Proceedings of the 10th Annual Meeting of Japanese Society for Alternatives to Animal Experiments

December 5-6, 1996
TOKYO

Overview of the 10th annual meeting of Japanese Society of Alternatives to Animal Experiments

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10th annual meeting was held in December 5-6, 1996 at Komaba Eminence. 114 active members of the society, 68 temporary attendants, 12 invited speakers, 51 invited attendants, and 22 secretariats participated. This meeting was supported financially also by 32 industries. About 130 persons attended reception. There were 40 poster presentation of original works by the members of our society. The number was the maximum for the space. We asked the counselors of the society and the top authors of the poster presentation to vote for the golden presentation prize. Posters selected were "Simultaneous evaluation of cytotoxicity in the neutral red, MTT, and crystal violet staining assays in the same micro plate" by Koji Kawakami, Ikuyo Makino, and Katsuyoshi Chiba (Yakuruto), "Experimental study using turkey as alternative animals" by Takashi Sugiyama, Kyoko Goto, Satoshi Ushimaru, Hiroyuki Miyazaki and Hideyo Shimada (Kitasato Univ.), and "Evaluation of an alternative to skin irritation test (2): Determination of pro-inflammatory cytokine gene expression" by Michio Shibata, Takanari Tsuda, Hiroshi Itagaki, Hideyuki Ichikawa, and Yoshihiro Morikawa (Shiseido) .

In response to the movement for animal right protection, many *in vitro* methods have already been developed as alternatives to existing toxicity test methods and the results of validations of those methods have also been accumulated. On the other hand, deadline of the EU Directive 76/768/EEC, aiming to prohibit the use of animal experiments for the safety evaluation of cosmetics until 1 January 1998, had been approaching. Even though the amendment made on 14 June 1993 admitted to postpone the date of implementation of the provision if there has been insufficient progress in developing satisfactory methods to replace animal testing, all reasonable endeavors was obliged to do. ICCVAM (Interagency Coordinating Committee on the validation of Alternative Methods) and OECD made sequential workshops on the criteria for the validation and regulatory acceptance of alternative toxicological testing methods (NTP workshop on validation and regulatory acceptance of alternative toxicological testing methods, December 11-12, 1995 and OECD workshop on harmonization of validation and acceptance criteria for alternative toxicological test methods, January 22-24,1996). Because those information seemed very important for our society, we asked Dr. William S. Stokes (NIEHS), major participants of both workshops, to make a special lecture on the issues. He

introduced the activities of those workshops and summarized the results. Final report of the OECD workshop described 1) the principles and criteria for the validation and acceptance of new or modified toxicological tests for purposes of risk assessment and other uses relating to the protection of man and the environment, 2) the validation process, and 3) principles of testing strategies and testing schemes for skin and eye irritation/corrosion testing. Criteria for regulatory acceptance were as followed. 1) Application of the method provides data that adequately predicts the end-point of interest in that it demonstrates either a linkage between (i) the new test and an existing test method or (ii) the test and effects in the target species. 2) The method generates data for risk assessment purposes that are at least as useful as, and preferably better than, those obtained using existing methods. 3) There are adequate testing data for chemicals and products representative of the type of chemicals administered by the regulatory program or agency. 4) The test must be robust and transferable and allow for standardization. 5) The test is cost effective and likely to be used. 6) Justification (scientific, ethical, economic) should be provided for the new method with respect to any existing methods available. He expressed that those report should encourage the development of new methods and improvement of existing test methods, provide effective guidance on the validation of new test methods, contribute to increased likelihood of regulatory acceptance of scientifically valid new test methods.

Three independent symposiums were held in this meeting. 1) Alternatives to toxicity tests for agricultural drugs, chaired by Dr. Yasuo Ohno (NIHS) and Professor Tomoyuki Shirai (Nagoya City Univ.). 2) Use of functional cells as alternatives to animal experiments, chaired by Professors Tetsuya Kamataki (Hokkaido Univ.) and Hitoshi Endou (Kyorin Univ.). 8) Education on the alternatives to animal experiments, chaired by Professors Kazutoshi Maejima (Keio Univ.) and Hiroshi Ninomiya (Azabu Univ.).

The first was planed to survey the development and application of alternative methods for the safety evaluation of agricultural drugs in the field of hepatotoxicity, neurotoxicity, mutagenicity, carcinogenicity and reproductive toxicity. Dr. Sunouchi (NIHS) classified the pesticide according to the toxic effects on isolated and primary cultured hepatocytes and non-liver cells (SIRC). She indicated several metabolites which were more toxic than the parent compounds. Dr. Inoue (NIHS) found some metabolites of permethrine, pyrethroid insecticide, inhibit dopamine release from PC12 cells, which retain neuron specific functions. Dr. Yamada (NIEIS) introduced transgenic mouse which was used to detect *in vivo* mutagenicity. Professor Shirai (Nagoya City Univ.) introduced median term carcinogenicity test system based on the 2 step carcinogenicity theory and the results of 272 chemicals. Professor Ezaki (Osaka City Univ.) introduced reproduction toxicity test methods using hen and

quail eggs and human embryo cells. It seemed that those alternative test methods were useful especially in the preliminary safety evaluation of the metabolites or degradation products, which were difficult to be assessed by ordinary animal toxicity tests.

