PROCEEDINGS OF THE 5TH ANNUAL MEETING OF JAPANESE SOCIETY FOR ALTERNATIVES TO ANIMAL EXPERIMENTS

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SPECIAL LECTURE

THE INTERNATIONAL MULTICENTER EVALUATION OF *IN VITRO* CYTOTOXICITY TESTS (MEIC) BASIC ASSUMPTIONS AND STRATEGIES

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Since 1989, the Scandinavian Society of Cell Toxicology is organizing an evaluation of *in vitro* toxicity. International laboratories are (still) invited to test 50 reference chemicals in their *in vitro* tests of toxicity or toxicokinetics. The results are evaluated for their relevance to various forms of human, non-genetic toxicity, such as acute and chronic systemic toxicity, eye and skin irritancy, etc. Later, technical reliability of methods will also be evaluated. The aim is to recommend batteries of methods as supplements or alternatives to animal tests. At the present, 80 laboratories from all parts of the world are taking part with around 200 methods. —We assume the all types of toxicity ultimately may be modelled by various combinations of elemental *in vitro* toxicity tests (basal and organospecific cytotoxicities, extracellular toxicities, etc.) and toxicokinetic tests (absorption, metabolism, distribution, excretion, etc.). We also assume that a basal cytotoxicity test will be the backbone of all batteries. —Typical traits of MEIC evaluation are: 1. Use of human toxicity data as the reference. 2. Quantitative and linear comparisons. 3. Parallel comparisons between animal and human toxicity for the same chemicals, to judge the effectiveness of *in vitro* tests. 4. Hard (biased) as well as soft (multivariate PLS) modelling. —Preliminary results with acute systemic toxicity have been very positive.

SIMPOSIUM I

NEW EXPERIMENTAL METHODS OF GENOTOXIC TESTS

INTRODUCTION

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Many different test methods have been developed to detect genotoxic potential of environmental chemicals, but only a few test system has commonly been used. Recently, biotechnological techniques have promoted to develop new test systems, of which representative interesting methods are introduced in this symposium.

Firstly, a Chinese hamster cell clone transfected with monky P-450IA1cDNA which is developed for the metabolic activation in cultured mammalian cells, is reported (Sawada et al.). Secondly, a *ras*-transfected BALB 3T3 clone is introduced as the two-stage transformation assay to detect promoters (Sasaki). To detect gene mutations in animals, a transgenic mouse which is integrared the *LacZ* gene of *E. coli*, has been developed and is thirdly reported (Myhr). Finally, the interspecific *in vitro* fertilization method between human spermatozoa and zona-free hamster oocytes in introduced as the monitoring method to detect human genetic damages (Mikamo). These new test systems are expected to contribute the great advance in the field of genetic toxicology.

S I-1 TRANSFECTION OF CYTOCHROME P-450 cDNA INTO CULTURED MAMMALIAN CELLS AND ESTABLISHMENT OF MUTAGEN SENSITIVE CELL LINES

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Although cytochrome P-450s are key enzymes for metabolic activation or detoxication of many kinds of mutagens and/or carcinogens, most of established cell lines used in cytotoxicity and mutagenicity tests are lacking in those activities. In order to make up for the disadvantage, postmitochondrial fractions prepared from rat liver (S9 mix) are generally added into the culture medium. However, there are some problems in the S9 mix system. In the present study, we tried to establish the cell lines which can stably express the P-450 activity by the method of cDNA transfection. Firstly, the expression plasmid for monkey P-450IA1 cDNA (MKah1) was constructed using SR_{α}-promoter. It was transfected into Chinese hanster fibroblast cell line (CHL) together with neo^{r}-carrying plasmid by the calcium phosphate precipitation method. After the selection with G-418, the expression

of MKah1 mRNA was confirmed by Northern blot analysis. These transformants showed much higher sensitivity to the cytotoxicity of aflatoxin B_1 (AFB_1) than the parental CHL cells. The hyper-sensitivity of MKah1-expressing cells was almost completely inhibited by α-naphthoflavone, a specific inhibitor of P-450IA, supportng that the hypersensitivity was caused by the introduction of MKah1. Furthermore, MKah1-expressing cells showed positive result in the mutagenicity assay of AFB_1 using HGPRT locus as a marker gene, while the parental CHL cells did not. These results showed that the products from the introduced P-450 cDNA have sufficient catalytic-activity whithin cells and that the method used here provides a useful model for mutagen-sensitive cell lines from a viewpoint of drug metabolism.

S I-2 DEVELOPMENT OF SCREENING ASSAY OF TUMOR PROMOTERS USING ONCOGENE-TRANSFECTED CELLS

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v-ha-*ras* oncogene transfected BALB 3T3 clone (Bhas 42) was established by cotransfectin with pSV2-*neo* drug resistant DNA. Bhas 42 cells were sensitive to contact inhibition, but were complete transformed after one week by treatment with 12-*O*-tetradecanoylphorbol-13-acetate (1000ng/ml)[1]. These observations suggested that Bhas 42 cells are a possible model for initiaed cells in the two-stage transformation process. Therefore, we plated 10^4 BALB 3T3 together with 100 Bhas 42 cells followed by treatment with known promoters and compared 3-methylcholanthrene (MCA) induced two stage transformation[2,3].

The Bhas 42 system showed several advantages over conventional MCA-induced transformation assay with regard to 1) objective judgment of transformed *foci;* 2) fewer effects of batches of serum in transformation frequency; and 3) high reproducibility of transformation frequency of each experiment[2,3]. These results suggest that transformation studies using oncogene transfected clones are a useful tool to screen promoters and to research interaction between oncogenes and tumor promoters.

S I-3 *IN VIVO* MUTATION ASSAY SYSTEM USING *LAC* Z TRANSGENIC MICE

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A *lac* Z transgenic mouse called MutaMouse offers, for the first time, the ability to measure mutations in any somatic or germinal tissue of choice caused by treatments with any number of agents, such as chemicals or radiation. This is possible because the inserted

genes can be recovered easily from the mouse genomic DNA and analyzed by a simple *in vitro* technique. Although animals are used in this procedure, the number is small for somatic tissue studies and potentially far less than the thousands of animals needed for specific-locus assays of heritable mutations.

MutaMouse was constructed by Gossen *et al* (1) and described as strain 40.6. Briefly, a bacterial *lac* Z gene was inserted into the single *Eco* R1 site of the bacteriophage lambda *gt* 10 DNA vector. This vector was the microinjected into fertilized hybried eggs (BALB/C × DBA/2) which were then implanted into pseudopregnant mice. Several founder animals were obtained and bred to homozygosity. MutaMouse was one resultant strain which contained 40 copies of the λgt10lacZ construct, inserted at a single site on both parental copies of an autosomal chromosome. There is no promotor for expression *in vivo*, and the insertion has not disrupted any known functions affecting the health of the animals. The 80 copies of *lac* Z gene per cell therefore provides a simple monitor of mutagenic events occurring at each cellular site.

The *lac* Z genes are recovered from the animal by first extracting the DNA from each tissue chosen for analysis. Then, in a one-step, *in vitro* process, the λgt10lacZ sequences are cut from the genomic DNA and packaged into empty lambda phage preheads. The resultant, infectious phage particles contain one nonmutant or one mutant *lac* Z gene. A typical reaction uses 7.5 μg of genomic DNA and produces some 150,000 to 1,000,000 phage, depending on the DNA preparation. Each lambda particle (or *lac* Z gene) is detected as a plaque on a lawn of *E. coli* C bacteria grown on agar. By including *X-gal* as a chromogenic substrate in the agar cultures, the phage with nonmutant *lac* Z genes produce dark blue plaques, whereas phage with a mutant *lac* Z gene will yield colorless plaques. The *lac* Z mutant frequency (MF) is the ratio of colorless to colored plaques.

A variety of known chemical mutagens have been surveyed for mutagenic activity in different tissues (2). The results show dose relatedness, the effect of elapsed time after treatment, the effect of dose fractionation, and tissue specificity in response. Among different tissues, the spontaneous MF was in the range of 8 to 28×10^{-6}. Among the tested chemicals, ethylnitrosourea, given as 5 intraperitoneal injections of 50 mg/kg each, induced a bone marrow MF greater than 100×10^{-6} but a much smaller testicular MF (42×10^{-6}). Diethylnitrosamine at 4×100 mg/kg induced a large response in liver (152×10^{-6}) but no detectable mutagenesis in bone marrow. Skin mutagenesis was demonstrated with N-methyl-N'-nitro-N-nitrosoguanidine at 300 μg/animal and with dimethylbenzanthracene at 10 μg/animal for single topical applications. These and other results showed the potential for quantitative studies of *in vivo* mutagenesis in any tissue of choice.

The use of animals for mutation studies will help provide a rationale for further chemical testing with cultured mammalian cells. Cell cultures may be established from MutaMouse tissues to investigate directly the relationship between *in vivo* and *in vitro* mutagenesis. For *in vivo* experiments, only 2 or 3 animals are necessary per dose condition because of small variability between animals and the high recovery efficiency of *lac* Z genes. The largest potential savings in animals will occur for studies of heritable mutations. Based on the presence of 40 *lac* Z genes per gamete rather than the 7 loci in T-strain animals used for specific-locus assays, the number of F1 progeny can be reduced by 80% for the same degree of resolution. Further, if an analysis of sperm from treated animals can suffice for F1 analysis, only a few animals will be required. Thus, mutatin studies with MutaMouse should contribute significantly to a reduction in animal usage for genetic risk studies.

S I-4 INTERSPECIFIC *IN VITRO* FERTILIATION SYSTEM TO MEASURE HUMAN SPERM CHROMOSOMAL DAMAGES

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Chromosomes of the spermatozoon are not visible untill they are replicated and condensed in the ootid as male pronuclear chromosomes. Therefore, human oocytes have been indispensable for chromosome study of human spermatozoa. Naturally, difficulty in obtaining human oocytes has been a severe restraint of the human sperm chromosome study. Fortunately, however, the interspecific *in vitro* fertilization became possible between human spermatozoa and zona-free hamster oocytes and it was confirmed that the haploid set of human chromosomes can be displayed in hamster ova.

In the present lecture, I shall explain briefly the procedure of our improved and efficient method, then report some results of our studies on spontaneous and radiation-induced human sperm chromosome aberrations. Ninety-eight sermen samples from 30 healthy Japanese men ranging from 21 to 44 years of age were used in the study of spontaneous chromosome aberrations, and 11,068 spermatozoa were karyotyped. The incidence of spermatozoa with numerical chromosome aberratins was 1.3%, while spermatozoa with structural aberrations occurred very frequently, 13.4%. Breakage was the most predominant type of structural aberration, being followed by fragment, exchange, gap and deletion.

