

# **The 20th Annual Meeting of the Japanese Society for Alternatives to Animal Experiments**

**Date:** December 8th and 9th, 2006 (Friday and Saturday)

**Venue:** Convention Hall and adjacent Foyer of General Research Experiment Building,  
Komaba II Campus, University of Tokyo  
(Komaba 4-6-1, Meguro-ku, Tokyo 153-8505, Japan).

**President at the Meeting:** Assoc. Prof. Yasuyuki Sakai  
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Center for Disease Biology and Integrative Medicine,  
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# **Abstracts**

# **Symposium 1**

**“Current Status of 3Rs in Asian Countries”**

## S1-1 Sophisticated *in vivo* research based on the 3R-principle

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### **【Purpose】**

Promotion of alternatives can be accomplished by implementing the 3Rs in a broad sense. At this symposium, we will introduce some examples of alternatives developed by us and define our basic concept on alternatives. Also, current information on Japanese regulations and guidelines on ethical animal experimentation will be briefly explained.

### **【Materials, methods, results and discussion】**

rasH2 mouse: Strict quality control of the rasH2 mouse resulted in a promising alternative for carcinogenicity testing. The newly developed test system using the Tg mouse was validated by ILSI/HESI (Toxicologic Pathology 2001, 29 Supplement), and competent regulatory agencies in the US, Europe and Japan have adopted the new method as an alternative to the current 2-year test. The testing period can be shortened by 1/4 and the number of mice involved can be reduced to less than 1/2.

TgPVR21 mouse: The Tg mouse introduced human poliovirus receptor DNA is susceptible to poliovirus. The mouse was validated by a WHO collaborative study (Bulletin of WHO 2003, 81(4), 251-260), and WHO accepted the mouse as an alternative to monkeys for neurovirulence testing of live oral polio vaccine.

Infrastructure: Genetic and microbiologic quality control is essential for a newly developed animal model to be a useful alternative, especially specifications of its quality and a monitoring system of the quality. An embryo cryopreservation system is also indispensable for stable large-scale supply. We consider such an infrastructure as one of the most important factors for refining animal experimentation.

### **【References】**

Kagiya N, Ikeda T & Nomura T (2006) Japanese guidelines and regulations for scientific and ethical animal experimentation. In: Stevenson C et al. (eds): *In vivo models of inflammation*, 2<sup>nd</sup> edition, Vol I. Birkhaeuser Verlag, Basel (in press).

## **S1-2 3Rs in India: Past, Present and the Future**

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The concept of animal welfare in India originated in the Prevention of Cruelty to Animals (PCA) Act of 1960. The first CPCSEA (Committee for the Purpose of control and Supervision of Experiments on Animals) a statutory body of the Government of India was constituted in 1964 to oversee the welfare of laboratory animals and to ensure that the experiments on animals were conducted in accordance with internationally accepted ethical norms, in line with the 3Rs concept of Russel and Burch. In spite of such an early beginning the real sting in the regulations came when in 1998 Ms. Maneka Gandhi, a former minister and an animal rights activist took over as the chairperson and highlighted the state of animals and animal houses all over the country. The CPCSEA has powers to make rules in relation to the conduct of animal experiments, to authorize any officer to inspect any laboratory, and to prevent an individual or institution from carrying out animal experiments. It is mandatory to have a representative from the CPCSEA on any Animal Ethics Committee (AEC). All laboratories carrying out animal experiments were required to be registered with and validated by the CPCSEA. The concept of the 4<sup>th</sup> R was officially accepted by CPCSEA in 2004.

The use of animals in medical education, production of immunobiological agents, vaccines and toxicity testing was also brought under these rules when 'Guidelines for the use of Animals in Scientific Research' were published by ICMR (Indian Council of Medical Research) and NCLAS (National Centre for Laboratory Animal Sciences) in 2000 and 'Guidelines for use of laboratory animals in Medical Colleges' by ICMR in 2001. To ensure proper education about alternatives and training in use of alternatives International Center for Alternatives in Research and Education (I-CARE) has been set up. The center conducted nation wide workshops for educators in the year 2004 and at present is actively engaged in propagation of the principle of the 4<sup>th</sup> R. Computer Aided Learning (CAL) software have become freely available all over India as alternatives to various animal experiments, at all levels of training, in medical, dental, pharmacy and veterinary colleges. The Chief Editor of the Indian Journal of Pharmacology, Dr. R Raveendran, has taken the initiative to develop software to serve as an alternative to animal experiments in medical colleges which are available free via the journal website and also distributed with the journal. As an outcome of all these efforts, in the year 2005, Bharathidasan University, Tamil Nadu became the first university in India to achieve a 95% reduction in use of live animals in teaching of Zoology. Additionally, "First Indian Congress on Alternatives to the use of animals in research, testing and education" is being organized in January 2007.

Thus, despite a slow start, considerable progress has been made in the field of animal welfare both at regulatory and individual levels with constant efforts being made to reduce, refine, replace and rehabilitate animals involved in experiments at all levels of education and research.

### S1-3 Current Status of 3Rs in China

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#### Abstract

In their 1959 book, *The Principle of Humane Experimental Technique*, William Russell & Rex Burch put up the theory of 3Rs. In the past more than 40 years, with the rapid development of biological technology, the people have been acquiring a better understanding about the concept of 3Rs, and have more understanding on the close relationship between the 3Rs and life science, and also have an intimate knowledge that application of 3Rs achievement is the motive force of scientific research and legal testing.

In China, more and more attention has been paid to the study on alternatives to animal experimentation in recent years and the development in this field is mainly reflected in the following aspects.

1. In 1997, the basic concept of alternative method was described for the first time in “the certain proposal concerning the development of laboratory animal science in Nine-Five plan” issued by State Commission of Science and Technology. In 2001, establishment of regulation for ensuring animal welfare, which is equivalent with international rule, is put forward clearly and definitely as an important content of legal management of laboratory animal in “national programme in the construction of condition for science and technological research in Ten-Five plane” issued by State Commission of Science and Technology. In “The Regulation for Laboratory Animal”, which is being revised, alternatives to laboratory animal as one part of animal welfare is added in the document. Beijing Legislation on Laboratory Animal had been revised in 2004 and issued in 2005. Animal welfare and alternatives to animal experiment are the important content in the document. The government attitude to 3Rs creates a beneficial developing environment for this work.

2. The alternatives to animal experimentation as a project in social public research has been brought into the state plan of scientific research management by Chinese government. In different levels and various fields, the government department of scientific research management offers funds to support the explorative research in this aspect by establishing research projects. Until now, some research projects, which have been accomplished, have brought us the satisfactory results.

3. Enforcing international academic exchanges for promoting the development of 3Rs research. Constructing the academic exchange platform by starting a special column in journal of laboratory animal science.

4. By TV, newspaper and journal, the scientists publicize the intension of 3Rs and significance of conducting research on alternative methods. The aim of doing those is to enable the public understanding the importance of animal experimentation in human life, and refine the social environment for the development of laboratory animal science.

Although the cognition to latent power of 3Rs on development of life science is not deepened, the scientists in China have been performing the significant attempt and active approach in the 3Rs field, for example, immunizing the poultry with special antigen and obtaining the antibody from eggs. By this method, a number of antibodies can be collected with reducing the number of animals used in production of antiserum in routine procedure. The animal testing is substituted with immuno-affinity-chromatography in the safety evaluation of biologicals. It should be said that the methods mentioned above belong to alternatives to animal experimentation, but they haven't been validated scientifically.

## **S1-4 Current Status of Alternative Study of NITR(National Institute of Toxicological Research) in Korea**

### **Kui Lea Park**

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On the global level, criticism of animal experiments is mainly directed at the toxicological safety tests prescribed by public agencies like the one stipulated in conjunction with the development of medicinal products, industrial chemical and cosmetics. National Institute of Toxicological Research (NITR) of Korea has been conducting alternative toxicity study for skin irritation test using fibroblast, immunotoxicity (local lymph node assay), phototoxicity (3T3 NRU assay), genetic toxicity(bacterial reverse mutation test, in vitro mammalian chromosomal aberration test, in vitro sister chromatid exchange assay, mammalian erythrocyte micronucleus test etc), reproductive toxicity (whole embryo culture system, mid-brain micromass culture system, limb bud organ culture system, limb bud micromass culture system) and endocrine disruptor (uterotrophic assay, Hershberger assay, estrogen receptor binding affinity assay etc). NITR also supports the research and development of alternatives to animal experiments such as skin toxicity, sensitization and ocular toxicity. Korean Society for Alternatives to Animal Experiments (KSAAE) was established in 2006 to promote research, development, and education of alternatives to animal experiments and assist to build national partnerships with governmental and non-governmental groups, including academia, industry, advocacy groups, and other stakeholders.

## **Special Lecture**

### **“ECVAM’S Role in Making Alternative Methods Available for New European Legislation”**

**T. Hartung (ECVAM)**

## **SL ECVAM's role in making alternative methods available for new European legislation**

### **Thomas Hartung**

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ECVAM was created in 1991 further to Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes which prescribes to use alternative methods whenever possible. As a service of the EU Joint Research Centre, ECVAM has pioneered the validation process and became a proactive facilitator for effective animal protection, especially in the field of regulatory toxicology. The field of alternatives is currently driven by the expectations from both cosmetics and chemicals policies: The 7<sup>th</sup> amendment to the Cosmetics Directive published in 2003 foresees to phase out animal experiments completely within 10 years. A timetable for the phasing out of the individual animal tests published by the European Commission in September 2004 had been supported by a taskforce of stakeholders chaired by ECVAM. The legislation is reinforced by an immediate testing ban for finished products and for all the human health effects, for which alternative methods have been validated by ECVAM. Furthermore, a testing ban and a marketing ban, which cannot be postponed, apply in 6 years for topical and acute systemic toxicity, while the animal tests for all the other human health effects should be phased out in 10 years with a possible postponement by co decision procedure.

The legislation for chemicals (REACH) is only emerging. It foresees data requirements for more than 30.000 substances produced at levels above 1 ton per year. Extensive in vivo data requirements are expected for a core of about 6.000 substances with highest production and concern. Alternative methods shall first be considered throughout the testing and be predominantly used for the largest group of chemicals, namely those produced between 1-10 tons per year. A Commission proposal as to the legislation has been handed to the European Parliament and Council, and is currently under consultation.

ECVAM has restructured its services directly targeting the animal tests to be replaced by in vitro and in silico approaches. Given the short time-lines to make available and implement validated methods, ECVAM is offering to steer the process of making validated alternatives available by bundling the inputs of stakeholders and by involving regulators in the process at an early stage. Steering groups composed of ECVAM's senior staff and complemented by external experts carry out the project management, which co-ordinates the various inputs. The collaboration with various stakeholders has also been reinforced. ECVAM is promoting the concept of an evidence-based toxicology, which aims to quality control toxicological tests in a structured manner. A series of activities including the initiation of and participation in research projects involving 300 partners and more than 80 million € of funding have put the tailored development and validation of alternative methods on a new scale.

The activities of ECVAM are embedded in the European Commission Action Plan for Animal Welfare published in January 2006. Furthermore, the EU is currently revising their animal welfare directive. The ECVAM activities are also closely linked to the European Partnership for Alternative Approaches to Animal Testing (EPAA), which was initiated at the 2005 Conference "Europe goes alternative".

# **Special Session 1**

**“Expected Roles of JaCVAM”**

## **T-1 The Evaluation of Alternative Test Methods by ICCVAM—Current Practices and Activities**

### **Leonard M. Schechtman**

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ICCVAM (the Interagency Coordinating Committee on the Evaluation of Alternative Methods), which began as an *ad hoc* committee in 1994, evolved into a permanent committee in December, 2000 as a result of Congressional passage of the ICCVAM Authorization Act of 2000. ICCVAM is comprised of 15 US Federal regulatory and research agencies and is scientifically and administratively supported by NICEATM (the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods). ICCVAM's purpose is to ensure that new/revised/alternative test methods are scientifically validated to meet agency needs, to increase efficiency and effectiveness of agency test method review, and to reduce, refine, or replace the use of animals in testing, where scientifically feasible. It does this by considering nominations/submissions from agencies and the public, reviewing and evaluating new, revised, and alternative test methods with potential regulatory use, coordinating technical reviews, forwarding test recommendations to Federal agencies, facilitating/providing guidance on various aspects of test method development, validation, acceptance, implementation and awareness of scientifically validated test methods, and interagency and international harmonization of those test methods. SACATM (the Scientific Advisory Committee on Alternative Toxicological Methods) was chartered in 2002 to provide advice to ICCVAM/NICEATM on its scientific activities. ICCVAM guidelines have been published to assist test method developers, individuals, organizations and end-users in the nomination, submission, validation and compilation of information for regulatory consideration of alternative methods; these guidelines and processes will be described. Details of the ICCVAM operating procedures, validation and regulatory acceptance processes and criteria will be reviewed and a summary of recent test method validation efforts and international collaboration activities will be discussed.

## **T-2 Development of alternative assay systems in the NEDO project**

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In developing toxicity safety tests for the vast number of existing chemicals which have unknown toxicity, the establishment of high through-put *in vitro* systems as alternatives to traditional animal tests is a very important and urgent issue for the protection of human health, the environment and animal welfare.

To address this problem, a new project for development of alternative assay systems sponsored by the New Energy and Industrial Technology Development Organization (NEDO) under the Ministry of Economy, Trade and Industry (METI) was initiated this year. In this project, high through-put *in vitro* test systems will be developed by collaborative studies with 8 institutions as alternatives to endpoints of carcinogenicity, teratogenicity and immunotoxicity. The concept is to develop sensitive, simple and mechanism-based assay systems. The outline of this project will be presented in the meeting.