The second symposium was planed to review the new approach of alternative researches. Studies on tissue distribution of drugs using cultured cells expressing peptide transporter and organic anion transporter were introduced by Dr. Hideyuki Saito (Kyoto Univ.). Cell lines permanently expressing S3 characteristics of renal tubules and its application to the toxicity study were introduced by Dr. Michio Takeda (Kyorin Univ.). Developments of *E. Coli* expressing human P450 (CYPIA2, 2A6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4) and its application to drug metabolism study were introduced by Dr. Hirotaka Kushida (Hokkaido Univ.). CHL cells expressing human CYP2E1 were sensitive to nitrosodimethylamine, hepato-carcinogen, especially in the case of cell lines deficient in DNA repair enzyme (Dr. Tsuyoshi Yokoi, Hokkaido Univ.). Dr. Charles L. Crespi (GENTEST Co.) introduced Caco-2 cell line expressing human CYP3A4 (principal P450 in the intestine) and a baculovirus infect cell expressing both human P450 and human NADPH cyt. P450 reductase.

The third symposium was planed to discuss about education of alternatives to animal experiments and to receive comments also from animal right protection groups. Professor Maejima, Professor Takemi Yoshida (Syowa Univ.), and Professor Hiroyoshi Ninomiya introduced the application of alternatives to the education of medicine, pharmaceutical sciences, and veterinary medicine. Examples were the use of audio-visual instruments, computer simulation, plastic models, placenta, internship in a expert veterinarian, education on the relief of animals from pain and stress. These seemed useful to decrease in the number of animals used in the experiments and also decrease the distress of experimental animals. However, the level of the incorporation into curriculum seemed to be quite different among universities. Mis Fusako Nogami (All Life in a Viable Environment) indicated the points from citizen and discussed on the expectation to the alternatives. There was a big discrepancy in notion between researchers and animal protection group. Continued discussion between us will help to have a common base our activities.

Professor Atsushige Sato (Tokyo Med. and Dent. Univ.) and Dr. Hiroshi Itagaki (Shiseido) chaired the session on "reports ' on the international trend of alternatives to animal experiments and on the 2nd World Congress on alternatives and animal use in the life sciences". Dr. Itagaki reported the current status of EU regulation on the cosmetics and future prospect. He indicated that the situation was flexible and the implementation of the EU Directive 76/768/EEC seemed to be later than 1998 and 2000 for cosmetic ingredients and endproduct, respectively. Dr Hiroshi Ono

(Hatano Institute) described the organization of the 2nd World Congress and reported that the next meeting would be held in Bologna, Italy, and the next would be in Boston, USA. Dr Tadao Ohno (Riken) summarized the results of validation project of our society. Dr. Y. Ohno explained how regulatory requirements in toxicity tests changed by the research on alternatives and strategy for the evaluation of eye and skin irritation indicated in OECD meeting mentioned above. Professor Atsushige Sato summarized the presentations in the Congress related to ethics and education. He indicated needs of strategy to level up the science of alternatives research, of international harmonization on what toxicity tests should be validated, and of expansion of our society to incorporate researchers from another field of science for the purpose to work with vaccines, immunobiologicals, skin tests, and philosophical issues. Dr. Yuznki Nakagawa (Hatano lhstitute), to whom our society granted part of his travel expenses for the World Congress, also summarized his presentation "Application of the comet assay for detecting the early events of photogenotoxicity" and told the impression of the meeting.

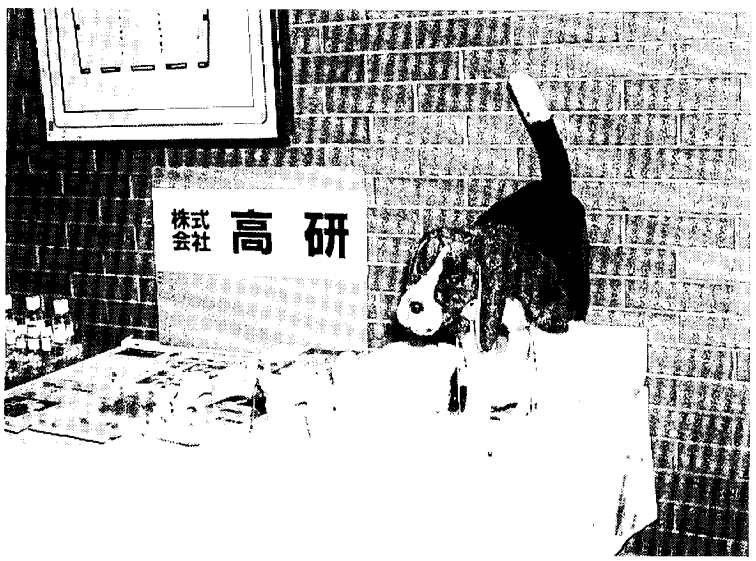

Photo l: Plastic models for the training of students

Photo 2(upper left): Golden presentation winner, Dr Michio Shibata (Shiseido)
Photo 3(upper right: Golden presentation winner, Mr Koji Kawakami (Yakulto)
Photo4(bottom): Golden presentation winner, Professor Takashi Sugiyama (Kitasato University)

Plenary Lecture

L-1.Criteria for the Validation and Regulatory Acceptance of Alternative Toxicological Testing Methods

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Background and Purpose

Toxicological test methods are being developed and revised with increasing frequency as scientists seek methods that will provide improved assessment of the potential toxic effects of chemicals and other agents to human health and the environment. The use of new or revised test methods for risk assessment purposes requires that that each method must first be validated for a specific purpose, followed by acceptance of the method for a specified purpose, i.e., it must be determined that the method will provide useful information for one or more regulatory agencies. The NIEHS, as a major component of the U . S . National Toxicology Program (NTP), develops and validates improved alternative toxicological test methods that will generate information more useful for risk assessment. Consistent with this responsibility, the NIEHS was recently directed by Public Law I 03-43 to establish criteria for the validation and regulatory acceptance of alternative testing methods, and to recommend a process through which scientifically validated alternative methods can be accepted for regulatory use.