Twenty-eight samples from 5 men were used in the study of X-rays, and 4,960 spermatozoa were analyzed. Twenty-three samples from 12 men were used in the study of γ-rays, and 6,581 spermatozoa were analyzed. Nine samples from 5 men were used in the study of β-rays, and 3,132 spermatozoa were analyzed. These radiations all induced chromosome aberrations linearly with increase of dosage and the majority of the induced aberrations were of breakage type.

In order to simplify the human chromosome study, both micronucleus test and use of cryopreserved hamster oocytes have been successfully incorporated into the technical procedures.

SYMPOSIUM II

USE OF PRIMARY CULTURES OF HEPATOCYTES IN TOXICOLOGICAL STUDIES

INTRODUCTION

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Liver is the main metabolic organ of the body and the main site of detoxication. However, it is hard to draw clear conclusions from *in vivo* studies, becaus so many indirect effects are involved. Yet use of perfused liver and established liver cell lines are not proper, because of limited time and secificity. Thus the most suitable *in vitro* experimental system for study of liver functions and toxic effects is primary cultures of hepatocytes, which maintain most *in vivo* liver functions and are regulated by various hormones. However, some differentiated functions of liver, including P-450 activity are unstable in conventional cultures. Therefore, improved methods have been developed to prolong the expressions of unstable functions, including addition of extracellular matrix, coculture with other cells and aggregate cultures. these improvements are due to changes of cell shape and contact, which mimics tissue architecture *in vivo*. Growth of these cells are markedly suppressed. Application of these aggregated form of primary cultured hepatocytes would open a new field in future toxicology.

S II-1 MAINTENANCE OF DRUG METABOLISM CAPACITY BY CULTURED HEPATOCYTES

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Isolated hepatocytes are now routinely obtained from the liver of various species including man by the two-step collagenase perfusion method (1). High yields of viable hepatocytes are isolated by perfusing whole organs, isolated lobes or wedge biopsies.

Since marked species differences exist in various functions and their response to endogenous and exogenous factors between animal species and man it is often difficult to extrapolate results obtained with experimental animals to man. These differences include both the rates and the routes of drug metabolism and susceptibility to toxins.

Therefore, isolated human hepatocytes represent the best *in vitro* approach to study human liver functions and diseases.

However, major problems are encountered when using human hepatocytes, i.e. erratic availability and the lack of reproducibility from batch to batch. Recent studies have shown that viable hepatocytes can still be obtained from organs preserved for 2 days in cold University of Wisconsin solution, the solution used to preserve organs before transplantation (2). Viable hepatocytes can also be hypothermically stored in the Leibovitz medium for a few days (3) or cryopreserved for weeks or months (4).

To survive longer than a few hours at 37°C parenchymal cells must attach to a support. However, rodent hepatocytes are unstable in culture and rapidly lose most differentiated functions. After 24 h of culture the rates of transcription of liver-specific genes drop between 1 to 10% of those found in the liver. Total cytochrome P-450 level decreases 50% or more during the first 24–48 h and cytochrome P-450 enzymes are differently affected.

By contrast human hepatocytes are more stable than their rodent counterparts. They still contain more than 50% of the initial cytochrome P-450 content after one week of culture and all the cytochromes P-450 hitherto analysed, namely P-450 IA, IIC, IIE and IIIA, and expressed and inducible by classical inducers such as 3-methylcholanthrene, phenobarbital and rifampicin.

In the early years it was assumed that phenotypic changes resulting in preferential loss of the most differentiated functions represent adaptative responses of hepatocytes to an inappropriate environment. A number of studies have dealt with identification of factors that affect cellular functions *in vitro*. It is known that three groups of factors can be involved: soluble factors, extracellular matrix components and cell-cell interactions (5). The detoxifying capacity of liver cells, i.e. the levels of phase I and phase II drug metabolizing enzymes, can be markedly modified by using more sophisticated culture conditions.

A number of soluble factors including physiologic and nonphysiologic ones enhance specific functions in hepatocyte cultures. Several hormonally defined serum-free media have been proposed, that favor maintenance of drug metabolizing enzymes and their response to inducers. Slight modifications of the composition of the nutrient medium can greatly alter glutathione S-transferase activity in cultured rat hepatocytes. The efficiency of attachment of parenchymal cells and longevity of cultures are increased when the cells are cultured on matrix protein substates. however, the substrates that promote cell spreading enhance alterations of liver gene expression. The most interesting results have been obtained by using a reconstituted basement membrane gel formed from components extracted from the EHS mouse tumor. This gel, termed matrigel, prevents hepatocyte spreading and various functions are well-preserved for a few days including phenobarbital induction of cytochromes P-450 IIB1 and IIB2. Cell-cell interactions are also critical. When cocultured with untransformed rat liver epithelial cells, hepatocytes from various species including man survive for several weeks and retain high functional activities including high cytochrome P-450 level, active during metabolism by phase I and phase II reactions and response to inducers.

Cultured hepatocytes have been widely used for drug metabolism studies and proved to be a valuable model to predict species differences. For a number of drugs a good *in vivo/in vitro* qualitative correlation has been observed whether phase I and phase II reactions are involved and whatever the species studied. By contrats striking quantitative differences are sometimes obtained. They may be only apparent depending on the expression of the results.

Since hepatocytes retain their detoxifying competence for several hours and even for several days in appropriate culture conditions

they have a great value as screening tests in theselection of compounds early during their development and in identifying chemicals with particular properties. Human hepatocytes in primary culture represent a unique model system for predicting metabolic pathways and cytotoxicity of new drugs in man and for the development of antifibrotic, antiviral and schizontocidal drugs.

S II-2 EVALUATION BY THE CHANGES OF DRUG METABOLIZING ACTIVITIES

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To establish *in vitro* screening methods for detecting the chemicals like TCDD, we studied the induction of drug metabolizing activities of primary cultured rat hepatocyte and compared with those of rat liver. Drug metabolizing activities in the hepatocytes, cultured on collagen coated culture dishes in Williams' E medium supplemented with insulin, dexamethazone, and antibiotics, were not induced by methylcholanthrene and the addition of trace amount of selenite (≧20 nM) was necessary for the induction. In the presence of 10% FBS in culture medium, the acitivities of ethoxycoumarine deethylation and aryl hydrocarbon hydroxylation and the contend of P-450c and P-450d in hepatocytes were increased by several methylcholanthrene type of inducers like *in vivo*. The dose which increased the ethoxycoumarine deethylation activity to two times of control correlated well between *in vitro* and *in vivo* experiments. On the other hand, the addition of nicotinamide (2MM) was necessary to observe the phenobarbital type of induction in this culture condition.

S II-3 LIVER MICROSOMAL ESTERASE AS A NOVEL MARKER OF HEPATOTOXICITY IN THE CULTURED HEPATOCYTES

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As an alternative toxicity testing of hepatotoxicity, we have used changes in the liver microsomal carboxylesterase activity when added test compounds to the cultured hepatocytes. Microsomal esterase activity is more susceptible and more stable, compared to cytochrome P-450. In addition, induction of esterase by phenobarbital is suppressed by addition of hepatotoxicants to the cultured hepatocytes. Release of β-glucuronidase from egasyn-β-glucuronidase (EG) complex in the liver microsomes into the medium is a good marker of the hepatotoxicity of the organophosphates and carbamate insecticides. The heterospheroid which we have tried to use a new type of hepatotoxicity testing is also discussed.

S II-4 STUDY OF FATTY LIVER INDUCTION BY XENOBIOTICS USING PRIMARY CULTURES OF ADULT RAT HEPATOCYTES

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Now a days, induction of fatty liver by xenobiotics was studied mainly using *in vivo* methods. Although *in vivo* studies are fit for obseration of whole body responses and pathological analysis, they have several problems in costs, times and quantitativeness. For developing a new method to assess fatty liver inducibility *in vitro*, we examined changes of cellular lipids by exposing primary cultures of adult rat hepatocytes to several drugs.

Primary cultures of mature hepatocytes retain many liver functions and various hormonal responses for a long period. A lipid loading caused 2 to 3-fold increases in hepatic triglyceride (TG) and non-esterified fatty acids (NEFA) in primary culture. Inhibitor of lipid metabolism, such as ethanol, puromycin, and cycloheximide, also increased cellular lipids. In case of the loading, lipids accumulation was prominent when the hepatocytes density was low in culture dish. In contrast, cultures in high cell density were favorable for retention of other liver-specific functions. This clearly suggests that the utilization of exogenous lipids by hepatocytes was invert proportion of cell density. Possible mechanism(s) may be affected by a cell density dependent manner in the apoprotein synthesis.

these results suggest that primary cultures of hepatocytes are useful for analysis of mechanisms, as well as toxicological assessment, of fatty liver *in vitro*.

S II-5 HORMONAL INFLUENCE ON THE *IN VITRO* EXPRESSION OF PHENOBARBITAL-INDUCIBLE CYTOCHROME P-450

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Cytochrome P-450, which catalyzes the oxidation of diverse chemicals, is mainly localized in liver of mammals. Although cytochrome P-450 belonging to the family of I to IV mainly participates in the metabolism of xenobiotics. The amounts of these enzymes in hepatocytes have been shown to decrease within a day after the isolation for the culture. Several attempts have been reported to maintain the drug metabolizing capability of livers, which include the changes in substrate and media and other biological components. Recent understanding on the mechanism of the regulation of cytochrome P-450 indicates the role of endocrine factor, especially pituitary hormone, on the expression of the hepatospecific forms. A family of this hemoprotein, P450IIB, is known to be induced by the treatment of rats cith phenobarbital (PB) or otehr drugs. The constitutive and PB-induced

levels of P450IIB1 and P450IIB2 have been shown by us to be regulated suppressively by pituitary growth hormone. These forms have also be found to be under the regulation of thyroid hormone in rats *in vivo*. Therefore, we examine the maintenance of hepatocytes and induction of P450IIB1 and P450IIB2 by PB-treatment, and examined the effects of growth hormone and thyroid hormone on these P450s. As the results, hepatic levels of P450IIB1 and P450IiB2 in hepatocyte culture were induced to more than 100 and 30 pmol/mg protein, respecgively 3 days after addition of PB. These induced levels were suppressed by the addition of growth hormone or triiodothyronine to the medium.

