups. The incidence of postimplantation loss per litter was increased in the DBTCl-treated groups, and a significant increase was observed at 15.2 mg/kg bw/day. A significantly lower fetal weight was found in males at 7.6 mg/kg bw/day and in both sexes at 15.2 and 30.4 mg/kg bw/day. One fetus with cleft palate in the control group and one fetus with a cleft palate and kinked tail in the 15.2 mg/kg bw/day group were observed. The placental weight in the DBTCl-treated

TABLE III. Maternal findings in mice given DBTCl by gastric intubation on days 4–7 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of females showing clinical signs				
Dead	0	0	1	0
Moribund condition (euthanized)	0	0	0	1
Vaginal discharge	0	0	4	4
Jaundice	0	0	2	6*
Decreased locomotor activity	0	0	0	1
Hypothermia	0	0	0	1
Initial body weight (g) ^a	28.1 ± 1.8	28.1 ± 1.8	28.1 ± 1.8	28.2 ± 1.7
Body weight gain (g) ^a				
Days 0–4	1.6 ± 1.0	1.9 ± 0.8	1.2 ± 1.2	1.6 ± 0.9
Days 4–8	3.1 ± 1.1	1.9 ± 1.6	0.5 ± 1.8*	-0.3 ± 2.1*
Days 8–18	24.9 ± 9.1	14.9 ± 8.9*	2.9 ± 6.3*	2.4 ± 2.4*
Adjusted weight gain ^b	8.3 ± 3.5	8.1 ± 4.3	3.2 ± 5.3*	3.8 ± 3.2*
Food consumption (g) ^a				
Days 0–4	18.5 ± 1.9	18.9 ± 2.4	18.4 ± 2.7	18.8 ± 1.3
Days 4–8	21.8 ± 1.9	19.2 ± 2.6	16.4 ± 3.3*	15.6 ± 3.5*
Days 8–18	74.5 ± 12.1	67.7 ± 9.9	55.2 ± 12.6*	57.2 ± 6.2*

^aValues are given as mean ± SD.

^bAdjusted weight gain refers to body weight gain excluding the uterus.

*Significantly different from the control, $P < 0.05$.

groups was not significantly different from that in the control group.

Administration of DBTCl on Days 4–7 of Pregnancy

Table III shows the maternal findings in mice given DBTCl on days 4–7 of pregnancy. One death was observed at 15.2 mg/kg bw/day, and one female that showed a moribund condition at 30.4 mg/kg bw/day was euthanized. The female mice in the DBTCl-treated groups showed vaginal discharge, jaundice, decreased locomotor activity, and/or hypothermia, and the incidence of females with jaundice was significantly increased at 30.4 mg/kg bw/day. The body weight gain on days 4–8 and adjusted weight gain, which indicates the net weight gain of female mice, at 15.2 mg/kg bw/day and higher, and on days 8–18 at 7.6 mg/kg bw/day and higher were significantly decreased. Food consumption on days 4–8 and days 8–18 was significantly lowered at 15.2 mg/kg bw/day and higher.

The reproductive and developmental findings in mice given DBTCl on days 4–7 of pregnancy are presented in Table IV. Although nonpregnancy was found in one, two, and one of the 12 females in the control, 7.6, 15.2, and 30.4 mg/kg bw/day groups, respectively, no significant decrease in the pregnancy rate was noted in the DBTCl-treated groups. In the successfully mated females, the number of implantations per female was significantly decreased at 15.2 mg/kg bw/day. In the pregnant females that survived until the scheduled sacrifice, totally resorbed litters were found in 2 of the 11 females at 7.6 mg/kg bw/day, 8 of the 9 females at 15.2 mg/kg bw/day,

and 10 of the 10 females at 30.4 mg/kg bw/day. At 30.4 mg/kg bw/day, no live fetuses were obtained. The numbers of corpora lutea per litter, implantations per litter, and preimplantation loss per litter, and the sex ratio of live fetuses in the DBTCl-treated groups were not significantly different from those in the control group. A significant increase in the number and incidence of postimplantation loss per litter, and a decrease in the number of live fetuses were found in the DBTCl-treated groups. The weights of male and female fetuses were significantly lowered at 7.6 mg/kg bw/day. One fetus with omphalocele, and one fetus with exencephaly and open eyelids were observed at 7.6 mg/kg bw/day. The placental weight was not significantly different between the control and the DBTCl-treated groups.

Serum Progesterone and 17 β -Estradiol Levels

The serum progesterone and 17 β -estradiol levels are shown in Figure 1. A significant reduction in the serum progesterone levels was noted in female mice given DBTCl on days 0–3 or days 4–7 of pregnancy. Although higher levels of serum 17 β -estradiol were observed after the administration of DBTCl on days 4–7 of pregnancy, no statistically significant difference in 17 β -estradiol levels were detected between the control and DBTCl-treated groups.