Materials and Methods

The NIEHS subsequently established the ad hoc Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) to develop a report recommending criteria and processes for validation and regulatory acceptance. The committee, consisting of representatives from fifteen Federal regulatory and research agencies and programs, developed the report "Validation and Regulatory Acceptance of Toxicological Test Methods" using information obtained from: a survey of participating agencies; a Federal Register request for information to be considered in preparing the report; presentations from government scientists; a review of the pertinent scientific literature; input from Federal Agencies; and a 1 995 open public workshop and Federal Register notice requesting comments on the draft report from interested stakeholders, including industry, academia, public interest groups, animal welfare organizations, and the international community. Additional input

was obtained from a 1996 Organization for Economic Cooperation and Development (OECD) Workshop on Harmonization of Validation and Acceptance Criteria for Alternative Toxicological Test Methods .

Results

The ICCVAM report describes: the validation and regulatory acceptance criteria that Federal agencies should employ in considering new and revised test methods; a series of recommendations to enhance the development, validation, and acceptance of new methods; and the establishment and proposed function of a permanent ICCVAM that will function to coordinate interagency validation and acceptance issues. The OECD Workshop developed internationally harmonized criteria for validation and regulatory acceptance that will form the basis for a future OECD Guidance Document for use b scientists in the 26 member countries.

Discussion

The ICCVAM and OECD reports provide information that should encourage the development of new methods and improvement of existing test methods provide effective guidance for scientists and regulatory staff on the validation and evaluation of new test methods, contribute to the increased likelihood of regulatory acceptance of scientifically valid new test methods, and encourage, where scientifically feasible, the reduction and refinement of animal use in testing, and the replacement of animals with non-animal methods and phylogenetically lower species. The use of validated and accepted improved toxicological testing methods is expected to provide enhanced protection of public health and the environment.

Symposium I
Alternatives to Toxicity Tests for Agricultural Drugs

SI-1.Studies on the Estimation of Hepatotoxicity of Agricultural Chemicals Using Hepatocytes

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To establish *in vitro* screening test system of hepatotoxicity, we studied the cytotoxicity of agricultural chemicals using freshly isolated and primary cultured rat hepatocytes (FIH and PCH). The effects on SIRC cells derived from rabbit cornea

were also studied to know the hepatocyte specificity of those effects. LDH leakage and GSH contents in FIH and LDH leakage and 7-ethoxycoumarin deethylation (ECOD) in PCH were measured as indicators of effects on hepatocytes. The insults to the hepatocytes were classified into four categories from the pattern of the effects. Type I including IBP, Erusan, Alanycarb, Benfuracarb, Prometryn (PRO), Ametryn (AME) and Mefenacet (MFC) caused LDH leakage from both FIH and PCH, type II, Swep, Chlornitrofen and Ethiofencarb (ETC) caused LDH leakage only from PCH, type 111, including Nitrofen and Chlomethoxyfen, increased ECOD activity and CYP1A at low concentration and enhanced LDH leakage from PCH at high concentration, and type IV, Permethrin, Cyfluthrin and Bifenox, caused no insults to hepatocytes. Loss of cell viabilities and decrease in GSH contents by PRO and AME were inhibited by metyrapone. PRO, MFC and ETC, which caused cellular toxicity in hepatocytes and not in SIRC cells, increased serum AST of rats. These findings suggest that the *in vitro* screening test system consisting of freshly isolated and primary cultured hepatocytes and SIRC cells is useful to estimate the hepatotoxicity of agricultural chemicals.

SI-2. An Alternative Test for Neurotoxicity of Chemicals Using Cultured Pheochromocytoma PC12 Cells

Kazuhide INOUE, Schuichi KOIZUMI, Tomoko OBAMA and Yasuo OHNO
Division of Pharmacology, National Institute of Health Sciences

In the present study, an *in vitro* culture system using neuronal cell line PC12 was proved as the evaluation method for neurotoxicity of chemicals. The method consists of 2 components: the detection of dopamine release and measuring of an intracellular calcium concentration. The method was very simple but was useful to find that the effect of mother compound on these 2 components was less than that of metabolite.

SI-3. Development of a New Evakuation Test of Genotoxicity Using Transgenic Mouse Technology

Takehiko NOHMI[1], Motoe KATOH[2], Hiroshi SUZUKI[3], Michiko MATSUI[1], Masami YAMADA[1], Masahiko WATANABE[4], Makoto SUZUKI[1], Naoko HORIYA[5], Otsuya UEDA[3], Toru SHIBUYA[5], Hideo IKEDA[6] and Toshio SOFUNI[1]
[1]Natl. Inst. Health Sci., [2]University of Chile, [3]Chugai Pharmaceutical Co., Ltd., [4]National

Cancer Center Research Jnstitute, [5]Hatano Research Institute (FDSC), [6]University of Tokyo

A new transgenic mouse mutagenesis test system has been developed for the efficient detection of point mutations and deletion mutations *in vivo*. The mice carry lambda EG10 DNA, which harbors *gpt* gene of *E. coli* as a reporter gene. When the rescued lambda phages are infected into an *E. coli* strain expressing Cre recombinase, the phage DNA is converted into a plasmid in the cell. The *gpt* mutants can be positively detected as colonies arising on plates containing chloramphenicol and 6-thioguanine . The EG10 DNA carries a *chi* site along with the red and *gam* genes, so that the wild-type phages display Spi^+ (sensitive to P2 interference -phenotype). Mutant phages lacking both red and *gam* genes can be positively detected as plaques that grow in P2 lysogens of *E. coli*. When the transgenic mice were treated with ethylnitrosourea, the *gpt* mutation frequencies were increased 4 - 7 fold over the background, in bone marrow. The spontaneous Spi^- mutation frequencies were 1.4×10^{-6} and 1.1×10^{-6} in bone marrow and sperm, respectively, that are, less than one tenths of the frequencies for *gpt* mutation. Spontaneous Spi^- mutants were not detected in spleen although 930,000 phages rescued from the untreated mice were screened. However, induction of Spi^- mutations was clearly observed in spleen at frequencies of 1.4×10^{-5} in gamma ray-irradiated (5 Gy) animals. These results indicate that the new transgenic mouyse could be useful for the efficient detection of point mutations and deletion mutations *in vivo*.