SIMPOSIUM III

DEVELOPMENTAL TOXICITY

S III-1 *IN VITRO* TESTINGS FOR DEVELOPMENTAL TOXICITY: APPLICATIONS TO RISK ASSESSMENT

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It is not an easy task to identify the developmental toxicity of environmental agents and to extrapolate experimental data to the human, since the end points of developmental toxicity and the process of teratogenesis are not simple. Recently sophisticated *in vitro* techniques have been developed that aim at simple, quick evaluation of developmental hazards or analytical studies of the mechanisms of teratogenesis.

In this symposium, some *in vitro* techniques will be introduced and their potential as an alternative to animal experimentation will be discussed. In addition, their advantages in developmental toxicology will be presented.

S III-2 AN ASSAY SYSTEM FOR DEVELOPMENTAL TOXICITY USING EMBRYOS AND LARVAE OF *XENOPUS LAEVIS*

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Xenopus laevis embryos and larvae were continuously exposed to Pb (lead acetate, lead nitrate), Cd (cadmium chloride, cadmium sulfate), Li (lithium chloride, lithium carbonate), Hg (mercuric chloride), Se (sodium selenite), caffeine, trypan blue, hydroxyurea, ethidium bromide, ethanol, amaranth, saccharin sodium, vitamin C and acetone. All of the chemicals induced some developmental toxicity on *Xenopus* embryos and larvae dose-dependently. Lowest concentrations to induce abnormalities of the metals were low (0.1–10 mg/l), those of caffeine, trypan blue, hydroxyurea and ethidium bromide, which are teratogenic in mammals or DNA synthesis inhibitors, were 100–500 mg/l. On the other hands, those of ethanol (weak teratogen), amaranth, saccharin sodium (non-teratogen) and acetone (teratogenicity is not determined) were very high (>10g/l). In the case of vitamine C (non-teratogen), the concentration to induce abnormalities was 250 mg/l, suggesting that further investigation is needed. Main abnormalities observed were abnormal neurulation and shortened body length with wavy fin. Evaluation of the results and predictability of this system as a prescreening system for developmental toxicity will be discussed.

S III-3 METHODS FOR EVALUATING TERATOGENIC ACTIVITIES OF CHEMICALS USING MICROMASS CULTURE SYSTEM

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In vitro micromass teratogen test using mouse and rat embryonic midbrain (MB) and limb bud (LB) cells has been investigating for several years in our laboratory. The cells in each micromass island differentiated to form small foci of neurons in MB cultures and chondrocyte in LB cultures. The number of individual foci was counted by use of a dissecting microscope.

The micromass teratogen test is simple, allowing numeroud compounds to be rapidly tested at a very low cost. Further, it is useful for the mechanistic studies and the monitoring of teratogenic activities in biologial fluid.

We validated the micromass assay using several tens of compounds. All data were assessed for sensitivity, specificity, and accuracy using several sets of criteria based on the IC50 values obtained. These results were also compared with those of other laboratories. The merits and demerits of micromass teratogen test will be discussed.

S III-4 SEARCH FOR A POSSIBLE CORRELATION BETWEEN TERATOGENICITY IN FETUSES AND TOXICITY IN PREIMPLANTATION EMBRYOS INDUCED AFTER TREATMENT OF MOUSE FEMALES WITH CHEMICALS AT THE PREIMPLANTATION STAGES

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Administration of mitomycin C (MMC) or methylnitrosourea (MNU) to female mice on days 0–3 of pregnancy caused significant increases of gross fetal abnormalities over control levels as assayed on day 18 of pregnancy. Both agents were effective in causing morphological abnormalities and chromosome aberrations at the preimplantation stages, as well. However, preimplantation losses were significantly induced only after MMC treatment. When embryos at the blastocyst stage from MMC-treated dams were transferred to untreated pseudopregnant females, resulting fetuses showed no evidence of induced abnormality. When embryos from untreated dams were transferred to MMC-treated, pseudopregnant females, abnormal fetuses were seen at a considerably higher than control. In marked contrast, no evidence of induced abnormality was seen at the fetal stage when embryos from untreated dams were allowed to develop in MNU-treated, pseudopregnant females. Abnormal fetuses were registered at a significant frequency when embryos from MNU-treated dams were transferred to untreated, pseudopregnant females. these results demonstrate that MMC treatment at the preimplantation stages induces abnormality in fetuses via damaging maternal environment, whereas MNU treat-

ment induces it via directly demaging preimplantation embryos, and lead to the hypothesis that preimplantation loss serves as a abortive mechanism against embryos bearing preteratogenic damage. We thus conclude that any sign of toxicity in preimplanation embryos can not be used as a predictor of direct teratogenicity of a chemical applied at the preimplantation stages unless nature of preteratogenic damage in embryos and assumed abortive mechanism are substantiated.

S III-5 TISSUE RECONSTRUCTION AND WHOLE EMBRYO CULTURE SYSTEMS

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In normal embryogenesis, a fertilized egg repeats exactly the cell division including the cleavage based on the genetic program which it is given, and finally forms a multicellular organism. There is unfolded an extremely complex and dynamic change which includes cell or tissue differentiation into a different form or fuction, and morphogenetic movement under the cell recognition system. Therefore, those in vitro test systems should be include the fundamentally important phenomenon as a marker of normal embryogenesis. In the present study, tissue reconstruction and whole embryo culture systems were employed in vitro alternatives to the use of whole animals as a primary screening test method for teratogens.

Animals used in this study were Slc: SD rats. In the tissue reconstruction method, fetal brain on day 14.5 of gestation were dissociated into single cell suspension with collagenase treatment and cultured for 48 hours in DMEM medium containg the test materials in various concentrations. On the other hand, embryos for the whole embryo culture method were collected on day 9 of gestation andcultured in 100% rat serum containing different concentrations of the test materials. After 48 hours, the embryos were examined with the morphological scoring system. The results from both in vitro culture systems were compared and discussed.

S III-6 ASSESSMENT OF DEVELOPMENTAL TOXICITY USING *IN VITRO* CULTURE OF FETAL MOUSE PALATES

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In vitro culture of fetal organs is useful for the detection of organogenetic malformations because whole embryo culture is unable to detect such anomalies as cleft palate and digit malformations. We developed a new organ culture technique of fetal mouse palates in a chemically defined serum-free medium and obtained successful fusion of fetal palates *in vitro* (Shiota et al., 1990).

We are now conducting studies to validate

this new organ culture method. We will present the results of experiments using some chemicals including those which induce cleft palate *in vivo*. The advantages and disadvantages of organ culture techniques in developmental toxicology will also be discussed.

SYMPOSIUM IV

ALTERNATIVES TO LOCAL IRRITANCY TESTINGS

INTRODUCTION

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An increasing number of *in vitro* tests are being proposed as alternatives for animals in safety testing. These *in vitro* systems use a variety of approachs and measure a broad range of endpoints, including cellular cytotoxicity and metabolism. Because each *in vitro* test in the battery will likely measure a specific endpoint related to part of the ocular injury process, currently, there are no *in vitro* assays that will replace the *in vivo* test procedure. In order to develop such batteries, more must be learned about the specific cellular and molecular mechanisms that lead to the damage observed in chemically induced injury. For example, the ocular surface, which may be relatively more simple than the other differential organs, is a complex system consisting of corneal and conjunctival epithelial cells, the underlaying stroma and associated cells. Exposure of the ocular surface to a foreign materials may result in a response ranging from mild, slight redness and itching to severe injury with loss of corneal epithelium, damage to the underlying stroma, severe inflammatory infiltration and loss of vision. This complexity makes the development of alternative tests capable of eliminating the need for animals very difficult. However, by using mechanistically-based, physiologically-relevant model systems, it may be possible to identify a battery of assays that will be useful in the ocular safety assessment process. In this view point, 4 new assay sytems including assay which assess membrane integrity (Ohno), assay which assess changing of intracellular ions (Miyakawa), assay which assess cell metabolisum by the extracellular pH (Wada), and quantitive structure-activity relationship study (Sugai) may ocupy a key place in any battery tests developed in the present and future.

S IV-1 ANALYSIS OF CYTOTOXICITY WITH AN ENZYMATIC MARKER

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As a strategy to develope an alternative method to animal experimentation, cultured cell lines were utilized to screen diverse toxicity of chemicals with a simplified marker, i.e., cell survival. However, methods developed to determine cell survival do not necessarily have sufficient sensitivity to detect cell death in lower dose range of chemicals. If one pick up a specific metabolic reaction depending on an enzyme activity, it may be relatively easy to construct a convenient biochemical screening system with higher

sensitivity to chemical toxicity than that of cell survival. We think it is desirable to establish an alternative method which can easily determine cell death with a high sensitivity comparable to that of an enzyme reaction.

We therefore searched for an enzymatic marker which can represent cell death. Lactate dehydorgenase (LDH) is a stable enzyme locating in cytosol. Its activity is easy to determine with commercially available assay kits. We reported in the IV JSAAE meeting that LDH is usful as a critical marker of cell death and survival. By determining LDH released from cells (LDH release assay), we here report that 1) LDH release assay will be an useful alternative to Draize rabbit eye irritation test for detergent-based cosmetics and chemical products, 2) ED50 does not necessarirly represent cytotoxicity of the tested chemicals in lower dose range, 3) for some chemicals, ED1 or ED10 is also required to understand their low dose cytotoxicity, 4) we developed screening systems for antitumor drugs and 5) for lymphokine-activated killer T cells.

S IV-2 MEASUREMTNT AND ANALYSIS OF INTRACELLULAR ION DISTRIBUTIONS IN SINGLE LIVING CELLS

Atsuo Miyakawa

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The distribution of intracellular pH, K^+, Na^+, Ca^{2+}, and Mg^{2+} concentration is measured using various type fluorescent indicator dyes. The measurements combining the fluorescent indicator dyes with a fluorescence microscopy are widely use in the research of life science. One application of this method is also estimate of cytotoxicity.

The intracellular pH and Ca^{2+} distribution was observed homogeneously distribution. But, the intracellular K^+, Na^+ and Mg^{2+} shows the levels in the cytoplasm and the nucleus was clearly different.

A problem remains to be solved to do accurate quantity of intracellular ions. The problem is due to the interaction between intracellular proteins and fluorescent probe dye. I try to resolution of the equilibrium reaction between Ca^{2+}, indicator dyes and intracellular proteins.