## T-3 JaCVAM topics

### Hajime Kojima, Ph.D.

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JaCVAM (Japanese Center for the Validation of Alternative Methods) was established in the Division of Pharmacology, Biological Safety Research Center, NIHS (National Institutes of Health Sciences) since a year, November 2005. The missions of this center are to perform research and develop new in vitro test methods, to use professional standards for the validation and evaluation of new alternative methods and to act internationally.

Within just one year, JaCVAM has been Supported by the Health and Labour Sciences Research Grants and continues to perform various collaboration with the Japanese Society for Alternatives to Animal Experiments (JSAAE), the Japanese Environmental Society, Mammalian Mutagen Study (JEMS-MMS), the Japanese Society for Dermatoallergology and Contact Dermatitis (JSDC), ICCVAM (Interagency Coordinating Committee on the Validation of Alternative Methods), ECVAM (European Center for the validation of Alternative Methods), Japanese Cosmetic Industry Association (JCIA) and many researchers in various companies.

In this presentation, I summarize the details, status and results of several validation assays and peer review as follows.

1. Non-RI LLNA (LLNA DA and LLNA-BrdU) -Validation study is currently on going.
2. In vitro sensitization test (h-CLAT)
  - Pre-validation study with several cosmetic companies is in progress.
3. In vitro corrosive test using 3-dimensional skin models -Peer review process is finished.
4. In vitro skin irritation test using 3-dimensional skin models -Peer review is expected soon.
5. In vitro photoirritation test (Shiseido method) -Catch-up validation is finished.
6. Endocrine disrupter screening
  - 1) HeLa reporter gene assay -Assist in the draft report of validation study
  - 2) Lumi cell assay -Phase I in the International validation study
7. Comet assay -Development of protocol in the validation management team
8. Human patch test -Planning of improvement in JSDC

## **Symposium 2**

**“Feasibilities of New Technologies  
for the Development of Alternative Tests”**

## **S2-1 Development of culture models for extrapolating the cell response *in vivo***

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### **【Objective】**

To create new culture models for extrapolating the cell response *in vivo*, we attempted to devise culture substrata of anchorage-dependent cells.

### **【Materials and Methods】**

Two different substrata, TOSHI (tissue/organ sections for histopathology)-substratum conserving both microarchitecture and components *in vivo*<sup>1)</sup> and collagen vitrigel membrane-substratum with excellent strength and transparency<sup>1)</sup>, were developed and utilized for the culture of various anchorage-dependent cells.

### **【Results and Discussion】**

TOSHI-substratum prepared from placenta induced unique cell behaviors to form a capillary network-like structure for CPAE cells (bovine pulmonary artery endothelia), and a neuronal network-like structure for PC-12 cells (rat pheochromocytoma cell line). Also, the substratum from regenerating liver efficiently induced the differentiation of mouse ES cells into hepatocyte-like cells. These data suggest that the analysis of interactions between different cell types and various TOSHI-substrata will play an important role for a novel approach to study both cellomics and histomics. Meanwhile, the collagen vitrigel membrane-substratum enabled the double surface-culture of different cells by the manipulation of two-dimensional cultures and consequently it resulted in reconstructing a three-dimensional organoid. An intestinal epithelial-mesenchymal model was reconstructed by co-culturing fibroblasts on the opposite side of the monolayered Caco-2 (human colon carcinoma cell line) cells on the substratum. Also, the substratum was useful for not only maintaining the function but also cryo-preservation of rat primary hepatocytes. These data suggest the vitrigel membrane-substratum has many advantages to reconstruct culture models.

### **【References】**

1. Takezawa T, et al. FASEB J. 16: 1847-1849, 2002.
2. Takezawa T, et al. Cell Transplant. 13: 463-473, 2004.

## S2-2 Micropatterning of Cell Spheroids with Tissue Like Function

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### 【Objectice(s)】

Micropatterned PEGylated substrates with two-dimensional arrays circular domains ( $\phi$ 100- $\mu$ m) were prepared by coating of hydrophilic poly(ethylene glycol) (PEG) on the substrate surface, followed by patterning through a mask pattern with circular holes. The PEGylation was studied using several methods such as self-assembled monolayer of mercapto-functionalized PEG, PEG based block and graft copolymer coating, and PEG-type gelation. The PEGylated region on the patterned substrate works to repel proteins, consequently, inhibits cell adhesion. Then the micropatterning of bovine articular chondrocytes or rat primary hepatocytes hetero-spheroids underlaid with human umbilical endothelial cells (HUVEC) was achieved on the plasma-etched circular domains, exposing the base gold surface. These arrayed spheroids was characterized for tissue and cell-based biosensors (TBB/CBB) as well as tissue engineering technologies.

### 【Materials and Methods】

PEGylated surfaces with micro-pattern were constructed by plasma etching (N<sub>2</sub>+H<sub>2</sub>) or photolithography through a mask pattern with  $\phi$ :100 $\mu$ m circular holes. After sterilization by ethylene oxide gas, HUVECs were cultured on micro-patterned PEG surface. Bovine articular chondrocytes or rat primary hepatocytes were seeded on the surface 1 day after culturing HUVEC.

### 【Results and Discussion】

Microfabrication of cell adhesion controllable surface was achieved using PEG brushed layer for the cyto-phobic region. Formed chondrocytes or hepatocytes hetero-spheroid array showed long term maintenance, depending on PEG chain density of the surface. The formation of hetero-spheroid is significantly modulated by surface properties, particularly non-fouling character of PEG region. These spheroids thus obtained may have high utility for the biomedical applications such as biosensors or tissue engineering.

### 【References】

1. Otsuka, H. et. al; Two-dimensional Multi-array Formation of Hepatocyte Spheroids on a Micro-fabricated PEG-brush Surface., *ChemBioChem*, 2004, 6, 850.
2. Otsuka, H.et. al; Characterization of Aldehyde-PEG Tethered Surfaces: Influence of PEG Chain Length on the Specific Biorecognition., *Langmuir* 2004, 20(26), 11285-11287.

## **S2-3 Functional analysis of hepatocytes differentiated from human mesenchymal stem cells**

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Mouse embryonic stem (ES) cells are capable of differentiating into any adult animal cell type. Recently, we have identified essential growth factors that allow direct hepatic fate-specification from mouse, rat and monkey ES cells using simple adherent monoculture conditions (Teratani T et al. 2005; Yamamoto Y et al. 2005) based on *in vivo* differentiation of hepatocytes in mice with liver injury (Yamamoto H et al. 2003). ES-cell-derived hepatocytes exhibited liver-specific characteristics including several metabolic activities. Thus, our novel *in vitro* culture system will help elucidate the precise molecular mechanisms of hepatic commitment. Furthermore, transplantation of ES-cell-derived hepatocytes showed significant therapeutic effects in mice with liver cirrhosis. What we have observed in ES cells, new method was established which allows direct differentiation of human mesenchymal stem cells (hMSCs) into functional hepatocytes *in vitro*. The ability to efficiently produce human hepatocytes from hMSCs is exciting, because such cells could potentially be used for hepatocyte transplantation, gene therapy, the generation of artificial liver devices, or even tissue engineering. Most importantly, these hMSC-derived human hepatocytes will allow screening of potential drug candidates by characterizing the metabolism and toxicology.

### **【References】**

Yamamoto H et al. Hepatology, 2003

Ochiya T, Pediatric Gastroenterology, Hepatology and Nutrition, 2004

Teratani T et al. Hepatology, 2005

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Banas A et al. Adv. Exp. Med. Mol. Biol., 2006

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## S2-4 *In vitro* and *in silico* phospholipidosis assay: utilization in the early stage of drug discovery

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Progress of drug discovery research, basically pharmacogenomics, and introduction of technologies for combinatorial chemistry have led to a large number of innovations in drug safety evaluation strategies; that is, the safety evaluation process has been vigorously incorporated into the screening cascade at early stages of drug discovery with the concept of High Throughput Toxicology (HTP-Tox) applied in developmental strategies. Accordingly, to perform safety evaluation for various kinds of small-lot compounds in a short period of time, technologies focusing on *in silico* and *in vitro* evaluation systems have been introduced and developed energetically. Development of *in vitro* screening systems has so far emphasized HTP Tox and has been facilitated to collect single endpoint information in a short period of time for a large number of compounds. This has enabled highly efficient evaluation of one aspect of toxicity profiles for a specific group of compounds. In addition, to perform good quality evaluation in consideration of a complicated toxicity mechanism, it is necessary to implement a multidimensional examination that concurrently detects several parameters within cells, and to establish a system that elucidates its mechanistic toxicity. In short, introduction of *in silico* evaluation systems linked to *in vitro* evaluation systems has been increasingly expected.

In this lecture, I would like to focus on phospholipidosis, with actual examples of *in silico* and *in vitro* safety evaluation systems at early stages of drug discovery.

Phospholipidosis is a disorder induced by metabolic disorders etc. of phospholipids in which excess phospholipids accumulate in various organs. The lung or liver is the main target organ for drug treatment, but phospholipidosis may also occur in various tissues and organs such as the adrenal gland, lymphocyte, pancreas and lens. In microscopic examination, multiple lamellar bodies are observed in the cytoplasm. In many cases, drug-induced phospholipidosis is observed for amphiphilic compounds, but it is mainly a secretory function disorder of phospholipids, chiefly induced by phospholipid metabolic abnormality. As there are cases in which phospholipidosis may become a great impediment to further drug development, it is of great significance to evaluate the possibility of phospholipidosis at early stages of drug discovery. In this sense, development and introduction of *in silico* and *in vitro* evaluation systems are highly useful tools in drug discovery, and are expected to contribute to risk evaluation, ranking, selection or optimization (design of synthetic compounds based on the mechanistic toxicity correlation), etc. of candidate compounds.

### **【References】**

Tomizawa, K., Sugano, K., Yamada, H. and Horii, I.: Physicochemical and cell-based approach for early screening of phospholipidosis-inducing potential. *J. Toxicol. Sci.*, in press.

## S2-5 Quantitative prediction of pharmacokinetic alterations caused by drug-drug interactions using a computer program

\*Yoshihisa Shitara<sup>1</sup>, Motohiro Kato<sup>2</sup>, Masato Kitajima<sup>3</sup>, Jose M. Ciloy<sup>3</sup>, Masaru Hirano<sup>4</sup>, Kunihiro Yoshisue<sup>4</sup>, Toshihiko Ikeda<sup>5</sup>, Satoshi Suzuki<sup>6</sup>, Toshiharu Horie<sup>1</sup> and Yuichi Sugiyama<sup>4</sup>

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Most drugs are cleared from the body by P450-mediated metabolism and transporter-mediated hepatobiliary elimination. In clinical situations, a number of drugs are often administered concomitantly. A coadministered drug (inhibitor) often inhibits the metabolism of another drug (substrate), resulting in a pharmacokinetic drug-drug interaction. Such interactions sometimes cause severe side effects. To avoid them, it is essential to develop an accurate method to predict the extent of pharmacokinetic interactions. The ratio of unbound inhibitor concentration ( $I_u$ ) to the inhibition constant ( $K_i$ ) is used as an index for the prediction of drug-drug interactions. The maximum unbound concentration of inhibitors in the circulating blood or at the inlet to the liver is used to make predictions with a low risk of “false negatives”. However, this method often produces “false positive” predictions. To avoid this, we attempted to predict the pharmacokinetic alterations caused by drug-drug interactions using a simulation analysis.

In the present study, we calculated the pharmacokinetic parameters of inhibitor and substrate drugs by fitting their plasma concentration-time profiles to a physiologically-based pharmacokinetic model and constructed an appropriate database. Plasma concentrations of substrate drugs coadministered with inhibitor drugs were simulated to quantitatively predict the extent of drug-drug interactions. However, the simulation analyses using  $K_i$  values reported in *in vitro* studies often lead to overestimations. Thus, we calculated the optimal  $K_i$  values for the prediction of drug-drug interactions by fitting the plasma concentration-time profiles of substrate drugs with coadministration of inhibitor drugs to a physiologically-based pharmacokinetic model and used these  $K_i$  values (*in vivo*  $K_i$  values). This allowed the degree of drug-drug interactions to be predicted accurately. We will show the recent progress in the presentation.

### [References]

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- Kato M, Tachibana T, Ito K, Sugiyama Y: Evaluation of methods for predicting drug-drug interactions by Monte Carlo simulation. *Drug Metab. Pharmacokinet.* 18:121-7 (2003)

# **Luncheon Symposium**

## **LS Development of nanobiodevices for alternative to animal experiments and their applications to evaluating and developing ‘life-care products’**

### **Ishikawa Sunrise Industries Creation Organization**

Topics and abstracts in this seminar are shown as follows.

#### **Development of cell-based biochips for alternative to animal experiments**

Eiichi Tamiya (Japan Advanced Institute of Science and Technology)

Biosensor and microchip technology has applied to development of alternative methods for animal experiments. Cell-based biosensors has consisted of microreactors for cell cultivation and detectors for bioassay. Mast cell and fluorescent assay were combined on microchips for detection of allergy response. We also developed melanoma cell-based chip for convenient melanin production assay for screening new cosmetics materials.

#### **Application of endoplasmic reticulum (ER) stress regulation to evaluating and developing ‘life-care products’**

Osamu Hori (Faculty of Medicine, Univ. Kanazawa)

It is well known that enhanced oxidant stress is involved in many pathologic conditions. Recently, however, another type of stress, endoplasmic reticulum (ER) stress has also been reported to be associated with different types of diseases. ER stress is characterized by accumulation of unfolded proteins in the ER that is caused by disturbance of the ER function. We recently reported that targeting disruption of Herp, a novel ER stress-related gene, caused F9 cells vulnerable to ER stress. Using these cells and other types of cells, we have searched compounds regulating ER stress. We have found some of these compounds could be candidates of novel ‘life-care products’.