DISCUSSION

The present study was designed to evaluate the adverse effects of DBTCl on the initiation and maintenance of

TABLE IV. Reproductive and developmental findings in mice given DBTCl by gastric intubation on days 4–7 of pregnancy

DBTCl (mg/kg)	0 (control)	7.6	15.2	30.4
No. of females successfully mated	12	12	12	12
No. of nonpregnant females	1	1	2	1
No. of pregnant females	11	11	10	11
No. of implantations per female ^{a,b}	12.6 ± 4.4	13.2 ± 4.6	7.5 ± 5.7*	11.1 ± 5.4
Pre-implantation loss per female (%) ^{a,b}	8.9	8.9	24.7	18.3 ^c
No. of pregnant females surviving until scheduled sacrifice	11	11	9	10
No. of litters totally resorbed	0	2	8*	10*
No. of corpora lutea per litter ^{a,d}	13.8 ± 2.1	14.5 ± 2.3	10.6 ± 5.2	13.9 ± 2.8
No. of implantations per litter ^{a,d}	13.7 ± 2.1	14.4 ± 2.2	9.4 ± 5.1	12.7 ± 4.1
Pre-implantation loss per litter (%) ^{d,e}	0.6	0.6	10.7	10.2
No. of postimplantation loss per litter ^{a,d}	0.6 ± 1.0	7.2 ± 6.1*	8.7 ± 4.8*	12.7 ± 4.1*
Post-implantation loss per litter (%) ^{d,f}	4.3	48.3*	94.4*	100*
No. of live fetuses per litter ^{a,d}	13.1 ± 2.0	7.2 ± 5.6*	0.8 ± 2.3*	0
Sex ratio of live fetuses (male/female)	82/62	50/29	4/3	
Body weight of live fetuses (g) ^a				
Male	1.45 ± 0.10	1.23 ± 0.10*	1.27	
Female	1.39 ± 0.10	1.18 ± 0.14*	1.18	
External examinations of fetuses				
No. of fetuses (litters) examined	144 (11)	79 (9)	7 (1)	
No. of fetuses (litters) with anomalies	0	2 (2)	0	
Omphalocele	0	1	0	
Exencephaly and open eyelids	0	1	0	
Placental weight (mg) ^a	102 ± 10	99 ± 12	114	

^a Values are given as mean ± SD.^b Values obtained from females successfully mated.^c Value obtained from 11 females, because corpora lutea were indistinguishable in one female.^d Values obtained from pregnant females surviving until scheduled sacrifice.^e [(No. of corpora lutea—no. of implantations)/no. of corpora lutea] × 100.^f (No. of resorptions and dead fetuses/no. of implantations) × 100.* Significantly different from the control, $P < 0.05$.

pregnancy following maternal exposure during early pregnancy in mice. The most striking finding in the present study is pregnancy failure, decrease in the pregnancy rate, and litters totally resorbed, in females given DBTCl during early pregnancy.

Death and/or moribund condition were observed after the administration of DBTCl at 7.6 mg/kg bw/day and higher on days 0–3 of pregnancy and at 15.2 mg/kg bw/day and higher on days 4–7 of pregnancy, and significant increased incidence of females showing clinical signs of toxicity were found after the administration of DBTCl at 15.2 mg/kg bw/day and higher on days 0–3 of pregnancy and at 30.4 mg/kg bw/day on days 4–7 of pregnancy. These findings indicate that more severe general toxicity was induced by DBTCl on days 0–3 of pregnancy than that on days 4–7 of pregnancy. However, adverse effects on body weight gain were detected after the administration of DBTCl at 30.4 mg/kg bw/day on days 0–3 of pregnancy and at 7.6 mg/kg bw/day and higher on days 4–7 of pregnancy. Although the recovery of body weight gain was observed after the administration of DBTCl on days 0–3 of

pregnancy, recovery by the end of the study was not found in females given DBTCl at 7.6 mg/kg bw/day and higher after the administration on days 4–7 of pregnancy. Following the administration on days 4–7 of pregnancy, a significantly lower adjusted weight gain was also noted in females given DBTCl at 15.2 mg/kg/day and higher. These findings indicate that more severe adverse effects on body weight gain were induced by DBTCl on days 4–7 of pregnancy than that on days 0–3 of pregnancy. More severe effects of DBTCl on body weight gain following the administration on days 4–7 may be attributable to the significant decrease in the number of live fetuses.

The earlier administration period, days 0–3 of pregnancy, corresponds to the period before implantation, and the later administration period, days 4–7 of pregnancy, corresponds to the period when implantation is in progress and the period shortly after implantation in mice (Rugh, 1968). We expected that DBTCl insult on days 0–3 of pregnancy might result in preimplantation loss of embryos; i.e., the absence or decrease of implantation sites, and DBTCl insult on days 4–7 of pregnancy might result in postimplantation loss of embryos; i.e.,