S IV-3 MEASUREMENT OF CELLULAR RESPONSES TO EFFECTOR AGENTS USING A SILICON MICROPHYSIOMETER

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The silicon microphysiometer monitors cell metabolism *in vitro* and has been used to detect cellular responses to various agents, including irritants and toxic substances (1). This nstrument uses a light addressable potentiometric sensor (2) to measure millipH changes in micro-flow chambers (120 μm depth×7 mm diameter) maintained at 37C. Cells are immobilized in the flow chambers and perfused with medium. When medium flow through the chamber is stopped, the cells acidify the medium. The rate of medium acidification is quantitated by the instrument during the stopped flow period. Subsequently, the flow is turned on to purge waste products and re-establish the physiologic pH of the micro-environment. The acidification rate is an indirect measure of catabolic metabolism, which results in lactic acid and carbonic acid production. Continual cycling through on and off periods provides a steady stream of non-destructive, metabolic measurements. When a cell-affecting agent is introduced into the fluid stream, using bolus administration or continuous infusion, a change in acidification rat eindicates the response of the cells to the agent. If cells are stimulated, rate increases, and if cells are damage or killed, rate decreases. Thus, rapid cellular responses to chemical agents can be detected within minutes. The potency of these agents can be quantitated by the speed and magnitude of acidification rate change. Recovery time after insult may also be a measure of irritancy/toxicity.

A panel of 17 test substances was used to evaluate the usefulness of acidification rate changes as a measure of in vivo ocular irritancy potential (3, 4). Human keratinocytes were grown on coverslips and loaded into the microphysiometer. When the acidification rates wre stable, test material was then pumped into the chambers using the lowest concentration first. The cells were exposed to test substance for 320s. the test material was then washed out, followed by a 200 s period when acidification rate is measured. The concentration at which rates were reduced by 50% was determined for the panel of test substances. When correlated with the rabbit Low Volume Eye Test (LVET), a modification of the Draize Ocular Irritation Test, a correlation coefficient $r=0.85$ ($p<0.001$) was observed. An interesting observation was that sub-inhibitory doses of irritating substance scaused increases in acidification rate although a larger dose caused a drop in rate. A similar result was observed when alterations in pH were used to as the irritant. Slight changes (+-2 units) caused an increase in rate, and extreme changes caused a drop.

Silicon microphysiometer acidification rates have been used as the indicator of cell viability (1, 5). This method has been used in several studies to demonstrate its use for chemotherapeutic efficacy, anti-viral drug, and neurotoxicity testing. A drug resistant line and a drug sensitive line of human uterine sarcoma cels were tested for susceptibility to vincristine, doxorubicin and cisplatin. Within 12 hrs, differential sensitivities to these drugs were

observed on the two cel lines. Viral cytotoxicity by VSV infection of L cells and inhibition ofviral replication by ribavirin could also be detected within 8 hus using acidification rate measurements. A panel of test compounds, including drugs used to treat HIV-1 viral infection, were tested for efficacy and toxicity using CD4 transfected HeLa cells which were infected or not infected with HIV-1 virus. Due to the slow replication cycle of this virus 6 to 7 days of treatment in the microphysiometer wre required to observe viral cytopathic effects (6), which were observed as a decrease in acidification rate, relative to controls. The inhibition of viral cytopathic effects was demonstrated using 0 to 5 μM concentrations of AZT (ED50%=0.23 μM). AZT toxicity caused an increase in rate at low levels and a decrease at higher levels in uninfected cells, relative to untreated controls (TD50%=67 μM). The rank order of *in vitro* anti-HIV efficacy was AZT >DDI >ChloramineT >mannitol.

Receptor activation by specific ligands, such as hormones, neurotransmitters, and growth factors, can be detected within minutes by acidification rate monitoring (7). This approach provides a means to responses due to exposure to substances could possibly be used as an indicator of cell type specific toxicity. Materials not necessarily producing general cytotoxic effects may modify receptor mediated intracellular signalling, and, consequently, disrupt vital physiological systems, i.e. immune response, cardiovascular control, CNS and motor functions, etc.. As an example of tissue specific toxicity, the neurotoxicity of the overstimulation of glutamate receptor inmouse hippocampal mixed cell cultures was detected by monitoring acidification rate for 16 hrs, after a 10 min. exposure to 2.5 mM kainate, a glutamate receptor agonist (8). These and other novel approaches to irritancy and toxicity testing, such as hepatocyte processing of substances prior to target cell exposure are possible using the microphysiometer method of metabolic monitoring.

S IV-4 A QUANTITATIVE STRUCTURE-ACTIVITY RELATIONSHIPS APPROACH TO PRIMARY EYE IRRITATION OF CHEMICALS IN RABBITS

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Quantitative structure-activity relationships (QSARs) are employed increasingly to screen and predict the toxicity and the mechanism of toxic effects of chemicals. QSARs contribute to the reduction of cost and animals for the safety assessment of chemicals and could be considered a kind of alternative.

The results of a QSAR for the analysis of te relation between the structural features of chemicals and primary eye irritancy evaluated by Draize test will be described. The Draize test has been used as an attempt to predict the human ocular irritancy of chemicals. This test, however, may be considered to be associated with unnecessary use of animals, irreproducibility and high cost. the inadequacies of this test have led to efforts developing alternatives.

Availability of QSAR in he prediction of eye irritation potential, investigation of the mechanism of eye irritation and development of alternatives will be discussed.

GENERAL PRESENTATION

WING SPOT TEST ON PROMUTAGENS BY USING HIGH METABOLIC SYSTEM

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Hikone R strain of Drosophila has RI gene, which increases P-450 dependent metabolic activation, on 2nd chromosome. Ryo et al. introduced this 2nd chromosome to wing spot test to made a high metabolic system and reporte ten-fold increase in induced wing spot frequencies by urethane. i compared this new system with usual one on the induction of wing spots by other three compounds, diethylnitrosamine (DEN), 2-acethylamino-fluorene (AF) and metronidazole (MTN). The wing spot frequencies did not change remarkably, althouth the mortalities by these comounds increased by four-fold. This system may have different property from the high metabolic system of Frölich adn Würgler (1989, 1990), by which the wing spots induced by DEN and polycyclic aromatic hydrocarbons increased remarkably compared to the usual system.

INTERSPECIFIC DIFFERNCE IN DRUG SENSITIVITY —CLASTOGENICITY OF NaF IN VARIOUS PRIMATE CELLS—

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Sodium fluoride (NaF) has been known to be clastogenic in human cells but not to rodent cells with a few exceptions. Using cells from several species of primates, which have been diverged into more than 200 species including rodent-like prosimians and human-like great

Table 1. List of primate cells investigated or to be investigated

Origin	Cells	Sensitivity	Origin	Cells	Sensitivity
Man	PB	+	New World monkeys		
	LCL	+	marmoset	PB	
	FC	+		LCL	-
Apes			squirrel monkey	PB	
pygmy chimpanzee	LCL	+	Prosimians		
common chimpanzee	PB	+	grand galago	PB	
	LCL	+		FC	-
orangutan	LCL	-	ring-tailed lemur	PB	
agile gibbon	PB			LCL	-
Old World monkeys			Rodents		
green monkey	PB		Chinese hamster	V79	-
Hamadryas baboon	PB			CHO	-
crab-eating monkey	PB			CHL	-
	LCL	-			
Japanese monkey	PB				

PB:peripheral lymphocytes; LCL:lymphoid cell line; FC:fibroblastic cells.

apes, interspecific difference in chromosomal sensitivity to NaF was investigated. Cell lines or peripheral lymphocytes from primates were treated with 0 to 6 mM NaF and their chromosomal sensitivity was evaluated.

Here we report that NaF demonstrate clastogenicity not only in human but primate cells (Table 1) and that the interspecific difference in NaF sensitivity might be derived from phylogenetic background. Furthermore, change in drug sensitivity by cell line establishment was also investigated.

DETECTION OF TUMOR PROMOTERS USING RAS-TRANSFECTED 3Y1 CELLS (HR-3Y1-2)

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As a simple and rapid test for promoters screening, we studied an ability of cell proliferation induction by test compounds in confluent state of HR-3Y1-2 cells.

Five milliliters of 5%FCS/DME containing 1×10^5 cells was plated in a 60-mm-diameter Petri dish and cultured for 7 days. Cells in confluent state were exposed to test compounds, and 3 days after cells were counted using a Microcell counter.

Cells increased significantly in the dishes treated with TPA, BHT, meserein and PMAAL, but did not increase with anthralin and okadaic acid which were known as tumor promoters. Proliferation of cells in the dishes treated with DNFB was not clearly observed, but cells increased significantly when the dishes were treated three times every 2 days. No increase of cells was observed in the dishes treated with phorbol, ethanol, acetone, DMSO and SDS which were not tumor promoters.

These findings suggest that the present method can use as a simple, rapid and highly sensitive test for tumor promoter screening.

USEFULNESS OF PREMATURE CHROMOSOME CONDENSATION (PCC) TECHNIQUE AS AN ALTER-NATIVES TO DETECTING MUTAGEN AND CARCINOGEN

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Conventional chromosome assay can not directly detect chromatin damage in interphase. On the other hand, premature chromosome condenstaion (PCC) technique is very sensitive method in detecting chromatin damage in interphase cells. Previous reports suggested that contribution of interphase cell death to express biological effects, such as mutation and malignant transformation, by heavy ions may be larger than low LET radiations. Therefore, in this study we detected chromosome aberrations as chromatin breaks in interphase cells using PCC and compared it with the data of those detected by

conventional methods. Results suggest that the technique of PCC is more sensitive in detecting chromosome damage induced by radiations and chemicals than the conventional chromosome analysis asn very useful in assesing genotoxic risk of by chemicals and radiations.

CONCOMINANT DETECTION OF GENE MUTATION AND MICRONUCLEUS INDUCTION *IN VIVO* IN *LAC Z* TRANSGENIC MICE

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We have developed a new assay system which can detect gene mutation and micronucleus induction simultaneously using *lacZ* transgenic mice (MutaMoust). Mitomycin C (MMC) and ethyl nitrosourea (ENU) were injected intraperitoneally and peripheral blood were colected from the tail for micronucleus assay. Then the mice were sacrificed and DNA was extracted from their organs for the mutation assay.