#### **Development of alternative methods to animal experiments for screening immunopotentiators and bioactive natural products**

Fumihide Takano (Faculty of Pharmaceutical Sciences, Univ. Kanazawa)

# **Poster Presentation**

## **P-01 Effects of Ginnema (GIM) on Rat Embryos Cultured *In Vitro***

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### **【Objectives】**

We have developed the screening system for drugs and chemical compounds in foods by the use of rat whole embryo culture. The advantages of whole embryo cultures are to examine the direct effects of drugs and chemicals on embryos and also to find the non-teratogenic agents in their analog compounds.

### **【Materials and Methods】**

As the testing agent, ginnema (GIM) was examined in the present study using the cultures of the rat embryos of day 11 to day 13 gestation. Rat embryos were on day 11 (plug day = 0) of gestation were cultured in vials (25ml) containing 5ml of rat serum under 95% O<sub>2</sub> and 5% CO<sub>2</sub> using improved rotator for 48hrs.

### **【Results and Discussion】**

The group of embryos treated with GIM was not changed in the heart beat, the crown-rump length, the embryo weight and the embryonic total somites from control embryos cultured without GIM. The malformation was also not observed in the embryos cultured with GIM.

## **P-02 Effects of Dvil Crow (DC) on Rat Embryos Cultured *In Vitro***

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### **【Objectives】**

The food additives and supplements to excrete efficiently the environmental hormones to the outside of the body are recommended to take for keeping our health.

### **【Materials and Methods】**

As the testing agent, devil crow (DC) was examined in the present study using the rat embryo cultured of day 11 to day 13 gestation. Rat embryos on day 11 (plug day = 0) of gestation were cultured in vials (25ml) containing 5 ml of rat serum with 95% O<sub>2</sub> and 5% CO<sub>2</sub> using improved rotator for 48 hrs.

### **【Results and Discussion】**

The group of treated embryos of DC was not exchanged in the heart beat, the crown-rump length, the embryo weight and the embryonic total somites. On the other hand, the malformation (short or curly tail) was observed in the embryos cultured with DC at a highest concentration of 500 µg/ml. The results obtained in the present study suggest that the safety concentration of DC used as an inhibitor of embryonic toxicity of environmental hormone may be less than 100 µg/ml.

## **P-03 Effects of Torikoenol (TRE) on Rat Embryos Cultured *In Vitro***

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### **【Objectives】**

In the United States, much amounts of food supplements are extensively used as their health. In the present study, the information on the safety of such supplements was collected by using the whole cultured system.

### **【Materials and Methods】**

As the testing agent, trikoenol (TRE) was examined in the present study using the cultures rat embryos of day 11 to day 13 gestation. Rat embryos were explanted on day 11 (plug day = 0) of gestation were cultured in vials(25ml) containing 5ml of rat serum under 95% O<sub>2</sub> and 5% CO<sub>2</sub> using improved rotator for 48 hrs.

### **【Results and Discussion】**

The group of embryos treated with TRE was not changed in the heart beat, the crown-rump length, the embryo weight and the embryonic total somites from control embryos cultured without TRE. The malformation was also not observed in the embryos cultured with TRE, although the number of somites in treated embryos was one somite less than that of the control embryos.

## **P-04 Development of *in vitro* embryotoxicity testing by differentiation of ES cells using glass fiber material**

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### **【Objective】**

We have proposed the embryotoxicity testing of mouse ES cells embedded in a collagen gel matrix<sup>1-3)</sup>. The rate of differentiation of ES-D3 cells into cardiomyocytes was measured using a glass fiber as a substitute material for collagen gel, in order to design a test method for embryotoxicity under an exposure condition where broader applications would be possible in various fields using a glass fiber as a non-animal-derived core material, making this procedure an alternative to an animal experiment.

### **【Materials and Methods】**

ES-D3 cells were maintained in DMEM supplemented with 20v/v% FCS, glutamine, penicillin, streptomycin, NAA and  $\beta$ -mercaptoethanol. ES-D3 cell suspension ( $3.75 \times 10^4$  cells/ml) was cultured by "hanging drop culture" for three days in a CO<sub>2</sub> incubator. Embryoid bodies (EBs) were transferred to a bacterial petri dish, then cultured for two days. EB was poured onto the collagen gel matrix (Nitta Gelatin, Japan) or glass fiber (Nippon Sheet Glass, Japan) based on three-dimensional cytotoxicity methods. Differentiation into contracting myocardial cell was determined by light microscopy after a further 5 days of culture. The percentage of wells of each plate in which contracting cardiomyocytes had developed was determined and compared to the percentage of wells in which cardiomyocytes developed on the negative control plate.

### **【Results and Discussion】**

Eighteen of 22 wells contained contracting cells, and the differentiation rate of the wells was 80.0% with glass fiber. There was no significant difference found with glass fiber, showing 81.0% in the cell differentiation rate of collagen gel matrix. Therefore, it was suggested that glass fiber could be used as the embedding material for embryotoxicity test.

### **【References】**

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- 2) Imai K et al., *In vitro* embryotoxicity testing of metals for dental use by differentiation of embryonic stem cell test. *Congenit Anom*, 2006;**46**:34-38.
- 3) Imai K et al., Use of an alternative for whole animal experiments: determining embryotoxicity of dental biomaterials by differentiation of ES cells. *Folia Pharmacol.Jpn.* 2005;**125**:335-342.

## **P-05 Anxiolytic effects of kava (*Piper methysticum*) in mice**

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**BACKGROUND:** Kava has been used for centuries by Pacific Islanders for its tranquilizing and sedative effects. Recent clinical trials suggest that kava has therapeutic value for the treatment of anxiety. Demonstration of kava's anxiolytic effects in animals under controlled conditions would provide additional support for its clinical potential as an anxiolytic and would facilitate investigation of its mechanism(s) of action.

**AIM:** This study systematically characterized the acute dosage-dependent anxiolytic and sedative effects of kava extract in well established quantitative murine behavioral assays and compared kava-and diazepam-induced behavioral changes.

**METHODS:** Various doses of an ethanolic extract of kava root or diazepam were administered intraperitoneally to BALB/cByJ inbred mice. Behavioral changes were measured in the mirrored chamber avoidance assay and elevated plus-maze assay. Reduced latency to enter and increased time spent in a normally avoided environment operationally defined anxiolysis. Sedation was defined by a significant decrease in locomotor activity in a circular arena.

**RESULTS:** Kava extract produced statistically significant dose-dependent anxiolytic-like behavioral changes in both assays of anxiolysis. ED(50) values for kava-induced increases in time spent inside the mirrored chamber and on the open arms of the plus maze were 120 mg/kg and 90 mg/kg, respectively. Kava extract also caused a profound decrease in locomotor activity (ED(50) of 160 mg/kg). Flumazenil, a competitive benzodiazepine receptor antagonist, blocked both the anxiolytic and sedative effects of diazepam, but had no effect on kava's behavioral actions.

**CONCLUSIONS:** Kava extracts produce significant murine anxiolytic-like behavioral changes and sedation that are not mediated through the benzodiazepine binding site on the GABA(A) receptor complex.

## **P-06 A Study of alternative method for the eye irritation test with reconstruction of multilayer system of rabbit corneal epithelial cells in vitro**

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### **【Objectives】**

We attempted to reconstruct the multilayer system of rabbit corneal epithelial cells using pig amnion as a basement membrane in vitro, and examined the possibility of this system being used as an alternative method for a corneal epithelial wound healing test, one kind of eye irritation test.

### **【Materials and Methods】**

The corneal explants separated from JW rabbit eyeballs were cultured on the collagen gel at 37 degrees centigrade with 5% CO<sub>2</sub>. Corneal epithelial cells proliferated from explants were collected and cultivated onto the pig amnion developed on the bottom of a culture insert adapted for 6-well type culture plates with 3T3 as the feeder layer. One week later, the cells were exposed to an air-liquid interface (air-lifted), and maintained the condition for 2-3 days, and promoted multi-layer construction of epithelial cells.

To make a model with an absence of epithelial cells, a sponge column of 8mm diameter with 1N NaOH was set on the center of the insert. After taking off the sponge column, the insert was washed with the culture medium, and maintained air-lifted culture with the medium containing the test articles. Benzalkonium chloride and sodium lauryl sulfate were used as test articles.

### **【Results and Discussion】**

A quantitative epithelial cell defect model with no damage to amnion was performed with sponge columns and 1N NaOH. The healing process was observed as the development of epithelial cells from a residual cell layer when it was cultivated with the medium without test articles. After 3 days, the defect disappeared completely. The addition of Benzalkonium chloride to the medium resulted in concentration-dependent inhibition of defect healing in the conc. from 10<sup>-4</sup> to 10<sup>-2</sup> %. The addition of sodium lauryl sulfate to the medium resulted in concentration-dependent inhibition of defect healing in the conc. from 10<sup>-2</sup> to 10<sup>-0</sup> %.

This method using amnion as basement membrane is an alternative to the total biological reaction on the corneal epithelium, because it is a reconstruction of the multilayer system of corneal epithelium in vitro. One hundred assay sheets can be prepared from one rabbit, so it achieves contemporaneous assay systems for multiple test compounds, and also it contributes to the reduction in the number of animals used in eye irritation tests.

## **P-07 Usefulness and Inter-Laboratory Study of Short Time Exposure (STE) Method for Evaluation of Eye Irritation Potential using 51 chemicals.**

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### **【Objectives】**

There is a greater urgency to develop non-animal safety tests in view of the forthcoming EU ban on in vivo testing. For the evaluation of eye irritation, some in vitro assays are used, but none are accepted as an OECD test. Previously we reported a good predictive capacity for eye irritation of personal care and household products using Short Time Exposure (STE) method<sup>1)</sup>. STE method involved assessing cytotoxicity with SIRC (rabbit corneal cell line) cells following a one 5 minute dose treatment. In the current study, the aim was to confirm the predictive capacity of the STE method for assessing eye irritation of ingredients (51 chemicals). As a second goal, we evaluated the transferability and reproducibility of the STE protocol in two laboratories.

### **【Materials and Methods】**

Materials: 31 cosmetic ingredients<sup>2)</sup> and 20 chemicals<sup>3)</sup> in the ECETOC database

Methods: SIRC cells were seeded in 96 hole microplates and treated with diluted test material for five minutes. Next, cell survival rate (%) was calculated by the MTT method.

Comparison with Draize: The results of the 51 chemicals were analyzed. Chemicals with a MAS  $\geq$  15 for Draize test or cell viability  $\leq$  70% for STE method were classified as irritants. Those chemicals outside of these parameters were classified as non-irritants.

### **【Results and Discussion】**

The accuracy of this classification for the 51 chemicals was about 78%. For our second goal, an inter-laboratory study was conducted with 31 cosmetic ingredients to confirm inter-laboratory reproducibility using same protocol, treatment dose, and cell lot in two laboratories. The two laboratories obtained identical data classifications from our STE method. These data suggest that the STE method has a good predictive capacity, easy transferability, and good reproducibility. The advantage of using this method as an in vitro eye irritation test is that it is an easy (5 min., one-dose) and fast method to use and it has the ability to evaluate eye irritation potential of both products and ingredients.

### **【References】**

- 1) Ito et al., (2001). *The Toxicologist* 75, abstract #476
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## **P-08 Species differences of skin permeation and in vitro permeation using human nail plate**

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### **【Objectice(s)】**

For drugs or cosmetics used by topical application, it is important to obtain quantitative information in process of a stratum corneum of skins to circulating blood. The data of skin permeation in rats or dogs could be obtained by in vivo experiments, but it is not easy to estimate the permeation in humans. In this study, species differences of skin permeation in humans and animals were investigated using antifungals, anti-inflammatory drugs and steroidal agents. In addition, the permeations of these drugs using human nail plates were investigated.

### **【Materials and Methods】**

[<sup>3</sup>H]Ketoconazole, [<sup>3</sup>H]Clotrimazole, [<sup>3</sup>H]Ketoprofen, [<sup>3</sup>H]Flurbiprofen and [<sup>3</sup>H]Estradiol were used in this study. We used a Franz-type diffusion cell for permeation experiments, adjusting the cell for the test of permeation into the human nail plates. Human skins were purchased by KAC Co., Ltd. and human nail plates were supplied by Human & Animal Bridging Research Organization.

The skins and nail plates were attached to the cells and the drugs were applied to the skins or nail plates. The cells were set in a CO<sub>2</sub>-incubator to keep the skin surface or nail plate temperature at approximately 32°C. An aliquot of the receptor fluid was taken at 0.5, 1, 2, 4, 6, 8, 10 and 24 hours in skin permeation test or 24-hour interval to 168 hours in nail permeation test after application and the radioactivity was measured. The stratum corneum was separated from the epidermis and the data was incorporated into a mass balance.

### **【Results and Discussion】**

The flux constant (ng/cm<sup>2</sup>/h) was calculated using the linearity of time-penetration curve and the permeability coefficient and cumulative absorption amount were calculated.

### **【References】**

1) Y. Kobayashi, T. Komatsu, M. Sumi, S. Numajiri, M. Miyamoto, D. Kobayashi, K. Sugibayashi, Y. Morimoto, 2004. In vitro permeation of several drugs through the human nail plate: relationship between physicochemical properties and nail permeability of drugs. *Eur. J. Pharmaceutical Sci.* 21, 471-477

## P-09 Evaluation of skin irritation using cultured cells

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### 【Objective(s)】

MTT assay has been accepted as a useful alternative to evaluate skin irritation. It may be better than Draize test and patch test from a point of quantitative determination. Toxicokinetics (TK) and toxicodynamics (TD) and their relationship are paid attention for evaluation of topical skin irritation of drug and cosmetic formulations, and therefore percutaneous absorption and skin concentration are very important to determine the skin irritation. We then started to study the TK/TD of a model irritant, cetyl pyridinium chloride (CPC) as a cationic surfactant<sup>1</sup>. Skin irritation of CPC was well correlated to its skin concentration, and their relation could be expressed by the Emax model. In the present study, butyl pyridinium chloride (BPC) and dedecyl pyridinium chloride (DPC) were also selected, and the effect of length of side chain of the cationic surfactant was evaluated from a point of TK/TD.