SI-4～SI-5 English abstructs were not submitted.

Symposium II
Use of Functional Cells as Alternatives to Animal Experiments

SII-1.Evaluation and Prediction of Tissue Distribution of Drugs Using Cell Culture and Atably Transfected Expression Systems

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In the present study, we examined functional expression and characterization of the

cloned rat peptide transporter(rPEPT1) and organic anion transporter(OAT-K1) using *Xenopus* oocytes and stably transfected epithelial cells. The results suggest that both *in vitro* expression systems are useful for evaluation of physiological and pharmacological roles of these transporters in the intestinal absorption or renal excretion of *xenobiotics*.

SII-2. Genetic Regulation of Cisplatin-induced Apoptosis in Mouse Proximal Straight Tubule(S_3) Cell Line

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Purpose

In order to clarify the intracellular mechanism for cisplatin-induced nephrotoxicity, we have elucidated the signaling pathway leading to cisplatin-induced apoptosis mainly in view of the role of various apoptosis-related genes on the process.

Materials and Methods

S3 cell line derived from transgenic mice harboring temperature-- sensitive SV40 large T-antigen gene was applied to the study. Cisplatin as well as staurosporine and H_20_2 have been used as stimuli for the induction of apoptosis. Gene-transfer was performed by liposome method. RT-PCR method was used to detect the changes in mRNA expression for apoptosis-related genes. Cell cycle analysis was performed by flow cytometry.

Results

1. Cisplatin (10^{-5}-10^{-4} M) and staurosporine (50-100 nM) induced morphological and biochemical changes characteristic of apoptosis. In contrast, H202 at high concentration (0.5-1.0 mM) induced oncosis, but that at low concentration (0.05~0.1 mM) resulted in apoptosis as well as oncosis. 2. Among various apoptosis-related genes screened, increase in *c-fos* mRNA expression occurred in concurrent with the induction of cisplatin-induced apoptosis. 3. Overexpression of *bcl-2* suppressed cisplatin-induced apoptosis, whereas overexpression of *bax* and *Ice* enhanced apoptosis. 4. The H202-induced oncosis was accompanied by increase in *c-fos* and *Fas* mRNA expression, and was suppressed by overexpression of *bcl-2*. 5. Staurosporine-induced apoptosis was preceded by decrease in *bcl-2* mRNA expression and blocked by *bcl-2* overexpression. 6. The role of interleukin-1β-

converting enzyme (ICE) family was elucidated by using peptide inhibitors for ICE family. Either Ac-Tyr-Val-Ala-Asp-H (Inhibitor for ICE) or Ac-Asp-Glu-Val-Asp-H (inhibitor for CPP32, one of ICE-like protease) inhibited apoptosis induced both by cisplatin and staurosporine. In contrast, Z-Asp-CH_2-DCB (Inhibitor for ICE and ICE-like protease) suppressed cisplatin-induced apoptosis but not staurosporine-induced one. These results indicate that ICE and ICE-like protease including CPP32 appear to be responsible for cisplatin-induced apoptosis, whereas ICE-like protease other than CPP32 may be involved in staurosporine-induced one. The contention was confirmed by the studies with the introduction of *crmA* gene, a cowpox virus gene which has been shown to inhibit the activity of ICE family. 7. Cell cycle analysis revealed that S3 cells treated with low concentration of cisplatin (10^{-5} M) represented the induction of apoptosis with G_2+M accumulation, whereas high concentration of cisplatin (10^{-4} M) resulted in the appearance of apoptotic cells without G_2+M arrest, suggesting that there exist multiple pathways for the induction of apoptosis depending on the cisplatin concentrations administered.

Discussion and Conclusion

Cisplatin-induced nephrotoxicity was associated with the induction of apoptosis, where various oncogenes, ICE family, and cell cycle regulation may be involved in this process. There seems to exist not only common but also multiple pathways for the induction of apoptosis in renal tubular cell line. In addition, distinctive types of cell death, apoptosis and oncosis, appear to share a mutual signaling pathway. The current result will lead us to the better understanding of cisplatin-induced nephrotoxicity and provide an opportunity for developing new therapeutic agents.

References

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SII-3. *In Vitro* Drug Metabolism Study Using Recombinant Human P450 Expressed in *Escherichia Coli*

Hirotaka KUSHIDA[1], Tsutomu SAKUMA[1], Tsuyoshi YOKOI[1], Ken-ichi NUNOYA[1], Yuko KONNO[1], F.J.Gonzalez[2], Toru ENDO[3], and Tetsuya KAMATSKI[1]

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It is difficult to predict *in vivo* drug metabolism in humans from studies with experimental animals. Human liver tissues or microsomes and expression systems of human drug metabolizing enzymes have been used to solve this problem. B-lymphoblastoid cells, insect cells, yeast and E. coli are used as expression systems. Since the expression of human P450 in E. coli is relatively easy and inexpensive, we expressed human P450s in *E. coli*. Thus, human P450 and P450 reductase were co-expressed in *E. coli* to evaluate catalytic capabilities and usefulness of E. coli expression system.