Both chemicals induced micronucleated reticulocytes which reveal the applicability of the MutaMouse to the micronucleus assay. ENU induced mutation in the *lacZ* gene strongly in bone marrow and moderately in liver. On the other hadn, MMC showed no increase in mutation frequency by single treatment and subchronic treatment slightly induced mutation only in bone marrow. These results showed that MMC has a weak potential in the induction of gene mutation although it has strong clastgenicity.

The concominant detection of mutagenicity and clastogenicity *in vivo* provides an important information for the evaluation of the genotoxicity of chemicals.

AN ALTERNATIVE TO THE SPECIFIC-LOCUS TEST IN MOUSE SPERMATOGONIA STAGE USING MUTA™MOUSE

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Muta™ Mouse test can detect chemical mutagenesis induced in somatic and germinal tissues (Myhr et al., 1991). When the whole testis is used in this test, it is difficult to find out in which stage the mutation have occured. The mutations which induced in spermatogonial stage, however, could be detected on testis of more than 4 weeks after treatment. The present study was undertaken to clarify whether Muta™ Mouse test can detect the gene mutations induced in mouse spermatogonia after treatment with N-ethyl-N-nitrosourea (ENU).

Muta™ Mouse were given single intraperitoneal injection of 150 mg/kg ENU. Testes were extracted at 3 and 61 days after injection

and Muta^{TM} Mouse test was carried ot by the methods of Myhr et al. (1991).

Hih mutation frequencies were detected at 3 and 61 days after injection with ENU. The mutation frequency in spermatogonia detected by Muta^{TM} Mouse test was lower than that detected by the mouse specific-locus test (Murota and Shibuya, 1983). Muta^{TM}Mouse test proved to be able to detect mutations induced in mouse spermatogonia and is an alternative to the specific-locus test.

MODIFICATION OF THE MILLIPORE FILTER DIFFUSION ASSAY AND EVALUATION OF THE ITS APPLICABILITY FOR SEVERAL CLASSES OF CHEMICALS

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We attempted to minimize the morphological alterations of L929 cells when cytochemical demonstration of succinate dehydrogenase activity was carried out on the millipore filter diffusion assay (MFDA). By Barka's or Pearse's staining method, the cells on the filter showed morphological changes like cytoplasmic shrinkage and/or spherical-shape formation. These alterations were reduced by an addition of 1 mM $MgCl_2$ into the reaction mixture but not by $CaCl_2$, and cells showed an almost normal morphological appearance.

Toxicity of 9 chemicals, which included a detergent, a corrosive agent and carcinogens, were examined by the MFDA with the modified cytochemical method. Only two chemicals, sodium dodecylsulfate and phenol, caused an unstained zone clearly. Carcinogens did not show any toxicity to the L929 cells, even under the microscopical observation. As previous reports, the results obtained concluded that the MFDA is useful to detect toxicity of detergents or corrosive agents, but less sensitive to the chemical carcinogens, even by the modified cytochemical technique.

EFFECTS OF DENTAL DRUGS ON CULTIVATED CELLS

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We examined effects of some dental drugs (guaiacol and sym-trioxane) on cell activity of BM cells, MC3T3-E1 cells and FBLM cells. Sym-trioxane was trimer of formaldehyde. BMcells were derived from human bone marrow; FBLM cells from human tooth ligamentium. MC-3T3-E1 cells were presented from Dr. Amagai, in the department of Oral Physiology of Oh-u University. After 18 hr preincubation with α-MEM, we added guaiacol to the cells, incubatedthen cells for 6hr, added ^{3}H-thymidine and incubated for 2 hr. Then, we measured the activity of proliferation of the cells after drug addition by liquid scintillation counter. In the case of trioxane, after 2 hr preincubation, we added trioxane to the cells and incubated for 22hr. Then, adding ^{3}H-thymidine and incubating

for 2 hr, we measured the activity of prolification of the cells. 1) In the case of BM cell: less than 10^{-10}M of guaiacol increased ^{3}H-tyymidine uptake, and more than 10^{-10}M decreased the uptake. The proliferation activity of cells was maximum at 10^{-12}M of guaiacol. 2) In the case of MC-3T3-E1: similar to BM cell, less than 10^{-8}M of guaiacol increased the uptake and the proliferation activity was maximum was at 10^{-10}M.3) In the case of fibroblast: less than 10^{-8}M of guaiacol increased the uptake of ^{3}H-thymidine. 4) In the case of trioxane: trioxane did not increase the uptake but decreased at 1M.

It was shown that guaiacol, one of the derivatives of phenol, had the potency of proliferation of cells. The experiments using cultivated cells have following ad-vantages: (1) It is easier and more useful for determination of effective doses for cell activation and cell toxication. (2) We can make many kinds of results in short while.

AN *IN VITRO* MODEL FOR ASSESSING MUSCLE IRRITATION OF PARENTERAL ANTIBIOTICS USING RAT CULTURED SKELETAL MUSCLE CELLS

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The muscle irritation is conventionally performed int herabbit by a injection of drugs into muscle, and evaluated by gross and histopathological examinations. The objective of our present study is to examine the notential of an *in vitro* model for assessment of muscle irritation.

The muscle irritation was evaluated *in vitro* using rat cultured skeletal muscle cells. The myoblasts derived from rat newborns were incubated at 37°C in an atmosphere of 5% CO_2 and 95% air. Electron microscopic observations revealed the myofibrils in muscle fibers on day 11 of culture and more than 70% of creatine kinase (CK) isoenzyme was MM type.

On day 11 of culture, the muscle fibers, which were developed from myoblasts, were exposed to cephaloridine (CER), cefazolin sodium (CEZ), flomoxef sodium (FMOX), cefamandol sodium (CMD), latamox sodium (LMOX) and cephalothin sodium (CET) for 1 hour (31.25–250 mg/ml). The CK activity in muscle fibers was measured and the depletion of CK relative to control cultures was utilized as the index of muscle irritation. The DC_{50} (depletion concentration 50%) values were estimated to be 406.7, 311.1, 211.6, 132.7, 114.2 and 56.6 mg/ml, for CER, CEZ, FMOX, CMD, LMOX and CET, respectively by the logit model. There was a good correlation between these DC_{50} values in our *in vitro* study and the irritation volumes on day 2 whch have been reported *in vivo* studies by other authors.

From these results, it was suggested that this *in vitro* model should be a useful method for assessment of muscle irritation.

STUDY OF THE ALTERNATIVE METHOD FOR ORAL MUCOSAL IRRITATION TEST

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We evaluated the effects of somo surfactants on the intra- and extra- cellular matrix of normal human keratinocytes from a human embryo by immunocytochemical staining.

Sodium lauryl sulfate (SLS) and sodium lauroyl sarcosinate (LS) caused more severe damage to the intra- and extra-cellular matrix than benzalkonium chloride (BC). On the other hand, SLS and LS indicated the inhibition of neutral red dye uptake less severely than BC.

In the hamster oral mucosal irritation test, Yoshikawa reported that BC induced severe inflammation with slight desquamation of the keratin layer, however, SLS and LS induced severe desquamation with less severe inflammation than BC in this test. This study suggests that the desquamation of the keratin layer induced in the hamster occur due to the damage of the intra- and extra-cellular matrix. It seems that the evaluation of the intra- and extracellular matrix is necessary in developing the *in vitro* alternative method for oral mucosal irritation test.

IN VITRO CYTOTOXICITY/IRRITANCY TESTING USING THE MARROW-TECH THREE-DIMENSIONAL SKIN^{2TM} HUMAN DERMAL MODEL AND SIX DIFFERENT ASSAY SYSTEMS

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Inger Kidd, Jan Rust and G.K. Naughton Marrow-Tech, Inc., La Jolla, Calif., U.S.A.

Summary

A three-dimensional human skin model has been developed in our laboratory and utilized as a substrate in a number of cytotoxicity and irritancy tests. The Skin^{2TM} Dermal Model consists of metabolically and mitotically active, neonatal foreskin-derived fibroblasts and naturally secreted collagen and extracellular matrix proteins (1). This model has beenused effectively to study the toxic effects of a variety of compounds and formulations. Utilizing six *in vitro* cytotoxicity/irritancy assays—Neutral Red vital dye uptake [lysosomal function] (2, 3, 4), MTT reduction [mitochondrial function] (3, 4, 5), lactate dehydrogenase release [membrane integrity] (3), Prostaglandin E_2 (3) and IL-6 release [inflammatory mediators] and glucose utilization [metabolic activity]—we have assayed and obtained dose-dependent toxicity curves for surfactants, shampoos, industrial solvents, alcohols, metals, cosmetic products, pesticides, antimicrobial preservatives and petrochemicals. Correlation of the *in vitro* data to existing eye and skin irritationdata is very good, demonstrating the utility of these hu-

man skin substrates used in conjunctin with six different assays as potential *in vitro* alternatives to animal testing for scrrening the toxicity of test agents from divers classes.

References

1) Slivka, S., Stevens-Burns, D., naughton, G. and Bartel, R. 1991. A novel method for colorimetric assessment of collagen matrix deposition in a three-dimensional dermal culture system. *In Vitro Cell. Dev. Biol.* 27(3): 159A.

2) Borenfreund, E. and Puemer, J. 1984. A simple quantitative procedure using monolayer cultures for cytotoxicity assays (HTD/NR-90). J. Tiss. Cult. Meth. 9(1): 7.

3) Triglia, D., Sherard Braa, S., Donnelly, T., Kidd, I. and Naughton, G.K. 1991. A three dimensional human dermal model substrate for in vitro toxicological studies. In Alternative methods In Toxicology, volume 8. Ed Alan Goldberg. New York: Mary Ann Liebert, Inc. (in press).

4) Triglia, D., Sherard Braa, S., yonan, C. and Naughton, G.K. 1991. Cytotoxicity testing using neutral red and MTT assays on a three-dimensional human skin substrate. Toxic. In Vitro (in press).

5) Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: applications to proliferation and cytotoxicity assays. J. Immunol. Meth. 65: 55.

CONFOCAL FLUORESCENT OBSERVATION OF LIVING NEURAL CELLS IN THE PRIMARY CULTURE AND THE CHRONIC ETHANOL EFFECT IN THESE CELLS

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Tissue cultures of dorsal root ganglia from the 18th day mouse fetus were stained with rhodamine123 to observe confocally by laser scan microscope (Carl Zeiss). Though young neural cells in the cultures show uniform fluorescent cytoplasm, nonuniformity in fluorescence develops with some cells three or more weeks after explantation. It is our intention to study the relationships among the phenomena, cell age and chronic exposure to ethanol.