### 【Materials and Methods】

BPC, CPC and DPC were obtained from Tokyo Kasei Kogyo Co., Ltd. Three dimensional cultured human skin (LSE- high, Toyobo), cultured human dermal model (HDF, Toyobo) and Hos:Hr-1 hairless rat and rabbit fibroblasts (HMDF, prepared in our laboratory and RDF, Dainippon Pharmaceutical Co., Ltd.) were used for *in vitro* skin permeation experiments and male Hos:Hr-1 hairless rat and Hartley guinea pig were used for *in vivo* tests. Skin irritation was evaluated by MTT assay, and the results were analyzed by Emax model.

### 【Results and Discussion】

Irritation on the LSE-high or hairless mouse skin was similar to that on the HDF and HMDF, respectively, suggesting that HDF and HMDF are useful alternatives to evaluate the skin irritation. Evaluation on the effect of side chain length of pyridinium chlorides showed that the skin irritation was closely related with skin concentration of the surfactant as well as the irritation activity. In conclusion, the *in vitro* test using HDF must be a great tool to evaluate the *in vivo* human skin irritation.

### 【Reference】

1) Kano S, Sugibayashi K, Kinetic analysis on the skin disposition of cytotoxicity as an index of skin irritation produced by cetylpyridinium chloride: comparison of *in vitro* data using a three-dimensional cultured human skin model with *in vivo* results in hairless mice., *Pharm Res.*, 23(2) 329-35 (2006).

## **P-10 Basic feature analysis of human 3-dimensional keratinocyte and melanocyte co-cultured epidermal model (LabCyte MELANO-MODEL)**

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### **【Objective】**

A study related to melanocyte is performed generally in development of whitening agent, basic medical studies concerning skin pigmentation etc. Testing for human and animal *in vivo*, and melanocyte mono-layered culture *in vitro* have been practically used for these studies. In these testing methods, tested materials are limited only to water-soluble reagents and skin cutaneous absorbency for *in vitro* study is not considered because of no epidermal keratinocytes. Moreover for *in vivo* research, high costs and time-consuming, individuals variability, and an ethical problem are the obstacles for these studies. 3-dimensional human reconstructed epidermal model that resembles to normal human epidermis has been commercially available, and its utility is gradually recognized. We developed 3-dimensional human keratinocyte and melanocyte co-cultured epidermal model [LabCyte MELANO-MODEL] applying the manufacturing method of [LabCyte EPI MODEL]. In this report, we evaluated the basic feature of this model.

### **【Materials and Methods】**

[Model fabricating] Both human keratinocyte and melanocyte were seeded into cell culture insert at the constant ratio and co-cultured at air- liquid interface for two weeks. And then, this tissue was cultured with the medium promoting pigmentation includes factors related to melanogenesis and melanin production.

[Evaluation for pigmentation] Measuring melanin content and morphological observations (macro-and microscopic observation and light microscopic observation of hematoxylin and eosin stained paraffin section) were performed at each cultivation time to evaluate its ability of pigmentation.

[Inhibition of pigmentation] Kojic acid which was general whitening reagent was applied on the surface of the model and evaluated its inhibition effect to give pigmentation of the model by melanin content and morphological observation (macro- and microscopically).

### **【Results and Discussion】**

After dissemination, LabCyte MELANO-MODEL made typical epidermic tissue structures including melanocyte in its viable cell layer in two weeks cultivation. The number of the melanocyte and melanin content was increased successively over a certain period of cultivation time using medium promoting pigmentation. And pigmentation of the model surface was macroscopically recognized as a dark color. Furthermore, it was confirmed that topical application of Kojic acid, tyrosinase inhibitor, to the surface of co-culture model decreased the number of the melanocyte and the melanin content.

These results suggest that the melanocyte of LabCyte MELANO-MODEL maintained its ability similar to that of normal tissue. In conclusion, LabCyte MELANO-MODEL is useful means for development of whitening reagent and basic medical studies concerning skin pigmentation.

## **P-11 Assessment of the Human Epidermal Model LabCyte EPI-MODEL for the in vitro Corrosion Testing According to OECD TG431**

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### **【Objectice(s)】**

New test guidelines 431 (TG431)<sup>1)</sup> for in vitro skin corrosion testing using human reconstructed skin model was adopted by OECD at 2004. In Guideline TG431, the criteria of general function and performance of applicable skin models are defined. In order to confirm that new 3-dimensional human reconstructed epidermal model, LabCyte EPI-MODEL (Japan Tissue Engineering Co., Ltd., JAPAN) is able to apply to the skin corrosion testing according to TG431, the adequacy and reproducibility of the model for the skin corrosion testing was evaluated.

### **【Materials and Methods】**

Twelve chemicals (6 corrosive chemicals and 6 non-corrosive chemicals) that had been described at table 2 in TG431 were selected as testing samples for evaluation of LabCyte EPI-MODEL. The testing was performed according to the protocol of the description to TG431. After the appropriate incubation periods, each testing sample applied to the surface of LabCyte EPI-MODEL was completely removed from the model. Viability of cells was measured by using MTT assay to evaluate the skin corrosively of LabCyte EPI-MODEL.

### **【Results and Discussion】**

Based on the knowledge that LabCyte EPI-MODEL is a epidermal model as well as Epiderm<sup>TM</sup> (MatTek Co.), we have decided to adopt the Epiderm<sup>TM</sup> skin corrosion testing protocol for the LabCyte EPI-MODEL. As a result, the distinction of non-corrosively/corrosively was completely corresponding. Therefore, it was judged that the testing protocol of Epiderm<sup>TM</sup> was able to apply to LabCyte EPI-MODEL. The reproducibility of this testing protocol with LabCyte EPI-MODEL was examined, and found that it was recognized highly reproducibility. In addition, reproducibility was examined between laboratories, and then it was concluded that LabCyte EPI-MODEL was applicable to the skin corrosive testing protocol according to TG431.

### **【References】**

1. OECD GUIDELINE FOR THE TESTING OF CHEMICALS, No431; In Vitro Skin Corrosion: Human Skin Model Test. Adopted: 13 April 2004.

## **P-12 The correlation between apoptosis and CD86/54 expression induced by contact sensitizers on THP-1 cells**

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### **【Objective】**

To evaluate the skin sensitization potential, animal test methods such as Guinea Pig Maximization Test (GPMT) have been used. For animal welfare, the establishment of *in vitro* skin sensitization test method has been expected.

Recently, an *in vitro* method using the human monocytic leukemia cell line, *i.e.* THP-1, which exhibits the enhanced CD86/54 expression when exposed to sensitizers, has been reported.<sup>1), 2)</sup> In these methods, some allergens such as dinitrochlorobenzene (DNCB) and nickel sulfate (Ni) exhibit the remarkable enhancement of CD86/54 expression at non-toxic concentration. In contrast some non-allergens such as sodium lauryl sulfate (SLS) don't exhibit the enhanced CD86/54 expression at same condition.<sup>3)</sup>

The purpose of this study was to determine whether the CD86/54 expression and the programmed cell death, apoptosis, are correlated each other.

### **【Materials and Methods】**

THP-1 cells were monitored apoptotic changes and CD86/54 expression using flow cytometry after exposed to two kinds of known allergens, DNCB and Ni, and a non-allergen, SLS. In addition, after THP-1 cells treated with DNCB, apoptotic induction and CD86 expression measured by the double labeling method with anti-Annexin V-FITC and anti-CD86-PE monoclonal antibodies.

### **【Results and Discussion】**

Two kind of known allergens, DNCB and Ni, induced apoptosis, whereas a non-allergen, SLS, didn't induce apoptosis. THP-1 cells treated with DNCB or Ni exhibited CD86/54 expression following induction of apoptosis. Moreover, the double labeling method indicated that DNCB treated cells expressed CD86 mainly on non-apoptotic cells, but not on apoptotic cells.

### **【References】**

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## P-13 Development of alternative photosensitization assay using human monocyte-derived cells

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### 【Objective】

Evaluation of the safety of ingredients is an important part of the development of cosmetics. Recently, the development of *in vitro* tests has been further inspired by the 7<sup>th</sup> amendment of the EU Cosmetics Directive. The flow cytometric method using human monocyte-derived cells (THP-1 cells) was evaluated with promising results as *in vitro* sensitization test<sup>1, 2, 3)</sup>. Meanwhile, there are few cell-based *in vitro* photosensitization test. In this study, new *in vitro* photosensitization test system using THP-1 cells has been developed and verified.

### 【Materials and Methods】

As the light source, a solar simulator was used. The expression of CD86/CD54 in THP-1 cells was measured by flow cytometry.

- 1) Optimization of irradiation intensities and doses: THP-1 cells were irradiated with no treatment or with a photoallergen, 6-methylcoumarin, with varying intensities and doses.
- 2) Distinction between photoallergic and phototoxic potential: We evaluated the activation capacity of test chemicals with the post-irradiation method and the pre-irradiation method. In the former, cells and test chemicals were co-irradiated. In the latter, cells were treated with irradiated chemicals.
- 3) Verification of an evaluation system: We developed a decision tree on the basis of the preceding results. The usefulness of this system was verified using several test chemicals.

### 【Results and Discussion】

- 1) The irradiation caused the activation of THP-1 cells dose-dependently. Moreover, the cell toxicity was observed at high intensity. Therefore, we conclude that 1.7mW/cm<sup>2</sup> and 5J/cm<sup>2</sup> was appropriate irradiation condition.
- 2) We confirmed the augmentation of CD86/CD54 expression in THP-1 cells irradiated with phototoxins. This CD86/CD54 expression in the post-irradiation method was attributed to reactive oxygen species derived irradiated phototoxins. On the other hand, the augmentation of CD86/CD54 was less in the pre-irradiation method.
- 3) Based on post-/pre-irradiation methods and non-irradiation method, we made the decision tree, which distinguishes between photoallergens and phototoxins, and verified this system using several test chemicals. As a result, this system might be useful to evaluate the photoallergic potentials of various test materials.

### 【References】

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## **P-14 Development of 3D-culture model of THP-1 cell for evaluating insoluble test samples**

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### **【Objective(s)】**

To meet the requirements of the EU 7th Amendment to the Cosmetics Directive, The development of non-animal test is needed to ascertain the safety of cosmetic ingredients and finished products. Therefore, we have been studied and validated an *in vitro* skin sensitization test focussed on the augmentation of CD86 expression on THP-1 cell. However, this assay is not always satisfactory to evaluate a wide variety of cosmetic ingredients, because most water-insoluble chemicals cannot be applicable to the assay suitably. The aim of this study is to develop a 3D-culture model of THP-1 cell that would be able to evaluate insoluble test samples.

### **【Materials and Methods】**

THP-1 cells in "thermo-responsive gelation polymer scaffold" were treated with chemicals for 2h. These cells were further cultured in normal medium (RPMI1640) without the test sample for 22h. After washing, these cells were stained with anti-CD86-FITC monoclonal antibody. Finally, the expression of CD86 on the THP-1 cell surface was measured by using flow cytometry.

### **【Results and Discussion】**

#### Setting the protocol.

When the cells were cultured in the hydrogel for 24h, the viability was 70-80%. It suggests that the cells were damaged by the shear stress during the culture. Therefore, the incubation time in hydrogel was shortened to 2h, and the cell viability improved to over 90%. Furthermore, we confirmed that DNCB, that is typical sensitizer, showed significant augmentation of CD86 after 2h exposure.

#### Performance of the test.

The expression level of CD86 was increased by the exposure to nickel sulfate or glutaraldehyde, that is a sensitizer. On the other hand, the exposure to sodium lauryl sulfate or lactic acid, that is a non sensitizer, did not induce the CD86. These data suggest that this 3D gel model would be suitable for evaluating sensitization potential of insoluble samples. Further studies will be required to confirm an usefulness of this test method with a wide variety of chemicals and formulations.

## **P-15 Results of a Japanese ring study of a human Cell Line Activation Test (h-CLAT) for predicting skin sensitization potential (2<sup>nd</sup> Report): A study of the criteria for THP-1 cell selection.**

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### **【Objective】**

In the previous Japanese ring study, it has been reported that the inter-laboratory transferability and reproducibility of the human Cell Line Activation test (h-CLAT) protocol is basically good<sup>1</sup>. The aim of this study is to define the criteria for THP-1 cell selection for h-CLAT.

### **【Materials and Methods】**

New THP-1 cells lots were obtained from three sources: a major American, European and Japanese cell bank. The new THP-1 cells were compared to those cells used in the previous Japanese ring study (ATCC; Lot.3364116). Cellular proliferation in subculture and CD86/CD54 expression on THP-1 cells following exposure to two known allergens (dinitrochlorobenzene (DNCB) and Nickel sulfate (Ni)) and one non-allergen (Sodium lauryl sulfate (SLS)) were evaluated. Test dose for each chemical was set at the concentration giving 75% cell viability (CV75) and a concentration of 5 µg/mL of DNCB was included as a positive control.

### **【Results and Discussion】**

THP-1 cells from two of the three sources showed similar results compared with the ATCC(Lot.3364116) in cellular proliferation and CD86/CD54 augmentation induced by DNCB and Ni. Meanwhile, cells from the third source showed low cell viability, which was below 90%, in subculture and h-CLAT. Additionally, Ni did not induce CD86/CD54 augmentation on cells from the third source. These results demonstrated that the variability of cellular responses depended on the cell source (i.e., the cell banks) indicated by the results from both laboratories. Therefore, further investigations for defining the criteria for THP-1 cell selection for h-CLAT will be necessary. This study was supported by a Grant-in-aid from MHLW.