SII-4.Establishment of Cell Lines Expressing Human CYP2E1 and Its Application for Toxicological Studies

Tsuyoshi YOKOI[1], Tetsuya NAKAGAWA[1], Minoru SAWADA[1], Ryoji ISHIDA[2], Frank J. GONZALEZ[3] and Tetsuya KAMATAKI[1]

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Most of established cells do not express cytochrome P450 (CYP). Because of the lack of CYPs in the cells, these cells are rather insensitive to promutagens or procarcinogens. In order to investigate the participation of human CYP2E1 in the metabolic activation of promutagens, the cell line expressing this cytochrome was established, designated as ER-181. ER-181 cells showed high sensitivity to N,N-dimethylnitorosamine (DMN) in cytotoxicity assays, while parental cells were insensitive. The hypersensitivity to DMN of ER-181 cells was almost completely suppressed by 3-amino-1 ,2,4-triazole, a known inhibitor of CYP2E1. O_6-methylguanine-DNA methyltransferase (MGMT) deficient cell line was also adapted to clarify the involvement of MGMT in the sensitivity of cells to the chemicals.

SII - 5. High Level Expression if Human Drug Metabolizing Enzymes-Integration into Drug Metabolism and Toxicity

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Research Purpose

Cytochromes P450 (CYP) are the principle enzymes responsible for the metabolism of many drugs and environmental pollutants. The study of xenobiotic (drug) metabolism and toxicity increasingly utilizes *in vitro* systems containing catalytically active cytochrome P450s. The amount and identity of the specific cytochromes P450 present in a system depends of the intended applications. Two different systems will be discussed: 1 . A Caco-2 cell line which has been developed to express human CYP3A4, the principal cytochrome P450 expressed *in vivo* in the intestine. This cell line has the potential to be a better *in vitro* model for human drug transport and metabolism. 2. A baculovirus insect cell expression system which co-expresses human cytochrome P450, human NADPH-cytochrome P450 reductase (OR) and in some cases human cytochrome b5. This system provides large quantities of active enzyme for efficient production of metabolites.

Materials and Methods

Caco-2 cells were propagated in DMEM supplemented to 10% with heat inactivated fetal bovine serum in plastic tissue culture flasks. Au cultures were refed with fresh media every 2 to 3 days. Selection for the vector was maintained by the addition of 100 mg/ml hygromycin B to the culture media. Baculoviruses containing CDNAS for cytochromes P450 and coenzymes were constructed using vectors and viral DNA from PharMingen (San Diego, CA) according to the manufacturer instructions. Enzymes were expressed in spinner cultures of baculovirus infected insect cells (BTI-TN-5B1-4) using standard procedures.

Results

We have developed a Caco-2 cell derivative which expresses high levels of cDNA-derived CYP3A4. The CYP cDNA was introduced into an extrachromosomal vector under control of the cytomegalovirus early intermediate promoter. Vector-bearing cells were selected via resistance to hygromycin B and exhibited high levels of cDNA-derived protein as measured by Western blot, spectrophotometric P450 determination and/or cytochrome P450 form-selective enzyme assay. cDNA-expressing cells were found to form tight monolayers and were suitable for study of xenobiotic transport and metabolism. The permeabilities of cephalexin,

phenylalanine, mannitol and propranolol across transfected monolayers were found to be similar to those across untransfected monolayers. The metabolism of the CYP3A4 substrates testosterone and nifedipine were measured in the appropriate transfected monolayers.

The following human cytochrome P450s have been expressed using the baculovirus system: CYP3A4, CYP3A5, CYP2C8, CYP2C9-Arg144, CYP2C9-Cys144, and CYP2C19. Catalyic constants, apparent Km and turnover number, were measured for these baculovirus-expressed enzymes and compared to the same enzyme in human liver or produced using other heterologous expression systems. Cytochrome P450 enzyme-selective activity values (pmoles of product per mg per min) were 4.5 to 9 fold higher for baculovirus-expressed enzymes compared to human liver microsomes. Comparison of cytochrome P450 holoenzyme levels (by spectrophotometric P450 content) to apoprotein levels (by Western immunoblot) for baculovirus-expressed and human lymphoblast-expressed proteins indicates a higher proportion of apoprotein with baculovirus expression.

Discussion

We have successfully developed Caco-2 cells expressing human CYP3A4. The episomal vector based on the OriP sequences and EBNA-1 gene product derived from Epstein Barr virus provided relatively high expression levels of CYP3A4. However, expression of CYP3A4 cDNA markedly affected cell growth. Cell reattachment frequency after trypsinization was reduced and cell growth rate also appeared reduced. Expression of cDNA-derived CYP3A4 was also somewhat unstable, but this degree of instability could be addressed by the use of a freezer stock of freshly transfected cells. Clearly it would be desirable to have more stable cell lines, however, at this time it is unclear whether instability of expression is a general property of this cell line/vector system or limited to the cDNA expressed in the present study. However, even with the limitations with respect to stability and cell propagation, the CYP3A4-expressing cells have been found to be useful for drug transport and metabolism studies. The cells form tight monolayers and expressed native transporters . The levels of catalytic activity in monolayers could be increased, without loss of membrane integrity, by pretreatment with the phorbol ester, TPA. The levels of CYP3A4 appear to be sufficiently high and cell growth adequate to permit many applications with the CYP3A4-expressing cells. Our studies of testosterone and nifedipine transport and metabolism support this conclusion.