C-13 AUTOMAGNETOGRAPHY (AMG) AS AN ALTERNATIVE TO C-14 AUTORADIOGRAPHY

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Automagnetography (AMG) is a created term for MRI (Magnetic Resonance Imaging). Performing ^{13}C-MRI of a rat head *in vivo*, we compared between *AMG* and autoradiography (ARG).

A C-13 image was obtained *in vivo* after

intravenous injection of one gram of [1-^{13}C] glucose per Kg body weight as a metabolic tracer. The machine used was a 7.05-Tesla MR system with a horizontal bore. A spinecho sequence employing a chemical shift selective pulse was used. The total acquisition time was 36 min. The spatial resolution of the image was 0.98 mm and slice thickness was 6 mm. Signals from the brain were minimal, while strong signals were observed from the tissues outside the brain. [1-^{13}C] glucose, metabolized to be changed in the chemical structure, appeared to have lost signals on the chemical shift image.

The possible features of *AMG* were supposed to be (1) non-radioactive, (2) non-invasive to animals, (3) feasible of time-trend study of metabolic changes in one living organism, (4) feasible of projection imaging, however, (5) low in signal intensity.

STUDY OF DOSE-RESPONSE RELATIONSHIPS IN CONTACT SENSITIVITY USING AN *IN VITRO* ASSAY

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Dose-response relationships in contact allergy were evaluated in guinea pigs using an in vitro assay. Guinea pigs were sensitized by different doses of 1-chloro-2, 4-dinitrobenzene (DNCB) and challenged by DNCB and 2, 4-dinitrobenzene sulfonic salt (DNBS). Lymph node cells from sensitized and control guinea pigs wree cultured in the presence of different dose of DNCB and DNBS at 8×10^5 cells/well, respectively. The sensitivity was evaluated by lymphocyte transformation test (LTT) which was assessed by uptake of ^{3}H-thymidine. The results indicated that there are significant correlations between the doses of sensitizers and values of LTT in both the induction and challenge phase of contact allergic reaction. The values examined by the in vitro assay correlated significantly with patch test readings. The *in vitro* assay reproduced the cross reaction between DNCB and DNBS which was confirmed *in vivo*.

From the results, it was evaluated that the present in vitro assay might assess dose-response relationships for contact allergy objectively and quantitatively.

MURINE LOCAL LYMPH NODE ASSAY TO PREDICT CONTACT SENSITIVITY: COMPARISON WITH THE GUINEA PIG MAXIMIZATION TEST

Yoshiaki Ikarashi, Toshie Tsuchiya, Akitada Nakamura

National Institute of Hygienic Sciences

The contact sensitivities of metal salts and rubber chemicals wrc evaluated by murine local lymph node assay. The test chemical was applied to ears, and ^{3}HTdR incorporation of draining lymph node cells was measured. Exposure of contact sensitizers, $NiSO_4$,

$CoCl_2$, $K_2Cr_2O_7$, N-isopropyl-N'-phenyl-p-phenylene-diamine, tetramethylthiuram disulfide and 2-mercaptobenzothiazole, caused increase of lymphocyte proliferation. $ZnSO_4$, $MnCl_2$ and $FeSO_4$ caused negative response. This test method requires small number of animals, consumes shorter time, and provides more quantitative information than the guinea pig sensitization test. The local lymph node assay may be a useful predictive test for the identification of contact allergens.

THE SOLATEX-PI SYSTEM, AN *IN VITRO* METHOD TO PREDICT PHOTOIRRITATION

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The Solatex-PI System is an *in vitro* method to predict the potential for photo-irritation of chemicals, plant and natural extracts, fragrance oils, and formulations. The Solatex-PI System incorporates the direct measurement of dermal irritation in a two compartment physico-chemical model in the presence and absence of UV light. Test samples are applied to the first compartment, a keratin/collagen barrier membrane. This is incubated in the second compartment, an organized macromolecular matrix. UV_A as well as UV_A and UV_B light were used for exposure of test samples in this system in a controlled temperature incubator for up to 24 hours.

The Solatex-PI System has been used to evaluate 103 materials and formulations. The photoirritancy of these materials was based on the *in vivo*response observed n humans or guinea pigs. Of 38 known photoirritants, thirty-five were correctly identified. Two negative photoirritants produced response in this system.

The Solatex-PI System can effectively provide a rapid screen to determine the potential for photoirritation of chemicals and formulations.

PREDICTION OF PHOTOTOXICITY BY RED BLOOD CELL HEMOLYSIS

Mariko Sugiyama, Hiroshi Itagaki, Takeshi Hariya, Noriko Murakami and Shinobu Kato

Shiseido Safety and Analytical Research Center

Red blood cell (RBC) hemolysis is phenomenon based on photodynamic effect of chemicals. The method was validated as a possible alternative to predict phototoxicity of chemicals. 990 μl of aqueous suspension of RBCs was mixed with 10 μl of test chemicals and the mixture was subjected to UVA radiation (25J/cm^2). And the absorbance at 540 nm of the supernatant following centrifugation (3000 rpm, 15 min) was measured and photohemolysis ratio was calculated. When the data for 22 substances were compared with those of phototoxicity test in guinea pigs, sensitivity, specificity, predictive value and equivalence were 75%, 64%, 60% and 68%, respectively. These results suggest

that RBC hemolysis be a reliable screening method to predict phototoxic nature of chemicals.

DEVELOPMENT OF *IN VITRO* TEST FOR DETECTION OF EMBRYOLETHALITY USING CELL CULTURE SYSTEM

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New herbicidal compounds were found to be embryolethal but not teratogenic in rats. The range of the embryolethal dose varied from 0.2 to >200 mg/kg. This broad range enabled us to validate whether proposed in vitro teratogen tests can detect the embryolethality of these herbicides. The IC50 values (inhibition concentration 50%) for both differentiation and proliferation of mid-brain and limb bud cells of rat embryos were determined and found to be above 60 μg/ml in all cases. No correlation was observed between the embryolethality in vivo and the activities in these cells. The inhibition of colony forming ability in V79 cells was determined in order to test whether the potential to cause embryol ethality could be detected as a general cytotoxic effect. The results indicated that cytotoxicity in V79 cells may be useful for preliminary testing of the embryolethal effect of herbicides.

CARDIOVASCULAR MALFORMATIONS INDUCED BY PHENOBARBITAL AND CAFFEINE IN CHICK EMBRYOS

Akane Kurokawa, Takashi Kobayashi and Fumio Ariyuki

Safety Research Lab. Tanabe Seiyaku Co., Ltd.

One, 2, 5, 10, 15 and 20 μ mol/egg of phenobarbital (PB) or 15 and 20 μmol/egg of caffeine (CA) were administered topically to embryos at day 4 of incubation. 50 nmol/egg of adenosine (AD) was administered concomitantly with the PB or CA to the embryos. After administration, the embryos were reincubated until day 12 and examined.

The embryos treated with AD+PB or AD+CA showed higher survival rates and lower frequencies of cardiovascular malformations than those treated with PB or CA alone.

The above esults suggest that the mechanism of cardiovascular malformation by PB or CA is related to the circulatory disturbance induced by them rather than to their direct cellular effects. Chick embryo was found useful for investigation of the mechanism of the cardiovascular malformation.

VARIANCE IN THE DEVELOPMENT OF CULTURED RAT EMBRYOS EXPLANTED FROM THE SAME LITTER AND THE DIFFERENT LITTERS

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In the whole-embryo culture, data analyses are usually carried out on the basis of the embryo as sample unit. We wonder whether it is always necessary to distribute the embryos from each litter equally between all dose groups or not. To know how much variance exist in the growth parameters of cultured embryos from the same litter or the different litters, we statistically analyzed historical control data of 48-hours cultured rat embryos, which were explanted on day 11.5 of embryonal age without chemical treatment, by the method of principal component analysis.

Morphological abnormalities were not observed. The first principal component of this analysis, which was mainy composed of protein concentration and crown-rump length, was regarded as the growth index of cultured embryos. The second component was mainly composed of heart rate and was assumed to be statistically different index from the growth one. As for the first component, the growth indices were varied much more in the embryos from the same litter than in that from the different litters, except that some of the different litters deviated to lower growth level from the others. When both growth parameters of the intact decidual mass and the visceral yolk sac were added to the principal component analysis, however, it was noted that the variance among the embryos from the different litters appeared to be definite. In these analyses, it was considered that at the start of whole-embryo culture without exception of the deiated litters whose embryos will demonstrate less growth indices, we must distribute the embryos from each litter equally between all dose groups including the control.

THE STUDY OF NEW PARAMETERS FOR DIFFERENTIATION PARAMETERS FOR IN WHOLE EMBRYO CULTURE

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Whole embryo culture is useful technique to examined the effects of chemical compounds on embryo without maternal factors. It is necessary, however, to establish suitable parameters of growth and fifferentiation for cultured embryos. In the present study the number of pigment cells in eyes of mouse embryos cultured for 24 hrs from 11 day of gestation was mesured by the computer graphic analzyer (Nikon COSMOZONE 1S).

In the begining of culture, 11 day embryos had 51±2 somites, crown-rump length of 7.4±0.2 mm, and pigment cells $735.6 \pm 121.3 \times 10^{-6} mm^2$ in the eyes. After

culture for 24 hrs in vitro embryos had the 59±1 somites, crown-rump length of 8.5±0.3 mm, and $1496.7 \pm 235.0 \times 10^{-6} mm^2$ pigment cells in the eyes.

These results indicate that the number of pigment cells in eyes of the mouse ebryos may be one of the parameter for diffiientiation in the culture embryos, as same as the total number of somites in the embryos.

THE SUITABLE STAGE FOR OPENING YOLK SACS IN THE LONG TERM CULTURE OF RAT WHOLE EMBRYOS

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Using awhole-embryo culture system, several teratogenic comounds wree tested for their potential to induce abnormal development. In this culture, the opening of yolk sacs is necessary for the normal development or differentiation of cultured embryos. After 12 day of rat embryos (35 somites), they can grow in culture with the yolk sac and amnion opened (Cockroft, 1973). In the present study the suitable period for opening yolk sacs for long term culture was eramined. The techniqe (the long term culture of rat embryo) is based on the method developed by New or Takakubo (New 1978, Takakobo 1988). 11-day-old rat embryos were cultured in one medium containing 5 ml of rat serum in votating culture system (20 rpm) at 38.0°C for 48 hr. At the end of culture the embryos were examined for the protein content, crown-rumplength, total number of somites and external malformation. These results of growth of cultured rat embryos were shown in fig. I.