### **【References】**

1. Ashikaga, T., et al. (2005); Alternatives to Animal Testing and Experimentation vol.11, suppl. p238

## **P-16 Results of a Japanese ring study of a Human Cell Line Activation Test (h-CLAT) for predicting skin sensitization potential (3<sup>rd</sup> Report): the effect of serum difference.**

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### **【Objective(s)】**

In the previous Japanese ring study, it has been reported that the inter-laboratory transferability and reproducibility of the human Cell Line Activation test (h-CLAT) is basically good<sup>1</sup>. In the present study, with the aim of collecting background data of h-CLAT, we have examined the effect of serum difference in h-CLAT.

### **【Materials and Methods】**

Three different lots of serums, which were obtained from three sources, were compared to the serum in the previous Japanese ring study (reference), in terms of cellular proliferation in subculture, cell toxicity, and CD86/CD54 expression on THP-1 cells following exposure to two known allergens (dinitrochlorobenzene (DNCB) and Nickel sulfate (Ni)) and one non-allergen (Sodium lauryl sulfate (SLS)). Test dose for each chemical was set at the concentration giving 75% cell viability (CV75) and a concentration of 5  $\mu$ g/mL of DNCB was included as a positive control. Each lab conducted the experiments.

### **【Results and Discussion】**

Among the four serums employed in the present study, there has been observed no clear difference in terms of cellular proliferation in subculture, cell toxicity and CD86/54 expression. From these results, it was suggested that the effect of serum difference is a little or negligible in h-CLAT. However, further investigation would be required to confirm this in both labs. This study was supported by a Grant-in-aid from MHLW.

### **【Reference】**

1. Ashikaga,T., et al. (2005); Alternatives to Animal Testing and Experimentation vol.11,suppl. p238

## **P-17 Results of a Japanese ring study of a human Cell Line Activation Test (h-CLAT) for predicting skin sensitization potential (4<sup>th</sup> Report): Effects of pre-culture conditions.**

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### **【Objective(s)】**

In the previous Japanese ring study, it has been reported that the inter-laboratory transferability and reproducibility of the human Cell Line Activation test (h-CLAT) is basically good<sup>1</sup>.

h-CLAT protocol defines that the inoculation cell concentration is approximately  $0.1\sim 0.2\times 10^6$  cells/mL and the cultivation time is 48 hr or 72 hr as pre-culture conditions. In this study, we investigated the effects of pre-culture conditions on the test results collaboratively.

### **【Materials and Methods】**

Nine different pre-culture conditions were compared in terms of cellular proliferation in pre-culture, and CD86/CD54 expression on THP-1 cells following exposure to two known allergens (dinitrochlorobenzene (DNCB) and Nickel sulfate (Ni)) and one non-allergen (Sodium lauryl sulfate (SLS)). Each lab decided test dose for chemicals and conducted the experiments. Cells were seeded at  $0.1 \times 10^6$ ,  $0.2 \times 10^6$  or  $0.3 \times 10^6$  cells/mL for 24 hr, 48 hr or 72 hr as pre-culture. THP-1 cell lot and fetal bovine serum used in this study were the same ones used in the previous ring study.

### **【Results and Discussion】**

To compare the results from each pre-culture condition, all conditions show similar results in two laboratories although CD86/CD54 augmentation induced by allergens tends to be lower as the inoculation cell concentration becomes higher and cultivation time becomes longer in one laboratory. It is suggested that this inter-laboratory difference is caused by different cell growth curves. This study was supported by a Grant-in-aid from MHLW.

### **【Reference】**

1. Ashikaga, T., et al. (2005); Alternatives to Animal Testing and Experimentation vol.11, suppl. p238

## P-18 Inter-laboratory validation study on LLNA-DA

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### 【Objective(s)】

The local lymph node assay (LLNA) is a well established alternative method using mice instead of the GPMT/BT test which uses guinea pigs to assess skin sensitization. The LLNA-DA is a modification method of the LLNA, in which ATP is measured for lymphocyte proliferation instead of <sup>3</sup>H-thymidine. We conducted the first inter-laboratory catch-up validation study for the assay. The primary objectives of this study are to evaluate reliability and relevance.

### 【Materials and Methods】

The standard protocol of the LLNA-DA for this study was prepared and fixed before it began. 3 chemicals were examined by all 10 experimental laboratories while 9 chemicals were tested by each of 3 different laboratories. Chemicals with the fixed 3 doses were distributed to each laboratory coded to disguise their type. The value of 3 was set as the cut-off point of the stimulation indices (SI), which summarize the ATP amount.

### 【Results and Discussion】

The results for the 3 chemicals examined by all laboratories and 5 of the other 9 chemicals were consistent and had small variances in the SI. There were 4 chemicals which produced inconsistent results between 3 laboratories. 2 chemicals showed the clearly dose response relationships. On the other hand, for the other 2 chemicals it seemed that the type of solvent in these chemicals caused the large variations. Sensitivity, specificity and concordance of the LLNA-DA compared to the GPMT/BT were 87.5% (7/8), 100% (3/3) and 90.9% (10/11), respectively. We conclude that, considering the published data of the LLNA, the results from this study are acceptable as a catch-up validation study, at least within the range of examined chemicals.

### 【References】

Yamashita K., Idehara K., Fukuda N., Yamagishi G. and Kawada N. (2005) Development of a modified local lymph node assay using ATP measurement as an endpoint. *AATEX* **11**, 136-144.

## **P-19 Effect of test condition of *in vitro* sensitization assay using changes of cell surface thiols as a biomarker (SH-Test).**

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### **【Objectice(s)】**

In the process of allergic contact dermatitis, dendritic cells(DCs) including Langerhans cells, which have potent antigen-presenting function, play crucial roles in the induction phase of skin sensitization. For predicting the sensitization potential of chemical *in vitro*, it is one of useful steps to detect the activation of DCs and monocytic cell lines by haptens. In 19<sup>th</sup> annual meeting of JSAAE, we reported about *in vitro* sensitization test using changes of cell surface thiols as a new biomarker (SH-Test) and appropriate indicator cells for SH-Test. In this study, we investigated about effect of test condition for the purpose of optimization of SH-Test.

### **【Materials and Methods】**

THP-1 (monocytic leukemia cell line) cells were treated with sensitizers (2,4-dinitrochlorobenzene (DNCB), nickel sulfate (NiSO<sub>4</sub>), diphenylcyclopropanone (DPCP)) and non-sensitizers (sodium dodecyl sulfate (SDS), methyl salicylate (MS)) for 2 h. Cells were washed and incubated with nonpermeable thiol reactive compound Alexa fluor C<sub>5</sub> maleimide (AFM) for 30 min. After having been washed again, cells were analyzed by flow cytometer (FCM). We investigated whether SH-Test was affected by difference among cell line lots, serum lots, passage period of cells, FCMs and solvents.

### **【Results and Discussion】**

SH-Test was not affected by difference among cell line lots, serum lots, period of passage, FCMs. Furthermore, we confirmed that the changes of cell surface thiols by lipophilic haptens did not have much difference among solvents such as DMSO, ethanol, acetone and acetonitrile. Taken together, SH-Test might be a useful *in vitro* sensitization test obtaining stable results easily.

### **【References】**

1. Suzuki *et al.*, 19<sup>th</sup> annual meeting of JSAAE, p108, 2005
2. Hirota *et al.*, 19<sup>th</sup> annual meeting of JSAAE, p110, 2005
3. Sasaki *et al.*, 19<sup>th</sup> annual meeting of JSAAE, p112, 2005

## **P-20 Construction of decision tree of *in vitro* sensitization assay using changes of cell surface thiols as a biomarker (SH-Test).**

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### **【Objective(s)】**

In the induction phase of skin sensitization, dendritic cells (DC) were reported to be activated by haptens. Furthermore, the activation is accompanied by the augmentation of CD86 expression, following intracellular redox imbalance and phosphorylation of p38 as upstream signals. In 19<sup>th</sup> annual meeting of JSAAE, we presented *in vitro* sensitization test using changes of cell surface thiols as a new biomarker (SH-Test) based on the hypothesis that a change of cell-surface thiols might be one of the triggers of cell activation. In this study, we investigated the dose setting condition such as maximum concentration treated to cells and the number of concentration for construction of decision tree of SH-Test.

### **【Materials and Methods】**

THP-1 cells (monocytic leukemia cell line) were treated with sensitizers and non-sensitizers for 2 h. Cells were washed and incubated with nonpermeable thiol reactive compound Alexa fluor C<sub>5</sub> maleimide (AFM) for 30 min. After having been washed again, cells were analyzed by flow cytometry (FCM).

### **【Results and Discussion】**

To decrease false-negative in SH-Test, we investigated the effects of the maximum concentration and the number of testing concentration. When the maximum concentration was set as 40000 ug/mL (for water-soluble chemicals) and 2500 ug/mL (for water-insoluble chemicals), and the number of testing concentration was changed from 3 concentrations (serial 3-fold dilution) to 8 concentrations (serial 1.2-fold dilution), some sensitizers previously shown as false-negative, were classified as positive. When this decision tree based on these results, was applied, the accuracy of *in vitro* assay vs. *in vivo* assay was 84 %. These results suggest that SH-test may be useful for an *in vitro* sensitization assay with low false-positives.

### **【References】**

1. Suzuki *et al.*, 19<sup>th</sup> annual meeting of JSAAE, p108, 2005
2. Hirota *et al.*, 19<sup>th</sup> annual meeting of JSAAE, p110, 2005
3. Sasaki *et al.*, 19<sup>th</sup> annual meeting of JSAAE, p112, 2005

## **P-21 Current studies on alternative methods in AMOREPACIFIC Corp.**

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AMOREPACIFIC corp. (AP), a leading cosmetic company in Korea, has been making an effort to use variety of alternative methods for many years. In this report, we introduce both present state of practical use of alternative methods and ongoing efforts to modify or optimize several new methods for additional applications.

AP has been making use of alternative methods on assessing phototoxicity of fragrances, corrosivity of hair care products, and ocular irritancy of eye creams and eye makeup products. We perform the *in vitro* phototoxicity test by battery of photohemolysis and 3T3 NRU PT test. In recent, we introduced MPE value as additional criterion on the test. Corrositex<sup>(R)</sup> is used for assessing corrosiveness of strongly acidic or basic materials. We do HET-CAM for predicting ocular irritancy of many materials and products. The test also contributes to reducing the number of rabbits by defining the suitable experimental concentrations prior to Draize test.

There are also several trials on improving and optimizing current alternative methods. We have intended to improve objectivity of HET-CAM and tried modifying the protocol by introducing CAM-TBS (Trypan Blue Staining) method. The modified protocol is now under study of the suitability for applying to cosmetic products. The MPE value is introduced to existing *in vitro* phototoxicity test protocol as additional criterion and we hope to overcome some existing limitations. We also make effort to get suitable alternative methods for predicting sensitizing potency of cosmetic components and products. The one of many candidates is h-CLAT that is under examination of its accuracy.

## **P-22 Construction of three-dimensional human skin model consisting of dendritic cells and *in vitro* evaluation of immune-sensitizers**

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### **【Objectie(s)】**

Recently, to study alternative animal testing of immune-sensitizing compounds, normal human dendrite cells were used. However, these models did not have many keratinocytes that so they induced immunoreactions with much less immune-sensitizing compound than real human skin and it was difficult to apply water-insoluble samples such as creams. We reported at the 19<sup>th</sup> Annual Meeting of the JSAAE that for alternative animal testing of immune-sensitizing compounds, we constructed a three-dimensional human skin model consisting of three different cells, dendritic cells, keratinocytes and fibroblasts (KDF-Skin), and immune-sensitizing compounds induced cytokine release and the expression of CD86 of KDF-Skin. In this study, other immune-sensitizing compounds and non-sensitizers were exposed to KDF-Skin and a new three-dimensional human skin model using collagen vitrigel membrane was constructed.

### **【Materials and Methods】**

Normal human skin fibroblasts (NHSF46) were seeded in collagen gel and cultured for 7 days or seeded in collagen vitrigel membrane and cultured for 2-h. After incubation, the normal human dendrite cells (NHDC) seeded in collagen gel were put into a glass ring on collagen gel containing NHSF46. Next, human epidermoid carcinoma (A431) was seeded in the glass ring. After 2-day incubation, the surface of the KDF-Skin was exposed in air and cultured for 13 days, and then immune-sensitizing or non-sensitizing compounds were exposed for 1 h. After 24 h incubation, 10% formaline neutral buffer solution was added to KDF-Skin and then it was stained with HE and CD86. The release of IL-1 $\alpha$ , IL-2 and IL-4 was measured by the method of surface plasmon resonance.

### **【Results and Discussion】**

Due to 1 mmol/L CoCl<sub>2</sub>, 5 mmol/L cinnamaldehyde and 100 mmol/L diethanolamine, the KDF-Skin significantly released cytokine and significantly expressed CD86. On the other hand, Tween20, Tween80 and DMSO did not induce IL-1 $\alpha$ , IL-2 and IL-4 release and the expression of CD86. These results suggest that KDF-Skin should be suitable for studying the alternative animal testing of immune-sensitizing compounds, and the incubation period for construction of the new skin model was 7 days shorter than that of KDF-Skin.

## **P-23 A novel technology for differentiating embryonic stem cells into hepatocyte-like cells by utilizing the section substrata prepared from regenerating liver tissues**

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### **【Objective】**

A novel method for animal cell culture was developed by applying histopathological thin sections to the substratum<sup>1)</sup>. On the other hand, it was reported mouse embryonic stem (ES) cells differentiated into hepatocytes in host liver after administration of carbon tetrachloride (CCl<sub>4</sub>) into mice<sup>2)</sup>. Therefore, we studied whether ES cells could be induced to hepatocyte-like cells on the section substrata prepared from CCl<sub>4</sub>-administrated mouse liver tissues.