Our results using a baculovirus expression system for the cDNA-directed expression of cytochromes P450 with co-enzymes support the following conclusions. Western immunoblotting data verified that all of the baculovirus-expressed P450s had mobilities equal to their counterparts in human liver microsomes . The Western

immunoblotting data also indicated that a significant portion of the baculovirus-expressed P450 enzyme was catalytically inactive apoprotein. Apparent Km values for baculovirus expressed P450s are reasonably close to those values recorded for HLM and the lymphoblastoid expression system. Turnover numbers for several baculoviruses, co-expressing a cytochrome P450 and OR, were lower than values for HLM or lymphoblasts. This data suggests further optimization of the interaction between OR and cytochrome P450 is possible. Cytochrome P450 activities (Vmax values per mg protein) for baculovirus expressed P450s were generally several fold higher than those found in HLM or expressed using lymphoblasts. Finally, baculovirus expression of P450 measured as activity per mg protein is therefore, superior to human lymphoblast and other cDNA expression systems.

Symposium III
Education on the Alternatives to Animal Experiments

SIII-1. Alternatives in Medical Education

Kazuyoshi MAEJIMA
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In Japan, most of medical and dental researchers are relatively inactive for alternatives to animal experimentation, testing and education. However, a few of laboratory animal researchers in medical schools have made to develop the plastic animal models(simulators) for training the animal experimentation techniques for beginners. The rat and rabbit models(KOKEN) can be used for training the techniques of the intubation into the stomach, trachea and urinary tract, injection to the tail vein, and so on. The development of the 3rd animal model, dog simulator for training of medical animal experimentation and veterinary clinical techniques have carried under collaboration with staffs of veterinary medical schools. The dog models will be manufactured by KOKEN at 1997.

SIII-2~SIII-4 Abstructs were not submitted.

SIII-5. What Concerned Citizens Expect of Alternative Methods to Animal Experimentation

Fusako Nogami
All Life In a Viable Environment/Japan Anti-Vivisection Association

In recent years, in response to mounting criticism of animal experimentation in Japan, there has arisen a movement among some of the involved parties to seek alternative methods to the use of live animals in experiments involving vivisection. If vivisection is to be reduced and ultimately eliminated. the following policies need to be implemented.

(1) The promotion of experimental alternatives to animal experiments involving vivisection (alternatives to animal experimentation in a narrow sense).

(2) A restructuring of the medical/biological research and health/ medical treatment systems .

(3) A shift in emphasis away from so-called "modern Western" medicine and toward alternative treatments and preventative measures. (alternatives to animal experimentation in a wide sense).

(4) The establishment of a legal framework in which items (1)-(3) can be realized.

1. Alternatives to animal experimentation in a narrow sense means methods of experimentation (such as in modern medical research) that do not utilize animals. Concerning this subject, many companies and research facilities are currently pursuing such alternative methods from the utilitarian viewpoint of cutting research costs. However, concerned citizens expect this subject to be approached also from an ethical viewpoint in which the welfare of animals is protected.

2. Simply by changing the current unreasonably expensive research system , the number of animals sacrificed in experiments could be remarkably reduced. For example :

(1)Full disclosure of the contents of research involving animal experimentation not only within the research community but also to the general public.

(2)The establishment of a disclosure system concerning the development of new drugs, chemicals, medical research techniques, etc. , and of an assessment system in which concerned citizens participate.

(3) More open exchange of information between research facilities in Japan and around the world in order to minimize the occurrence of overlapping tests.

(4)The performance of detailed follow-up surveys and the establish- ment of a information collection and analysis system.

(5) The establishment of a legislative framework for effective animal protection and welfare.

3. In order to reduce the huge financial cost to the nation, the sacrifice of an enormous number of animals, and the tragic consequences of the mistaken medical/social system, it is essential to look for new ways of carrying out medical research. The alternatives to animal experi- mentation in a wide sense that we advocate involve reexamining both the system of research which involves vivisection behind closed doors and without independent scrutiny, and the current medical research ideology in which living things are considered as mechanical. We wish to see the subject of ethics concerning life and the environment tackled within the framework of general education, and we also favor the introduction of medical concepts in which due consideration is given to the welfare both of human beings and of other creatures.

World Report

Reports on the International Trend of Alternatives to Animal Experiments and on the 2nd World Congress on Alternatives and Animal Use in the Life Sciences

Trends in Animal Alternatives and overview of 2nd World Congress on Alternatives and Animal Use in the Life Sciences

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The present status of animal alternatives in scientific community, industry and regulatory agencies, and tends of alternatives will be reviewed in the panel discussion. A global overview on the result of 2nd World Congress on Alternatives and Animal use in the Life Sciences will be provided.

POSTER PRESENTATION

P-1.Modifying Effect of Anti-oxidation Agent on Photogenotoxicity

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For detecting the photogenotoxic materials, we have developed the *in vitro* assay systems covered three levels of genotoxic damages: single cell gel (SCG) assay, micronucleus (MN) test and mutation assay using mouse lymphoma L5178Y cells. In the plesent study, we assessed the photogenotoxicity of anti-oxidation agents: L-ascorbic acid (VC) and Butylated hydroxytoluene (BHT) using SCG and MN assays. Furthermore, the modifying effect of VC and BHT on photogenotoxicities of Titaniurn dioxide (TiO_2) were examined. VC did not represent positive results at 10mM in both SCG and MN assays. On the other hand, BHT induced the DNA migrations in the SCG assay at the concentrations showing growth inhibition. However. MN was not induced at the same concentrations, therefore. we concluded that the DNA migrations observed in the SCG assay was caused by cell killing effect of BHA. Phototoxicities was not observed in both VC and BHT, In the experiment designed for the modifying effect with ant-oxidants on photogenotoxicity, VC strongly suppressed the genotoxicity and growth inhibition caused by photo-excited TiO_2. Lipophilic BHT showed somewhat different modifying effect from hydrophilic VC. BHT suppressed genotoxicity of photo-excited TiO_2, but growth inhibition was not shown.