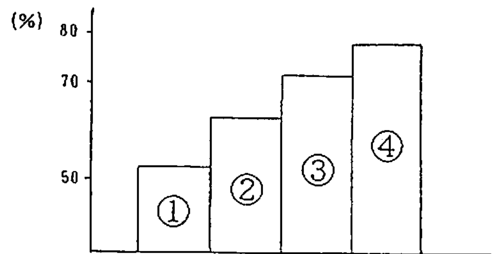

Fig. 1

EFFECTS OF MICROINJECTION OF SALICYLIC ACID OR GENTISIC ACID INTO YOLK SAC OF RAT EMBRYOS IN WHOLE EMBRYO CULTURE

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In the previous studies it was reported that there were some differences between effects aspirin and that of its metabolite, salicylic acid, in the malformationsthey caused. In the present experiment it was found indicated that salicylic acid-induced malformation was less severe than that produced by aspirin. This difference might be due to the metabolism of the drug in the yolk sac. We measured the concentration of salicylic acid in the placenta, yolk sac and embryo. Salicylic acid contents were 72 ug per placenta, 20 ug per yolk sac and 12 ug per embryo. Cultured embryos were injected with 20 μg/10 μl/embryo of salicylic acid or 20 μg/10μl/embryo of gentisic acid into the yolk sac. This microinjection of salicylic acid produced edematous malformation of the whole body, cleft lip, and anomalies of the tail. Thiss teratogenicity was as severa as that produced by aspirin.

On the other hand, embryos injected with gentisic acid did not differ from those in control culture, but treatment with gentisic acid decreesed int he heart beat for 10 hours in culture period. Thus, the results indicated that the yolk sac disturbed the teratogenic action of salicylic acid.

A CHEMICALLY-DEFINED MEDIUM FOR RAT WHOLE EMBRYO CULTURE 1. EFFECT OF 5FU ON 9.5-DAY OLD RAT EMBRYOS

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The purpose of this study is to examine the possibility of mixture of rat I.C. serum and a chemically-defined medium, Dulbecco's Modified Eagle's Medium (DMEM), as a medium for rat whole embryo culture. 9.5-Day old rat embryos were cultured for 48 h in the rat I.C. serum mixed with DMEM in the proportion of 0, 25, 50 or 75%.

5-Fluorouracil (5FU) was examined qualitatively and quantitatively for its ability to produce developmental toxicity in whole embryo culture system using the mixed medium, to compare with the results obtained by 100% rat I.C. serum. Embryos cultured in the 25% or 50% DMEM were not different in the developmental parameters when compared to those cultured in 100% rat I.C. serum. However, embryos cultured in the 75% DMEM showed growth retardation, and mortality and malformations at a considerably high frequency.

Embryos cultured in 100% rat I.C. serum,

25% DMEM or 50% DMEM, containing 5FU at the various concentrations showed retardation of development and gross malformations in a concentration-dependent manner. These results suggest that rat I.C. serum mixed with DMEM at the proportion of 50% or under can be used as a medium or rat whole embryo culture to estimate the teratogenicity of a chemical.

AN APPROACH TO *IN VITRO* TERATOGENESIS SCREENING USING WHOLE EMBRYO CULTURE: EFFECT OF TWO BASE ANALOGUES, 5FU AND BUDR, ON 9.5-DAY-OLD RAT EMBRYO

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The purpose of the present study is to obtain basic data for estimating *in vivo* teratogenicity of chemicals by *in vitro* experiments using whole embryo cultures. The study was assessed with two base analogues 5-fluorouracil (5FU) and 5-bromo-dexyuridine (BUDR). Embryos were studied either after direct exposure to the compounds during incubation of embryo cultures or maternal 5FU or BUDR dosing and subsequent embryonic development *in utero* with a view to assess the similarity of these two system to produce malformations.

Sprague-Dawley 9.5-day-old rat embryos were cultured for 48h in rat serum containing 5FU or BUDR. They were examined morphologically and their protain content were determined.

The cultured embryos showed mainly hypoplasia of the optic vesicles, at both compounds. In *in vivo* studies, hypoplasia of the optic vesicles or anophthalmia was observed when dams, intraperitoneally dosed with 5FU or BUDR on day 9.5 of gestation, were 48h following dosing to exposure of embryos or killed at term to evaluate fetuses. *In vitro* 5FU or BUDR exposure of embryos as well as *in vivo* maternal exposure caused dose-dependent significant increases in the incidence of malformations.

The slopes of dose (conc.)-responce curves for induction of hypoplasia of the optic vesicles *in vivo* and *in vitro* were not significantly different in respective comounds, consistent with the hypothesis that 5FU or BUDR may exert its adverse effects on embryo development in rats *in vivo* and *in vitro* through a common mechanism to produce adverse developmental outcomes.

These results suggest the usefullness of the whole embryo culture system as a suitable tool for estimating the embryonic/teratogenic risks of chemicals.

INTERLABORATORY VALIDATION STUDY OF THE MARROW-TECH SKIN^{2TM} DERMAL MODEL AND MTT CYTOTOXICITY ASSAY KITS

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Summary

Two factors of critical importance to the adoption of *in vitro* alternative assays are the predictive potential of the assay system and its intra- and interlaboratory reproducibility. Many *in vitro* alternatives to animal testing have been proposed, but only a few of them have been subjected to interlaboratory validation studies in order to assess their reproducibility and relevance to *in vivo* data. The study described in this paper was designed to assess the shippability, reproducibility and relevance to *in vivo* data of the Marrow-Tech skin^{2TM} Dermal Model substrate in concert with Marrow-Tech MTT Cytotoxicity Assay Kits. This three-dimensional Dermal Model, which consists of several layers of human, foreskin-derived, metabolically active fibroblasts grown to form a tissue equivalent on nylon mesh, was used as the substrate to test the toxicity of twelve chemicals proposed by the Commission of the European Communities (i.e., toluene, n-hexane, 1-butanol, chloroform, sodium dodecyl sulfate, benzalkonium chloride, silver nitrate, tributyltin chloride, dibutyltin dichloride, 2-butoxyethyl acetate, acetaldehyde, and 2-methoxyethanol). Fibroblasts growing within this three-dimensional framework secrete a number of known growth factors and extracellular matrix proteins(1). This substrate has been recently utilized to study the *in vitro* toxicity of a number of compounds including detergents, shampoos, cosmetics, alcohols, metals, antimicrobial preservatives and an ionophore using the neutral Red (lysosomal-based) viability assay (2, 3, 4), the MTT (mitochondrial function) assay (3, 4, 5), Prostaglandin E_2 (PGE_2), release (inflammatory mediator) assay (3) and the lactate dehydrogenase (LDH) release (membrane integrity) assay (3). *In vivo: in vitro* comparative toxicity data gathered to date have proven very encouraging.

Dermal Model Kits and MTT Assay Kits were manufactured by Marrow-Tech in the U.S. and shipped to Janssen Pharmaceutica in Belgium. Teo runs of each of the twelve chemicals were performed in each lab one week apart and MTT-50 values (i.e., the concentration of test agent which reduced the tumover of water-soluble, yellow MTT to a blue water-insoluble formazan precipitate to 50% of untreated control or vehicle control lelvels) were determined for each compound. Rank-ordered comparisons of toxicity were prepared for each laboratory's data. Linear regression analysis of interlaboratory reproducibility was calculated to be 97%. The *in vitro* data were also compared with existing *in vivo* rabbit skin irritation data (6) and also found to be highly correlative. The high degree of interlaboratory reproducibility demonstrated that the Marrow-Tech skin^2 human Dermal Model prouct remains stable at ambient temperature during intercontinental shipmenta and that the MTT Assay Kits, also supplied by Marrow-Tech, provide a standardized method for assessing the toxicity of test agents.

Materials and methods

MTT Assay procedure: This procedure has been detailed elsewhere (3, 4). Briefly, 11×11 mm mesh squares containing fibroblasts were

placed into 24-well plates and treated with 2 ml of five concentrations of test agents (two replicates per concentration) diluted in complete DMEM containing 2% fetal bovine serum [Assay Medium] for 20 hours at 37°C in 5% CO_2(≥90% humidity). Two untreated control mesh squares were incubated with the appropriate vehicle control. After overmight incubation with test agents, the spent media were aspirated and replaced with 1 ml (per well) of Assay Medium containing 50 μg/ml of MTT. The cultures were incubated for 2 hours, then washed twice with 1 ml PBS. Blue formazan precipitate was extracted from the mitochondria using 2 ml isopropanol on a shaker platform at room temperature for 1 hour. Aliquots (200 μl) of the extracted MTT solutions were transferred to 96-well plates and the optical density at 540 nm (OD_{540}) wad determined using a microplate reader making a blank correction to pretreated nylon mesh (without cells) which had been similarly treated with MTT. The mean OD_{540} of the duplicate vehicle control wells was set to represent 100% viability. Results for each concentration were plotted, as percentage of vehicle control, against the concentration of test agent (mM) on a log scale, and an MTT-50value was determined directly from the graph for each chemical.

THE SCIENTIFIC BASIS, BALIDATION AN APPLICATIONS OF THE EYTEX™ METHOD

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The EYTEX™ Method is an *in vitro* method to predict the potential of ocular irritation of chemicals and formulations. The scientific basis of the EYTEX Method is that changes in a highly organized macromolecular matrix which occur upon exposure of the matrix to chemical irritants are similar to changes which occur in the cornea *in vivo*. Changes in the matrix result in turbidity which is quantitated spectrophotometically to establish the EYTEX/Draize Equivalent and Irritancy Classifications of test samples.

The EYTEX Method incorporates four protocols. The major protocol is a broad screening protocol. A second protocol is specialized for low irritation test materials. Alkaline materials and surfactants and surfactant based samples require specific protocol adaptations. Utilization of these protocols in an evaluation of 1000 diverse chemicals and formulations demonstrated a 92% equivalence of *in vitro* results to Draize *in vivo* results. A sensitivity of 91% and specificity of 87% were determined in this evaluation. All chemicals and formulations could be analyzed neat except twenty surfactants analyzed at 10%.

The EYTEX Method provided a comprehensive system of four protocols to analyze chemicals and formulations of varying degrees and mechanisms of ocular toxicity.