### **【Materials and Methods】**

Section substrata were prepared from livers in various stages after administrating CCl<sub>4</sub> into mice. Excised liver tissues were rapidly frozen with liquid nitrogen. The frozen livers were cut into sections with thickness of 5  $\mu$  m. Mouse ES cells suspended in a culture medium containing 20% FBS and leukemia inhibitory factor (LIF) were seeded and cultured on the section substrata for 2 hours, and subsequently the medium was changed to a fresh serum-free medium without LIF. For analyzing the attachability and growth of ES cells, the area of the regions occupied by cells on each substratum were measured by NIH Image software. To evaluate hepatic-differentiation, albumin secretion levels in culture media were analyzed.

### **【Results and Discussion】**

The substrata derived from regenerating livers enhanced cell attachment, supported growth and induced differentiation to cells expressing albumin although the substrata from injured livers did not. Morphological observation revealed that ES cells cultured for 24 hours on the substrata from injured liver showed round shape. In contrast, those on the substrata from regenerating liver did polygonal shape. In particular, some colonies on the substrata from the most proliferative liver showed the reconstruction of hepatic cord-like structure and binucleated cell was found in them. These findings demonstrated a new culture system to efficiently induce the differentiation of stem cells into hepatocyte-like cells. We hope that the technology utilizing section substrata could be applied to the preparation of tailor-made hepatocytes *in vitro* in future.

### **【References】**

- <sup>1)</sup> Takezawa, T. *et al.* Cell culture on thin tissue sections commonly prepared for histopathology. *FASEB J.* **16**, 1847-1849(2002).
- <sup>2)</sup> Yamamoto, H. *et al.* Differentiation of embryonic stem cells into hepatocytes: biological functions and therapeutic application. *Hepatology* **37**, 983-993(2003).

## **P-24 Cell behaviors of two different cell lines on the section substrata prepared from rat organs and their mathematical models**

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### **【Objective】**

Thin tissue sections spread on a glass slide were applied to the culture substrata of anchorage-dependant cells, resulting in the induction of cell behavior corresponding to the region of the tissue as previously described<sup>1,2</sup>. In this study, we prepared tissue array substrata composed of histological sections derived from rat mature organs and cultured two different cell lines (RIN5F cells of rat insulinoma and HepG2 cells of human hepatoma) to compare their behaviors on the substrata.

### **【Materials and Methods】**

For the preparation of section substrata, cerebrum, thymus, heart, liver, kidney and testis of normal adult rats were excised and rapidly frozen with liquid nitrogen. The frozen organs were cut into sections with thickness of 5  $\mu$  m. RIN5F cells and HepG2 cells were seeded on section substrata to analyze cell behavior. The cell attachment and cell growth were evaluated by measuring the area of alive cells showing calcein fluorescence after incorporating calcein-AM. To evaluate cell-specific function, insulin or albumin secretion levels in culture medium were determined.

### **【Results and Discussion】**

RIN5F cells showed high attachability onto the section substrata prepared from heart and kidney, standard growth on the substrata from heart, kidney, and liver, and high level of insulin secretion on the substratum from thymus. Whereas HepG2 cells showed low attachability onto the section substrata prepared from liver, and kidney and not only standard growth but also standard level of albumin secretion on the substrata from cerebrum, thymus, heart, liver, kidney and testis. Also, the time-course data for cell growth and secretion level of insulin or albumin on each section substratum were successfully converted into a three-dimensional graph chart, i.e. a mathematical model. In near future, the database accumulating such mathematical models will contribute to investigate the unknown characteristics of cells and/or tissues.

### **【References】**

- 1) Takezawa, T. *et al.*: *FASEB J.* **16**, 1847-1849(2002).
- 2) Takezawa, T.: *Biomaterials* **24**, 2267-2275(2003).

## P-25 The Possibility of Chemical Computation Approaches to Chemical Photosafety Evaluation

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### 【Objectice(s)】

The in silico chemical computation of energy difference between the highest occupied and the lowest unoccupied molecular orbital (HOMO-LUMO gap) is considered as a method for predicting chemicals phototoxicity potency. The purpose of this study was to evaluate its prediction through investigating the correlation among the results of HOMO-LUMO gap analysis, in vitro 3T3 neutral red uptake phototoxicity test (NRU PT) and in vivo phototoxicity test in guinea pigs.

### 【Materials and Methods】

Using the software Jaguar 5.5, the HOMO-LUMO gaps were computed for 134 in house compounds and 30 currently marketed medicines which had been known the phototoxicity or not in human. All of these compounds were tested in the in vitro 3T3 NRU PT and the data were analyzed by the software Phototox. Ver 2.0. In the in vivo phototoxicity test, guinea pigs were given single oral dose of Ciprofloxacin (CPFX, 200mg/kg), Lomefloxacin (LFLX, 200mg/kg) or 8-methoxypsolaren (8-MOP, 25mg/kg). 30min after dosing, the animals were exposed to UVA for 40 min (10J/cm<sup>2</sup>). The phototoxicity was evaluated based on the degree of erythema and edema symptoms.

### 【Results and Discussion】

When the HOMO-LUMO gap values of 134 in house compounds was divided into three areas as  $A < 10.5$ ,  $10.5 \leq B \leq 11.7$  and  $C > 11.7$ , the phototoxicity correlation rates of the compounds, which the positive results were identified by the in vitro 3T3 NRU PT, were found as A=100%, B=44%, and C=17%, respectively. Both the phototoxicity medicines which had been known in human and the phototoxicity results of CPFX, LFLX and 8-MOP from the in vivo test also showed the correlation with their HOMO-LUMO gap energy ( $< 10.5$ ). The results from this study suggested that the chemical computation of HOMO-LUMO gap energy can reduce the phototoxicity risk and increase the throughput for screening the new drug candidates at the early stage of drug discovery.

### 【References】

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## **P-26 Development of the *in silico* prediction system for the safety of chemicals (the first report) -Prediction of the skin sensitization potential and the risk assessment using local lymph node assay (LLNA)-**

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### **【Objectice(s)】**

Towards the development of a prediction system for the skin sensitizing potential of chemicals, many attempts have been made by examining the structure-activity relationships<sup>1)</sup>. However, the results were not always satisfactorily enough in terms of predictability. Following are some of the reasons: 1) Only simple parameters such as the presence/absence and number of specific atoms, bonds, or partial structures in the molecules were examined; 2) Since the toxicity of chemicals was induced by complex living mechanisms, it was difficult to simply predict with a conventional linear regression analysis model; 3) Most of the studies were focused on only the presence/absence of the skin sensitivity.

In the present study, therefore, we have attempted to develop an *in silico* skin sensitization model to predict the sensitizing potential and strength of the chemicals, by using molecular orbital calculations in combination with the artificial neural network system.

### **【Materials and Methods】**

The skin sensitivity data of 110 chemicals by LLNA assay were collected from the past publications<sup>2-9)</sup>. The structures of the chemicals were constructed by using the Chem3D Ultra Ver. 10.0 software (CambridgeSoft Co., Cambridge, MA, U.S.A.) and stabilized in their neutral forms. Molecular orbital calculations were performed by MOPAC 2002. Out of molecular orbital descriptors obtained, those considered to be independent and useful for the prediction of the skin sensitization potential and the risk assessment were selected. Then, they were correlated with the LLNA data by using the artificial neural network system. Throughout the study the QwikNet Ver.2.23 software was used for the calculations.

### **【Results and Discussion】**

A combination of molecular orbital calculations and the artificial neural network system enabled us for the prediction of the skin sensitizing potential of the chemicals and their risk assessment.

### **【References】**

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## **P-27 Development of the *in silico* prediction system for the safety of chemicals (the second report) — Prediction of the skin irritation potential and risk assessment using human patch test—**

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### **【Objectice(s)】**

Towards the development of a prediction system for the safety of chemicals, many attempts have been made by examining the quantitative structure-activity relationships. However, the results were not always satisfactorily enough in terms of predictability. Following are some of the reasons: 1) Only simple parameters such as the presence/absence and number of specific atoms, bonds, or partial structures in the molecules were examined; 2) Since the toxicity of chemicals was induced by complex living mechanisms, it was difficult to simply predict with a conventional linear regression analysis model; 3) Most of the studies were focused on only the presence or absence of the toxicity.

In the present study, therefore, we have attempted to develop an *in silico* skin irritation model to predict the irritative potential and strength of the chemicals, by using molecular orbital calculations in combination with the artificial neural network system.

### **【Materials and Methods】**

The skin irritation data of 161 compounds by human patch test were collected from the past publication<sup>1)</sup> and experiments in our laboratory. The structures of the compounds were constructed by using the Chem3D Ultra Ver. 10.0 software (CambrideSoft Co., Cambridge, MA, U.S.A.) and modeled in their neutral forms. Molecular orbital calculations were performed by MOPAC2002. Molecular orbital descriptors obtained, those considered to be independent and useful for the prediction of the skin irritation potential and risk assignment were selected. Then, they were correlated with the human patch test data by using the artificial neural network system. Throughout the study the QwikNet Ver.2.23 software was used for the calculations.

### **【Results and Discussion】**

A combination of molecular orbital calculations and the artificial neural network system enabled us for the prediction of the skin irritation potential of the chemicals and their risk assessment.

### **【References】**

1. D.A. Basketter et al., Contact Dermatitis, 51, 1-4 (2004)

## P-28 A measure for inter-laboratory variation of the stimulation index

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### 【Objective(s)】

The local lymph node assay (LLNA) is a well established alternative method using mice instead of the test using guinea pigs to assess skin sensitisation. In this method, incorporation of <sup>3</sup>H-methyl thymidine is measured by  $\beta$ -scintillation counting as disintegrations per minute (DPM). The stimulation index (SI), obtained from the mean DPM for the examined chemical group divided by the mean DPM for the solvent control group, is an endpoint for the assay to assess stimulation of examined substances. In the case of assessing an inter-laboratory variation, the variability of only the SI has, usually, been taken into account. However, since the SI is a measure calculated from each DPM, the SI also has variation and each SI varies between laboratories. Therefore, when evaluating reliability on the LLNA, we should consider the variations for each SI. We have developed an approach to evaluate the inter-laboratory variation taking variation of the SIs into account.

### 【Materials and Methods】

Let Mean(Y) and Mean(X) be the mean DPM for the examined chemical group and one for the solvent control group, respectively. SE(Y) and SE(X) are the standard error of the DPM for each group, respectively. Using these notations, the SI is shown as Mean(Y)/Mean(X), and the variance of the SI, Var(SI), is calculated as  $\exp((1/\text{Mean}(Y)^2) * \text{SE}(Y)^2 + 1/\text{Mean}(X)^2 * \text{SE}(X)^2)$  approximately by using the delta method. Next, we consider data from an inter-laboratory study. Let SI<sub>i</sub> and Var(SI)<sub>i</sub> be the SI and the variance of the SI from the i<sup>th</sup> laboratory, respectively. Under the assumption for the estimate of log(SI<sub>i</sub>) to follow N( $\theta_i$ , log(Var(SI)<sub>i</sub>)) and each  $\theta_i$  follows N( $\theta$ ,  $\tau^2$ ), we can estimate the value of the  $\tau^2$  by using the restricted maximum likelihood method.  $\exp(\tau^2)$  indicates the variance between laboratories eliminating variance within each experiment, and then we can use this as a measure of an inter-laboratory variation.

### 【Results and Discussion】

Loveless et al. reported the mean DPMs and the SEs of the DPMs for the examined substances and for these solvents from three laboratories in their study. The values of the SIs (the value of its variances) were 8.8 (1.07) in laboratory C, 12.8 (1.04) in laboratory D and 7.08 (1.21) in laboratory E for 0.1% Dinitrochlorobenzene. In this case, the  $\exp(\tau^2)$  is estimated as 1.02 while, if the variation within each experiment is not taken into account, the variance is 8.61 and it is possibility of overestimation for the inter-laboratory variation.

### 【References】

Loveless S E., Ladics G S., Gerberick G F., Ryan C A., Basketter D A., Scholes E W., House R V., Hilton J., Dearman R J. and Kimber I. (1996) Further evaluation of the local lymph node assay in the final phase of an international collaborative trial. *Toxicology* **108**, 141-152.

## **P-29 A statistical method for evaluating transferability of an alternative test method to animal experiments**

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### **【Objective(s)】**

The inter-laboratory transferability is one of the most important modules in the validation of an alternative test method to animal experiments as is indicated in the OECD guideline No. 34<sup>[1]</sup>. The transferability is defined in the glossary as “the ability of a test method to be accurately and reliably performed in independent, competent laboratories” (GD 34). Up to present, there has been no proposal of practical procedures to examine the transferability of test methods. We tried to devise a statistical method to evaluate the transferability based on the data in a validation study.

### **【Materials and Methods】**

We assume that the data obtained in a validation study performed in several laboratories under a well-established SOP and the data obtained in the standard laboratory which developed the test method are available. These data are distributed as normal under a mixed effect model with random effects of experiments and laboratories. The proposed method is composed of two steps with “Step 1: Outlying non-competent laboratories are detected and eliminated” and “Step 2: The inter-laboratory variability is evaluated among competent laboratories.” Step 2 presents an index for transferability.

### **【Results and Discussion】**

The proposed method was applied to the data in the validation study of LLNA-DA assay, which lead the conclusion that outlying non-competing laboratories were not detected out of 9 participating laboratories and the inter-laboratory variability was not so great that the transferability of LLNA-DA assay was denied. The applicability of the proposed method will be examined further by the succeeding second validation study.

### **【References】**

[1]. OECD Environment, Health and Safety Publications Series on Testing and Assessment, No, 34. 2005.