P2. Embryotrophic Activity of the Third Component of Complement in Post implantation Rat Embryo Culture

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We previously showed that the third component of complement (C3) functioned as an embryotrophic factor for rat embryos cultured in rabbit Serum. In the present study , embryotrophic activity of C3 was confirmed as selective Consumption by cultured rat embryos in the medium composed of rat Serum. Synthetic peptides of CR2 binding region in C3 had no effects on the growth of cultured embryos, suggesting that this region was not involved in the embryotrophic activity of C3.

P3. Whole Embryonic Culture Method as an Alternative of Developmental Toxicity Test(1)- In the Case of Aminopyrene

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Aminopyrine (AM) is metabolized to 4-alminoantipyrine (AA) in the liver of maternal animals, which reaches to embryos by passing through the placenta with the unchanged compound (AM). After intra-amniotic administration to rats on 11 days of gestation in an *in vivo* study, the mortality of embryos was 60% and the incidence of cleft lip was 20% by treatment with AM at a concentration of 200 μg/embryo. No change was observed by treatment with M at a concentration of 250 μg/embryo. We examined the independent direct effects of AM and J~ on rat embryos by using the whole embryonic cultures. In the whole embryonic cultures, rat embryos on the I I days of gestation were cultured for 72 hours exposed to AM or AA at 200 μg/embryo and 250 μg/embryo respectively for 2 hours.

Heart beating rate of embryos was decreased by 20% in the AM treatment group, leading to a decrease in blood circulation system. The crown-rump length was significantly decreased by 13%, and the total number of somites was also significantly decreased by 4%. The incidence of malformation was 100%, including 90% curved short tail, 80% inhibited elevation of head, 10% dead embryo and 40% cleft lip as the types of malformations. From these findings, it was confirmed that AM had a potent direct toxic effect on rat embryos. On the other hand, AA showed no difference between the experimental groups and the control groups.

P4. Effects of Salicylic Acid on the Nasal Placode in Cultured Rat Embryos

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The snout of rodents (rat, mouse) elevates from the 12 day of gestation. Inhibition of elevation by drugs was prominent in bilateral cleft lip. In the present study, in order to observe this correlation, we treated rat embryos in whole embryonic cultures an *in vitro* test system, with salicylic acid (SA) and observed the pattern of elevation of snout.

In whole embryonic cultures, was perfonned rat embryos of 11 days of gestation

were cultured rotatorily for 72 hours. The following indices were determined. a. heart beating rate of embryos, b. systemic blood circulation, c. crown-rump length and d protein contents (face). The dose of SA used was 600 μg/ml (2-hour treatment) showing the incidence of malformation of more than 85%. (a) The heart beating rate in embryos was significantly decreased during the period of treatment with SA compared with the control group (b) There was no difference in systemic blood circulation. (c) There was no difference in crown-rump length (d) The protein contents in the face was decreased by 47%after 60-hour culture and by 32% after 72-hour culture Although the cleft lip after this treatment was unilateral the incidence was 94%.

P5.Effects of Vitamin B or C on Cultured Rat Embryos

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In a series of our experiments, we are examining the effect of over dosages of vitamins on mammalian embryos. In the present study, we carried out a paradoxical experiment to examine whether over dosages of vitamins ineffective *in vivo* may show no effect *in vitro* either. The embryos were obtained on 1 1 days of gestation and cultured for 48 hours. Vitamins C and B I were administered during cultivation (48 hours) at concentrations of O, 500, 1000 and 2000 μg/ml. The results were as follows: (1) The heart beating rate was 190 $\pm$ 14.7 beats/min. in embryos treated with the highest dose (2000 μg/ml) of vitamins, showing no change. (2) There was no difference in the systemic blood circulation of the embryos between each vitamin group and the control group . (3) When the crown-rump length and the protein contents were compared as the indices of growth, no difference was observed. (4) The external morphology was normal, and no anomaly was observed. From the above results, there was no detectable effect of over dosages of vitamins on the cultured embryos.

P6.Effects of Thalidomide on Cultured Rat Embryos in Newborn Calf Serum(2)

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We have been examining the alternatives of rat serum for cultivation of mammalian whole embryos, to solve the major problem in embryonic culture. We have carried out whole embryonic culture using pre-colostrum fetal calf serum(PFCS) as one of the alternatives_ As a result, we have could established the stage of embryos to allow the cultivation using PFCS to test the effects of drugs.

Rat embryos on 12 days of gestation were cultured for 24 hours. As media tested, 100% PFCS(Mitsubishi Chemical, Inc.) was used, compared with 100% rat serum in control cultures. As a drug tested, thalidomide was added to the culture medium at 700 μg/ml and incubated for 24 hours after 2 hours of pre-incubation. After cultivation for 24 hours, embryos in thahdomide containing medium were compared with those in embryos in control cultures.

The heart rate of embryos treated with thalidomide showed no significant difference, compared with that in embryos in control cultures. In embryos in control cultures after incubation for 24 hours, the crown-rump length was 7.9 mm and the total number of somites was 15, while in embryos treated with thalidomide, the embryonic blood circulation tended to be inhibited. There were no differences between embryos treated with thalidomide and those in control culture, in the crown-rump length, the total number of somites and the morphological anomaly. From the above results, it was considered that, when embryos were cultured in medium with PFCS, the effect of thalidomide may be lowered to appear compared with the control cultures with rat serum. As one of the reasons, there may be some differences in the components between rat serum and PFCS. An effective dose of thalidomide may due to the content of drug binding protein such as albumin in serum.