A VALIDATION OF *IN VITRO* ALTERNATIVE METHODS (EYTEX™ AND SKINTEX™) TO *IN VIVO* IRRITATION TEST AND THESE APPLICABLE AREA IN DRUG DEVELOPMENT

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EYTEX/SKINTEX is one of usuful alternative methods to *in vivo* Draize. We conducted EYTEX/SKINTEX tests of 34 and more substances to validate. The equivalence of EYTEX was 93.9%, the sensitivity was 100% and the predictive value was 83.3%, while the equivalence of SKINTEX was 71.4%. We used these *in vitro* methods for two practical assessments of drugs. One was a preliminary test for *in vivo* Draize test, the other was general assessment for relatives of a known medicine for external use.

We think that these *in vitro* irritation tests are applicable to; 1. Screening of new substances, 2. Preliminary test for *in vivo* main test, 3. Temporary or general assessment of relatives of a known substance, 4. Reference for pharmaceutical designing and 5. Assessment of chemical hazard level of environmental chemicals.

NATIONAL VALIDATION PROJECT OF ALTERNATIVES TO THE DRAIZE EYE TEST IN GERMANY

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Since 1988 ZEBET has coordinated a national German interlaboratory study on the validation of two alterntive methods to the Draize rabbit's eye test, the neutral res/kenacid blue (NR/KB) cytotoxicity assay and the hen's egg chorioallantoic membrane (HETCAM) test.

During the first two years the two methods were established in twelve laboratories to ensure intra- and interlaboratory reproducibility. Testing 32 chemicals from a variety of chemical classes indicated a better correlation between data from HET-CAM test and both human and Draize test data than between cytotoxicity and the *in vivo* data.

During the final experimental stage of "data base development"[1] 150 chemicals were tested in both test systems to provide information whether and to what extent the *in vitro* tests might be able to replace the Draize rabbit's eye test. The test chemicals selected are representing a broad spectrum of both chemical classes and *in vivo* eye irritating properties.

A preliminary evaluation of the final data indicates a good correlation between HET-CAM and Draize test data and a poor correlation between cytotoxicity and *in vivo*

data.

References

1) Balls et al. 81990) *ATLA* 18, 313–337.

INTER-LABORATORIES VALIDATION OF *IN VITRO* ALTERNATIVES FOR OCULAR SAFETY TESTING

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Two *in vitro* assays were evaluated to determine if any were useful as screening in ocular safety assessment. Twelve test chemicals were tested in both assays. *In vivo* ocular irritaiton scores for the materials wree obtained from existing rabbit eye test data. There were a significant correlation between the *in vivo* irritant potential and *in vitro* data for both colony forming assay and neutral red assay used in this study. The procedure of both tests were tranfered well among 12 independent laboratories. These result suggested it may be possible to classify materials into board irritancy categories with *in vitro* methods.

INTER-LABORATORIES VALIDATION STUDY OF CYTOTOXICITY TEST USING SKIN² DERMAL MODEL AS AN ALTERNATIVES TO OCULAR SAFETY TEST

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Skin² Dermal Model was evaluated to determine if it was useful as screening procedures in ocular safety assessment. Six tes chemicals were tested in this assay. *In vivo* ocular irritation scores for the materials were obtained from existing rabbit eye test. For the six materials used in this study there was a significant correration between the *in vivo* irritant potential of the test materials. The result of this study suggested it may be possible to classify materials into broad irritancy categories with Skin² Daermal Model system. This would allow its use as screen prior to limited *in vivo* confirmation in the ocular safety assessment process.

QUANTITATIVE EVALUATION TO PREDICT THE EYE IRRITATION USING HEMOGLOBIN

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Based on the hypothesis that the damage of cellular plasma membrane and proteins plays an important role in eye irritation of chemicals, hemoglobin denaturation was examined for its applicability to predict the eye irritaion of chemicals. 100 μl of aqueous surfactants at the concentration ranging from 0% to 2% were mixed with equal amount of 0.05% buffered hemoglobin solution in 96-well microplates and incubated at 25°C for 5 min. The absorbance at 418 nm was measured and the hemoglobin denaturation ratio (HDR%) for each test material was calculated. Correlation analysis, Factor analysis and Multiple linear regression analysis were aplied in this study and following results were obtained. 1) Correlations between HDR%s and Draize scores are reasonably high. 2) Factor analysis suggested that there are 2 factors of controlling HDR%, Low concentration (less than 0.25%) factor and High concentration (more than 0.5%) factor. 3) Higher correlation coefficient could be obtained by multi-regression analysis using HDR% of low concentration (0.031%) and that of high concentration (1.0%). The correlation coefficients between Draize score of both cornea and total for twelve surfactants were r=0.84 and r=0.87 respectively, and

they were high enough for primary screening test. We believe this method contributes a lot for the product researcher because it does not require any sophisticated instruments or facilities even though it remains to be validated with more materials.

AN ALTERNATIVE METHOD TO THE DRAIZE EYE IRRITATION TEST—USE OF CHORIOALLANTOIC MEMBRANE OF THE CHICK EMBRYO (II)

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the applicability of the chorioallantoic membrane (CAM) test using fertile hen's eggs as an alternative method to predict the eye irritancy was examined for a wider variety of chemicals. The judgement of the injurious effects of twelve chemicals was carried ot by trypan blue staining method that was developed to overcome disadvantages based on the lack of objectivity and quantitativeness in original CAM test (Luepke's method). The amounts of pigment adsorbed with the CAM showed a good correlation with the scores obtained by Draize eye irritation test (r=0.92). The results suggest that the trypan blue staining method be useful as an alternative model of in vivo eye irritation test.

ALTERNATIVE METHOD FOR DRAIZE TEST II RAPID AND SIMPLIFIED ASSAY FOR DEAD CELLS BY DETERMINING LDH RELEASED FROM SUSPENSION CULTURED CELLS

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We have been proposing that LDH released from cultured cells is a sensitive marker of dead cells and that LDH release assay could be an alternative to the Draize eye irritation test, especially for detergent-based cosmetics. To simplify the assay method, we replaced target cell lines from anchorage-dependent cells to suspension cltured cells. Ten detergents tested showed sigmoid dose-response curves. ED_{50} values of each detergents observed in human myeoma cell line HL60 were slightly lower than those observed in the anchorage-dependent cell line SQ-5. Values of ED_1 or ED_{10} were also easily determined in the susension cultured cells. Crossing of several sigmoid curves in this low dose range was observed. Merits obtained by utilizing suspension cultured cells were

1) Preculture in microculture plate was not required.
2) Short treatment period of detergents (20 min) allowed us to handle the culture plate in unsterlized condition during the assay.
3) Fully automatized processing of the assay may be easily constructable.

APPLICATION OF NORMAL RABBIT CORNEAL EPITHELIAL CELLS IN SERUM-FREE MEDIUM AS AN ALTERNATIVE TO THE DRAIZE OCULAR IRRITATING TEST

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We evaluated an alternative to the Draize ocular Irritating Test using normal rabbit corneal epithelial cells (NRCE) grown in serumfree medium (RCGM, Kurabo). Test compounds were added to each well of 96-well tissue culture plate, in which cells had been cultivated for 3 days. Growth and viability of the cells were estimated with the uptake of neutral red, which could be incorporated only by teh viable cells. Twelve chemicals including detergent, antiseptic were tested with this method.

First, we examined the amount of the uptake of neutral red by the viable cells (NRCE). We found that the uptake of neutral red was affected by the calcium concentration in RCGM. And 0.15 mM calcium was most suitable for *in vitro* test. Therefore, we used RCGM containing 0.15 mM calcium for medium.

The correlation coefficient between *in vivo* Draize score and the values with *in vitro* test using NRCE was 0.711. And the correlation coefficient between the values with this method and those with *in vitro* test using NHEK was 0.967.

These results suggested that the cytotoxicity test using NRCE is useful as an alternative to *in vivo* Draize Ocular Irritating Test.

References

1) J. Draize et al. (1944) J. Pharm. Exp Ther., 82, 377–390.
2) H. Torishima et al (1990) Altern. Anim. Test. Exp., 1, 20–26.

CORRELATION BETWEEN IN VIVO DRAIZE RABBIT EYE IRRITATING TEST AND IN VITRO TEST WITH VARIOUS CULTURED CELLS

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We compared the cell sensitivity among 5 types of cells, established Chinese hamster lung (CHL) cells, established HeLa cells, primary rabbit corneal (RC3) cels, normal human skin fibroblast (NB1RGB) and normal human skin epidermal keratinocytes (NH-EK), and compared each cell types in vitro on the cytotoxicities of 8 surfactants and the Draize rabbit eye irritating test in vivo. Cytotoxicities of the surfactants were assesed by neutral red uptake reduction after 48 h of treatments. NHEK were tested in a serumfree medium but CHL cells were tested both serum-free medium and medium with 10%

fetal Calf Serum (FCS). Other cells were tested in medium with 10% FCS.

there was a good correlation between the cytotoxicities of each cells in vitro and the Draize scores in vivo. NHEK and CHL cells cultured in serum-free medium were more sensitivity than other cells cultured medium with 10% FCS.

These results suggested that the cytotoxicity test in vitro using one of 5 types of cells is useful as an alternative to the Draize eye irritating test in vivo on the suffactants. Especially, pre-test of cytotoxicity on the chromosome aberration test using CHL cells, one of Mutation tests, can be useful as an alternative to Draize eye irritating test in vivo.

PREDICTION OF IN VIVO DRAIZE RABBIT EYE TEST BY *IN VIVO* PRIMARY RABBIT SKIN IRRITATION TEST AND *IN VITRO* CYTOTOXICITY TEST

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(Biochemical Research Institute, Nippon Menaed Cosmetic Co., LTD.)

We compared the correlation between in vivo Draize rabbit eye irritation scores and primary rabbit skin irritation scores, and also compared between in vivo Draize eye irritation scores and in vitro cytotoxicties of Chinese hamster lung (CHL) cells and nomarl human skin fibroblasts in response to the 16 surfactants, in order to predict precisely of Draize eye scores.

Primary skin irritation scores and in vitro cytotoxicties showed a good correlation with Draize eye scores, but each correlative coefficient was low score.

The results obtained the combination of primary skin irritation scores with in vitro cytotoxicties corresponded completely with Draize eye scores.

These results suggested that combination of primary skin irritation scores and pre-test of cytotoxicity on the chromosome aberration test using CHL cells are possible to predict precisely of Draize eyescores.