## **P-30 Study on the optimality of material allocation design in the validation study of alternative assay to animal experiments**

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### **【Objectives】**

One aspect of the validation study on an assay is to evaluate the inter-laboratory variability. The study with this objective usually requires many laboratories to conduct experiments for assessing the target toxicity of specified test materials. Although a good allocation design of test materials to laboratories is desirable to make the study effective, the methodology to actualize such an optimum design has not yet been established. The objective of this study is to propose a methodology for addressing this issue and examine its practicability through the evaluation of the allocation design in the validation study for LLNA-DA.

### **【Proposed Allocation Method】**

The endpoint to assess the target toxicity is assumed to obey a linear normal model with fixed effects of laboratories and test materials. D-optimality, i.e., to minimize the determinant of variance covariance matrix of estimators for fixed effects is adopted as the optimality criterion. The proposed method searches the optimal allocation design by examining all possible cases which satisfies the actual restriction condition. The difficulty lies in the list up of all possible cases.

### **【Results and Discussion】**

In the validation study for the LLNA-DA, 9 specific materials in addition to 3 common materials were allocated to 10 laboratories for evaluating the skin sensitization potential. The application of the proposed methodology to evaluate the adopted allocation design showed that the adopted design had a good quality in the D-optimality criterion. The methodology for evaluating the optimality of any allocation design based on the mixed effect model should be investigated in future studies.

### **【References】**

- [1] OECD Environment, Health and Safety Publications Series on Testing and Assessment. No. 34. 2005.
- [2] Yamashita K. et al. Development of a modified local lymph node assay using ATP measurement as an endpoint. Alternatives to Animal Testing and Experimentation. 2005; 11: 136-144.

## **P-31 A statistical method for estimating ET50 using alternatives to skin irritation testing**

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### **【Objective(s)】**

The time score for 50% cytotoxicity (50% effective time; ET50) is the conventional index of skin irritation evaluated by alternatives using a three-dimensional human skin model, such as TESTSKIN<sup>TM</sup> or Vitrolife-Skin<sup>TM</sup>. ET50 is estimated as the time corresponding to 50% cell viability using linear interpolation of observations at two time points including 50% time point. This simple method is problematic in that a biased estimate is obtained occasionally as a result of linear approximation for the time-response curve and the precision of the ET50 estimate cannot be evaluated. We proposed and evaluated statistical methods for estimating ET50 applying a logistic model, a log linear model or a simple linear model to absorbance as the time-response curve, under the assumption of small sample sizes due to the experimental restrictions.

### **【Materials and Methods】**

We evaluated the performance of the proposed method for estimating ET50 through a Monte-Carlo simulation within the similar conditions of the validation study for TESTSKIN<sup>TM</sup> (2002) and Vitrolife-Skin<sup>TM</sup> (2004). We constructed a confidence interval for ET50 using a delta method by applying a logistic model, a log linear model or a simple linear model to virtual data based on a logistic curve on the time-response. As measures for performance, we used a bias in estimates, a proportion of estimable cases, and a coverage probability in which each interval contains the true ET50 values. We also evaluated the performance of the two-stage estimation method using a log linear or a simple linear model as a local approximation of the logistic model if the logistic model could not construct a confidence interval for ET50.

### **【Results and Discussion】**

The logistic model provided almost unbiased estimates, although the proportion of estimable cases decreased to 85% with increase of the true ET50 values. On the other hand, a log linear model and a simple linear model provided 100% proportions of estimable cases, although the positive biased estimates were obtained. The coverage probabilities were always less than the nominal level. We recommend using a logistic model for estimating ET50 at first. If the logistic model cannot construct a confidence interval for ET50, we recommend using a log linear model or a simple linear model for estimating ET50 as an alternative to the logistic model.

### **【References】**

Shiraishi A, Hyodo Y, Sozu T, Hamada C, Yoshimura I. A statistical method for estimating ET50 under the condition of small volume of data. *Alternatives to Animal Experimentation* 22 Special Issue 2005; 166.

## **P-32 Neutral Red Uptake Phototoxicity Assay of Methylparaben in Balb/c 3T3 Mouse Fibroblasts**

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### **【Objective(s)】**

Recently, the safety of parabens, used as the preservatives for cosmetics, has caused concern. In 2005, Handa et al. reported that the cell death rate induced by ultraviolet rays increased in the presence of methylparaben in the human keratinocytes.

In the present study, we evaluated the influence of methylparaben and ultraviolet rays on skin according to the OECD Guidelines (OECD TG 432) for a phototoxicity study which was adopted by international validation examination.

### **【Materials and Methods】**

The *in vitro* phototoxicity study using the Balb/3T3 clone A31 was executed according to the OECD Guidelines. The IC<sub>50</sub> values, the PIF value, and the MPE value were calculated without or with irradiation of UVA with a 6500-watt Xenon Arc Solar Simulator.

### **【Results and Discussion】**

In the presence of methylparaben at concentrations of 12.0~1000.0 μg/mL or 12.0~1200.0 μg/mL, the IC<sub>50</sub> values without and with of UV irradiation were 688.9 μg/mL and 681.4 μg/mL, the PIF value and the MPE value were 0.990 and -0.027, respectively, indicating that methylparaben did not reduce the viability of Balb/c 3T3 mouse fibroblasts, and that the IC<sub>50</sub> values were not modified by the UV irradiation. These results suggest that methylparaben had no phototoxicity. On the other hand, in the presence of CPZ in the concentration range from 0.1~62.0 μg/mL, the IC<sub>50</sub> values without and with UV irradiation were 25.56 μg/mL and 0.9378 μg/mL, the PIF value and the MPE value were 27.272 and 0.536, respectively, which clearly demonstrated phototoxicity. The difference in the present results and those by Handa et al. may be attributable to the fact that Handa et al. adopted an extremely severe condition which was different from the method recommended by the OECD guideline.

In addition, although the phototoxic substances damage the cell by absorbing light and producing free radicals, methylparaben has an absorption peak at 250-260nm, and it does not absorb light of wavelength longer than 300nm, including UVB, UVA and visible rays. This result also suggests that methylparaben has no phototoxicity.

Parabens have been used as preservatives in cosmetics, pharmaceuticals, and foods for more than 70 years in many countries including Japan, USA, and Europe. They have a broad spectrum of activity in a wide pH range and are especially effective against molds and yeasts, also gram-positive bacterium.

There have been many reports concerning the safety of parabens, and in 2004, the report suggesting connection with breast cancer by Darbre et al. was denied by an inspection of the EU authorities. In 2006, the Cosmetic Ingredient Review has finally evaluated the numerous reports concerned with parabens and reconfirmed its safety, which is consistent with the present results.

### **【References】**

- 1) Final Report of the Cosmetic Ingredient Review : Amended Safety Assessment of Methylparaben, Ethylparaben, Propylparaben, Isopropylparaben, Butylparaben, Isobutylparaben, and Benzylparaben, June 13, 2006, USA
- 2) SCCP/0874/05 EUROPEAN COMMISSION health & consumer protection directorate-general

## **P-33 A Genotoxicity test system based on the p53 transcriptional activity in a human lung epidermal cell line**

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### **【Objectives】**

Recently, Ohno et al. developed the genotoxicity test system based on a luciferase (luc) reporter gene assay depending on p53 transcriptional activity in a human breast cancer cell line, MCF-7. One of the important advantages of this assay is that it can be applicable to other cells derived from various organs. We therefore investigated the applicability of this method to a human lung epidermal cell line for detecting the genotoxicity of atmospheric environmental samples.

### **【Materials and Methods】**

A normal human bronchial epidermal cell, BEAS-2B, was transfected transiently with the reporter gene for p53 transcriptional activity. Intracellular p450 1A1/2-dependent metabolic capacity of the cells was induced by 3-methylcholanthrene (3MC). For the extracellular metabolism, chemicals were preincubated with a rat liver S9 fraction (S9 mix). Adriamycin (ADM), whose genotoxicity is not affected by such metabolism (direct genotoxic chemical), and Benzo-[a]-pyrene (B[a]P), whose genotoxicity is activated by metabolism (indirect genotoxic chemical), were exposed to the cells at various concentrations. Intracellular metabolic activity was measured by ethoxyresorfin-O-deethylase (EROD) activity assay. Genotoxicities were evaluated by the increase in luciferase activity with or without extra- or intra-cellular metabolism. The cell viability was quantified by the acid phosphatase activity (AP) assay.

### **【Results and Discussion】**

The reporter gene assay successfully detected the metabolism-independent genotoxicity of ADM. Genotoxicity of B[a]P was also detected when it was preincubated with S9mix or when the intracellular p4501A1/2 activity was induced with 3MC in advance. All the increases in luciferase activities were observed in lower concentration ranges than those caused cell death. These results showed the applicability of this reporter-gene assay to lung epithelial cell-based cytotoxicity tests for detection of genotoxicity of atmospheric environments. In addition, these results suggest the possibility for the detection of different genotoxicities that depend on different metabolic activities of various human organs.

### **【References】**

Ohno, K. *et al.*, *Mutat. Res.*, **588**, 47-57 (2005)

## **P-34 Development of the alternative method for renal drug excretory mechanism using *Xenopus* oocyte expression system**

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### **【Objectice(s)】**

Organic anion transporters (OATs) play an important role in the pharmacokinetics in our body by mediating the renal transport of endogenous substrates and several anionic drugs.<sup>1)</sup> To date, we had succeeded in the development of *in vitro* pharmacokinetics prediction system by establishing the cell lines stably-expressing several OATs. This system is suitable to evaluate the drug uptake into the cells, but it is not completely appropriate to examine the drug efflux from the cells. Here, we tried to develop the novel high-throughput system to evaluate the drug efflux by expressing renal transporter hNPT1<sup>2)</sup> that is localized at the apical side of the renal proximal tubules and is suggested to function as a drug efflux pathway.

### **【Materials and Methods】**

hNPT1 cRNA was *in vitro* transcribed and injected into *Xenopus* oocytes by automatic injection machines. Transport function of hNPT1 expressed in the oocytes was confirmed by measuring the uptake of radiolabelled compounds such as *p*-aminohippurate (PAH). To evaluate the drug efflux, we injected the radiolabelled compounds directly into the oocytes that express hNPT1, incubate oocytes in the buffer for 15 min, and measure the radioactivities of the buffer using liquid scintillation spectrometry.

### **【Results and Discussion】**

hNPT1 cRNA-injected oocytes showed significantly higher count of [<sup>14</sup>C]PAH than water-injected control oocytes, indicating the drug efflux function by hNPT1. Introduction of automatic oocyte injection machines enabled us to achieve the equalization of hNPT1 expression level. These results indicated that it seems likely to develop the alternative system to evaluate the renal drug excretion by using the automatic oocyte injection machines in addition to the use of transporter stably-expressing cell lines instead of excised mammalian kidney slice methods.

### **【References】**

- 1) Anzai N, Kanai Y, Endou H. (2006) *J Pharmacol Sci.* 100(5):411-426.
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## P-35 Gene expression in transformed cells

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### 【Objective】

The *in vitro* cell transformation assay is recommended for screening potential carcinogenesis. However, it is difficult to maintain cells in a culture for 4 to 6 weeks, and to score morphologically transformed foci in the cell transformation assays. We considered it important to attempt further improvement of the method. We studied altered gene expressions in BALB/3T3 cells treated by chemicals.

### 【Materials and Methods】

BALB/3T3 clone A31-1-1 cells at passage 6 were obtained from the HSRRB and maintained in Eagle's MEM (Invitrogen) supplemented with 10% heat-inactivated FCS (Japan Bioserum) at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. BALB/3T3 cells in the log-phase were dissociated enzymatically and plated in 35mm dishes at a density 5 x 10<sup>3</sup> cells/dish (Day 0). After one-day of cultivation, cells were treated with a fresh medium containing 100 ng/ml MCA. Culture medium was replaced with 10%FCS-MEM without the chemical on Day 4, and with 30 ng/ml TPA on Day 7, Day 11, Day 14, and Day 18. Thereafter, the medium was changed twice a week with fresh 10%FCS-MEM without the chemical. Total RNA was isolated from cells harvested from culture dishes on Day 4, Day 8, Day 14, Day 21, and Day 28. Mouse genome 430 2.0 array (Affymetrix) was used for the analysis of gene expression in cells treated with the chemicals. Data were analyzed using GeneSpring GX 7.3 (Agilent) software.

### 【Results and Discussion】

We analyzed gene expression to evaluate the change of cell transformation at the molecular level by treatment with chemicals. In this study, about 25,000 genes were “present” or “marginal” in BALB/3T3 cells. We evaluated the changes in gene expression by chemicals on their genes that were “present” or “marginal” in the cells. A total of 1,062 and 474 gene expressions changed at least twofold to MCA alone on Day 4 and 8, respectively. Similarly, a total of 723, 576, and 190 gene expressions changed to MCA plus TPA on Day 14, 21, and 28, respectively. CYP1A1, which was reported to have been induced by MCA, were induced by MCA alone on Day 4, remarkably. Jun and Fos genes families, which belong to immediate early response genes, were increased by MCA on Day 4 and 8. The expressions of Cyclin A2, B1 (Ccnb1) and B2 gene, which were cell cycle-related genes, were increased by MCA plus TPA on Day 14 and 21. We were certain that they were characteristic of initiation or promotion process of transformation.

### 【References】

IARC/NCI/EPA Working Group. 1985. Cancer Res. 45: 2395-2399.

## P-36 Automated *In Vitro* Micronucleus Analysis

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### **【Objective】**

Micronucleus induction is a key characteristic of genotoxic compounds. Analysis of micronucleus formation resulting from DNA strand breakage (clastogens) or interference with chromosome segregation (aneugens) is an important component of toxicology screening of new drug candidates. Manual scoring of micronucleus assays is time consuming and subject to operator variance, bias and error. Automated analysis of micronucleus assays allows very significantly faster analysis and consistently objective scoring. In this paper demonstrates use of the IN Cell Analyzer 1000 Micronuclei Formation Analysis Module.

### **【Materials and Methods】**

Micronucleus assays were carried out in CHO-K1 cells grown in imaging grade 96 well plates. Cells were exposed to solvent or test compounds for 24 hours, Cytochalasin B was added and cells incubated for a further 24 hours. Following fixation in ethanol for 30 minutes at room temperature, total cellular protein was stained with FITC, cells washed PBS and nuclear and micronuclear DNA stained with Hoechst. Plates were imaged on IN Cell Analyzer 1000 (20X objective) and were analyzed using the Micronuclei Formation Analysis Module.

### **【Results and Discussion】**

The Micronuclei Analysis Module successfully detected and quantitated micronuclei formation in response to three genotoxic compounds. Correlation analysis demonstrates that results obtained using automated analysis agree well with those obtained by manual scoring.

### **【References】**

1. Kirsch-Volders M. *et al.* Report from the *in vitro* micronucleus assay working group. *Mutat. Res.* **540** (2), 153–63 (2003).
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## **P-37 Development of Japanese medaka embryo cDNA microarray and its application in risk assessment of endocrine disrupting chemicals**

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### **【Objective(s)】**

Recently, we developed a medaka embryo cDNA microarray (target genes were randomly selected out of 1-3 days post-fertilization stage embryos), which included 2,222 independent target cDNA fragments. Considering the Cosmetics Directive, REACH, and European regulations for the protection of laboratory animals, we thought that utilization of medaka embryo (which does not comply with the European regulatory definition of a laboratory animal) and gene expression analysis with this cDNA microarray would be one powerful way to look for molecular signatures to evaluate toxicity of the chemicals as well as to look for biomarker candidate genes that are responding to the tested chemicals. Therefore, functionality confirmation test of this microarray was performed.

### **【Materials and Methods】**

Embryos of ER $\alpha$ -overexpressing transgenic d-rR strain of Japanese medaka (*Oryzias latipes*) at 10-hour post fertilization were exposed to 10ng/l and 10 $\mu$ g/l of 17 $\beta$ -estradiol (E<sub>2</sub>) for 12, 24, 36, and 48 hours. Then, pooled total RNA samples from 50 embryos per exposure condition were extracted and used for medaka embryo cDNA microarray gene expression analysis.

### **【Results and Discussion】**

The E<sub>2</sub>-induced blood clotting phenotype observed in the transgenic medaka embryos could be analyzed at gene expression level by using our medaka embryo cDNA microarray. Our microarray data revealed that chriogenin L (*chgL*), vitellogenin-1 (*vtg1*), angiotensin converting enzyme-1 (*ace1*) were significantly up-regulated. On the other hand, type 2 keratin E2 (*ck-E2*) was significantly down-regulated. The up-regulation of *chgL* and *vtg1* in the early-stage developing embryo was very curious, but the up-regulation of *ace1* seemed reasonable because it is known to function as a strong vasoconstrictor in other vertebrate species. Additionally, the down-regulation of *ck-E2* seemed also reasonable as it was easily assumed to function as structural component of the epithelium of the yolk vein. Further analyses will be carried out to identify novel molecular biomarkers for assessing estrogenicity of the endocrine disrupting chemicals.

### **【Reference】**

Kawamura T, et al. (2002) Estrogen inhibits development of yolk veins and causes blood clotting in transgenic medaka fish overexpressing estrogen receptor. *Zoological Science* 19: 1355-1361.

## **P-38 Application of DNA microarray to search for new sensitization markers**

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### **【Objectice(s)】**

Recently, several in vitro skin sensitization tests using cell lines have been reported; however, none of these methods has been completely established. In this study, to develop an in vitro sensitization test with high sensitivity and accuracy, we searched for new sensitization markers using DNA microarray technology.

### **【Materials and Methods】**

Human monocytic cell line THP-1 cells were treated with a sensitizer or non-sensitizer (1-chloro-2,4-dinitrochlorobenzen:DNCB, nickel sulfate:Ni, sodium lauryl sulfate:SLS) for 8 hours, and then total RNA was extracted from cells. RT-PCR was performed by CyScribe GFX Purification Kit and CyDye-labeled cDNA (Amersham) was hybridized with human cytokine chip (Takara) containing 553 genes related to cytokines. After selecting several genes from among these 553 genes, expressions of the selected genes were analyzed by quantitative RT-PCR.

### **【Results and Discussion】**

Expressions of 56 genes were up-regulated relative to the control and both DNCB and Ni increased the expression levels of only 20 genes. These genes were related to antigen-presentation or stress response, for example, CD54, macrophage inflammatory factor-1 $\alpha$  (MIP-1 $\alpha$ ), MIP-1 $\beta$ , interleukin-8, CD44, NF- $\kappa$ B, heme oxygenase-1 and thioredoxin reductase-1.

Because of the complex mechanism involved in sensitization, not one but several markers should be analyzed simultaneously to improve the accuracy of evaluating chemical sensitization potentials.

### **【References】**

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## **P-39 Modification poly-dimethylsiloxane (PDMS) surfaces suitable for the culture of primary rat hepatocytes**

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### **【Objectives】**

Liver tissue microdevices with primary hepatocytes using a highly-O<sub>2</sub>-permeable material, poly-dimethylsiloxane (PDMS), are expected to be applied to screen biotransformation and toxicities of various chemicals. Therefore, the microdevices are required to maintain functions of the hepatocytes. However, there have been few reports achieving that by a direct culture on the PDMS surface because of the detachment of cells from the PDMS. The objective of this research is to develop modification of PDMS surfaces suitable for the culture of primary rat hepatocytes.

### **【Materials and Methods】**

Cell culture plates which had the PDMS surface at the bottom were prepared. The PDMS surfaces were treated by oxygen plasma (PDMS-plasma), subsequently coupled with aminosilane (PDMS-NH<sub>2</sub>), and then induced cross-linkers under the UV light (PDMS-UV) to immobilize the collagen covalently on the surface. These surfaces were coated by the collagen type 1-P (PDMS-CN, PDMS-plasma-CN, PDMS-NH<sub>2</sub>-CN and PDMS-light-CN). In addition, PDMS-light-CN-O<sub>2</sub>-blocked plates, in which the oxygen supply to hepatocytes through the bottom PDMS surface was blocked, were prepared. The traditional collagen-coated polystyrene (PS) plates (PS-CN) were used as a control. The surface modification steps were confirmed by X-ray photoelectron spectroscopy (XPS) and contact angle measurements. Primary rat hepatocytes were cultured on these surfaces for two weeks and the albumin production was compared.

### **【Results and Discussion】**

XPS and contact angle measurements showed that the collagen was covalently immobilized only on the surface of PDMS-UV-CN. On this surface, rat hepatocytes first formed a complete monolayer, then organized themselves into stably attached spheroids, and were subsequently maintained with remarkably higher albumin production at least for two weeks of the culture. In contrast, hepatocytes on the other types of PDMS surface formed suspended spheroids or island shaped monolayers that had low albumin productivities. In addition, we confirmed that the direct oxygen supply through the bottom PDMS surfaces is crucial for the formation and function of such attached spheroids. These results show that the PDMS-light-CN is a material suitable for the development of liver tissue microdevices using primary hepatocytes.

### **【References】**

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## P-40 Cell Transfer Printing Technology

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### **【Objective(s)】**

The objective of this study is to develop promising cell engineering technology for alternatives to animal experiments and regenerative medicine. Recently, much attention has been paid to apply a variety of micro-patterning technologies to tissue engineering. We have achieved the cell transfer printing technology based on classic lithography and surface chemistry. We have demonstrated to make capillary blood vessels-like structure, micro-cell-patch array, and micro-cell-spheroid array embedded in gels derived from natural extracellular matrix (ECM).

### **【Materials and Methods】**

Cytophobic coating was prepared by coupling reaction of  $\gamma$ -glycidoxypropyltrimethoxysilane to glass substrate and then addition of tetraethyleneglycol to epoxy ring. The coating was etched to make cytophilic patterns by means of the photocatalytic lithography<sup>1)</sup>. Surface of the cell printing plate was characterized by water contact angle measurement, x-ray photoelectron spectroscopy, scanning white-light interference microscopy, and atomic force microscopy. The capability to transfer geometrically arranged cells to ECM-derived gels was evaluated by using bovine aortic endothelial cells, human microvascular endothelial cells, and human dermal fibroblasts with growth factor reduced Matrigel<sup>TM</sup> and porcine type I collagen gel.

### **【Results and Discussion】**

Cell transfer rate was affected not only the surface nature of the printing plates but also the type of cells and the culture conditions. Surface characterization data were shown that the cytophobic coating was etched gradually during photocatalytic lithography. This means that hydration capability of the cytophobic coating was also gradually decreased with the patterning. Such surface allows protein molecules to adsorb. Namely, cell adhesive nature was induced due to the decrease of hydration of the cytophobic coating. Experimental data also suggest that cells in sandwich configuration increase adhesion to the matrix and may simultaneously decrease adhesion to the printing plate. Thus, we assume that cell polarity change is a central role of the cell transfer mechanism.

We have achieved a promising alternative method to make three-dimensional culture systems with defined-micro-patterns of single and collective cells. Biological utility of this method should be verified in the near future.

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## **P-41 Application of inkjet printing technology for 3D cell patterning**

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### **【Objectice(s)】**

Engineering of the functional tissue model that closely mimic the native tissue architecture, is considered integral to the development of alternative methods. However, Tissues and Organs are composed of a variety of components, multiple type cells and extracellular matrices proteins. In many tissues, the position of the cells is critical to their function. For engineering their tissues in vitro, it is necessary to arrange their components onto targeted position with corresponding resolution. Therefore, many technologies are currently being adapted to applications in tissue engineering. For example, inkjet printing technology can eject small droplets of the ink at high resolution. Recently, this technology has been used to print various biomaterials such as nucleic acid, growth factor and cells instead of the ink. In this study, we tried spatially controlled patterning of the biomaterials in three dimensions using inkjet technology.

### **【Materials and Methods】**

HeLa cells were suspended in 0.8% alginate sodium solution. Then, the suspension was loaded into ink tank attached to the customized inkjet system that we originally developed for three-dimensional printing. Since sodium alginate constructs hydrogel under the existence condition of calcium ion, the cell suspension was printed into the calcium chloride containing solution for cell patterning.

### **【Results and Discussion】**

When the lines with the suspension were printed into calcium chloride solution on the slide glass, micro gel fiber containing cells could be made. By embedding in the alginate gel, the cells were arranged in programmed patterns such as a line or circle. In addition, we succeeded in the construction of a tubular structure of 1mm in diameter by laminated printing of the circle toward z direction.

We established a method, inkjet technology-based 3D cell patterning using alginate gel. These results suggested that inkjet technology will provide much possibility as a tool for 3D tissue engineering.

## P-42 Development of Cell-Based Array Chips Immobilizing Different Numbers of Liver Cells

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### 【Introduction】

We proposed a cell-based array chip to simultaneously evaluate minimum cell numbers for functional tissues. Using photocatalytic lithography,<sup>1</sup> cell attachment areas with varied sizes were formed on a single glass plate, and Hep G2 cells were immobilized onto these attachment areas.

### 【Experimental】

A 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer<sup>2,3</sup>-modified glass plate was faced to the Pt particles-TiO<sub>2</sub>-modified glass plate with a 12.5 μm intervening gap, and UV light was irradiated from the backsides of the Pt-TiO<sub>2</sub>-modified glass plate through a photomask (Fig. 1). MPC polymer was oxidized and decomposed by active oxygen species generated from the Pt-TiO<sub>2</sub> coating, resulting in the formation of cell attachment areas. After treated with collagen, Hep G2 cells ( $2.0 \times 10^5$  cells cm<sup>-2</sup>) were cast to the glass plate surface. Patterned cells were observed by a phase inverted microscope.

### 【Results and Discussion】

Hep G2 cells were successfully immobilized only on the patterned cell attachment areas exactly similar to the photomask configuration (Fig. 2). Single Hep G2 cell was immobilized in the narrowest area having a diameter of 20 μm, whereas cells formed a island shaped monolayer in the areas having diameters of over 200 μm. During 7 days of culture, cells immobilized in areas having diameters 20 μm and 63 μm were alive but did not grow, whereas cells immobilized in areas having diameters of over 200 μm actively grew and were sometimes connected with adjacent monolayer. In summary, it is expected that the cell-based array chip fabricated here can evaluate the minimum cell number necessary for functional tissues. We are now evaluating various functions of Hep G2 cell units having varied sizes.

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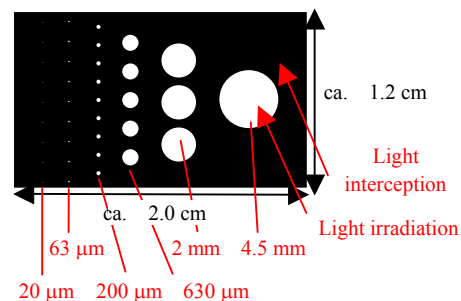


Fig. 1 Illustration of the photomask

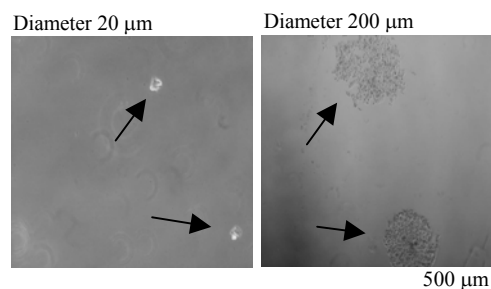


Fig. 2 Microscopic images of the array chip surface immobilizing Hep G2 cells