P7.Effects on Thalidomide on Cultured Rat Embryos- Cultured in Medium with Human Lympoblast Microsomes (Human UGT1*6)

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When rat embryos were treated with thalidomide in culture, the degree of malformation produced was slight, but more severe disorders developed when embryos were pretreated with human P-450 containing S-9. From these results, it was considered that human-type (derived) P-450 may have a marked effect on the expression of drug effects. In the present experiment, we examined the effect of pretreatment with human glucuronate conjugating enzyme in rat embryo culture.

Rat embryos on 12 days of gestation were cultured for 48 hours. Thalidomide was

added to culture medium (100% rat serum) at a concentration of 700 μg/ml. Before 2 hours of treatment with thalidomide, human-type glucuronate conjugating enzyme (Dai-ichi Kagaku Yakuhin Co., Ltd.) was added to the culture medium to examine its effect on the expression of thalidomide effect.

In embryos treated with this enzyme, the heart beating rate was 160 to 190 beats/min. from the start to the end of culture, and the blood circulation was also good. No abnormality was observed in the development of morphology at 24 hours of culture, but after 48 hours of culture, the embryos treated with thalidomide alone showed a decrease by about 200I. in the total number of somites, compared with the untreated group with a decrease by about 15% in the total number of somites. The edema and hematoma of the forefeet of embryos showing delayed growth of the whole body were also observed. However, embryos exposed to thalidomide after pretreatment with glucuronate conjugating enzyme showed the same results as the untreated embryos, in the crown-rump length, the total number of somites and external morphology. In addition, the embryos treated with glucuronate conjugating enzyme alone showed no significant difference from those in untreated embryos. From the above results, it was considered that the metabolite of thalidomide by glucuronate conjugating enzyme had no detectable effect on embryos cultured under these conditions.

P8. Whole embryo Culture for Assessment of the Developmental Toxicity Induced by the Drug Interactions

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The present study was performed to assess the developmental toxicity induced by the drug interactions *in vitro*, using 5-Fluorouracil (5-FU) and competitive inhibitors of 5-FU degradation, uracil. Rat embryos (Crj:CD) on embryonic day 9 (ED 9; plug day = ED 0) were cultured for 48 hours in media containing rat S-9 (10μl/ml), 5-FU (0 - 0.25 μg/ml) and uracil (0 - 0.5 μg/ml). Two hours before starting culture, rat S-9 and uracil were added to media and I hour after, 5-FU was added. Embryos cultured were examined for morphological development under a dissecting microscope. To compare the developmental effect of the co-administration *in vivo* with that of the coexposure *in vitro*, uracil (s.c.) and 5-FU (i.p.) were administered to dams on gestational day 9 (GD 9; plug day = GD 0) and embryos were examined on GD 11 . In *in vivo*, co-administration of 5-FU with uracil increased markedly the incidence of malformations and embryonic mortality. In *in vitro*, the incidence of malformations

induced by 5-FU in media containing S-9 was lower than that without S-9. Co-exposure of 5-FU with uracil under the existence of S-9 showed a higher incidence of embryonic malformations than that after a 5 -FU exposure with S-9. Uracil has no adverse effect on embryonic development in *in vivo* nor *in vitro*. In summing up all these results and other data[1)], it is suggested that uracil inhibits the 5-FU degradation through the same metabolic pathway in whole embryo culture as well as *in vivo*. Thus, the whole embryo culture can detect the developmental toxicity of co-administration of 5-FU with uracil.

P9. Evaluation of *In Vitro* Toxicity of MEIC Compounds on Human Natural Killer Cell Function

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To search for a method of detecting immunotoxicity of chemicals, effects of MEIC compounds were examined on cytotoxic function of a human natural killer (NK) cell-rich population cultured *in vitro*. Thirty two of 50 MEIC compounds showed apparent inhibition on NK cell-mediated cytotoxic activity, and their IC50 Values were highly correlated (r=0.92) with previously reported ED50 values for the inhibition of human squamous carcinoma cell growth. In comparison with reported IC50 values of hepatocyte, neuron and MDBK cells, the compounds were grouped into three categories; 1) effective equally to all types of cells (7/30), 2) effective preferentially to NK cells (4/30), 3) ineffective to neuron (5/30).Thus this study points to the possibility that a combination of *in vitro* assays with differentiated cells may be beneficial in establishing the alternative method for animal testing. This research was in part by the special coordination fund from the Science and Technology Agency.

P10. Effect of Hapten Application on Langerhans Cells Migration from Skin Explant

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Using skin explant cultures of C3H/He mice, we studied the effect of a hapten and an irritant on Langerhans cells (LC) migration. After 24 - 72h of culture , migration

of various cells, including la' LC, from mice ears was observed. This cell migration (LC migration) was significantly increased when mice were treated with 3% TNCB at 6 - 72h before the ears were taken. Among these migrating cells, only la; cells appeared to function as antigen-presenting cells because they were capable of initiating a delayed type hypersensitivity response when injected into the foot pat of syngeneic mice. The enhanced cell-migration was constantly observed when mice were treated with TNCB . In contrast, no significant increase in cell migration was observed by SLS treatment. These results suggest that only hapten treatment but not irritant treatment may stimulate the migration of LC which is capable of initiating the delayed type hypersensitivity.

P11. Evaluation of *In Vitro* Methods as an Alternative to Phototoxicity Test

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Many *in vitro* phototoxicity methods have been developed by both academic and industrial laboratories. In this study, we selected four phototoxicity methods ; 1) neutral red uptake phototoxicity method using BALB/3T3 and NB1RGB , 2) red blood cells photohemolysis method, 3) hemoglobin photooxidation method, 4) measurement of singlet oxygen; and evaluated as an alternative method to phototoxicity test. More than thirty substances were tested and the results of those substances were compared with *in vivo* phototoxicity in guinea pigs. The correspondences of four *in vitro* methods and *in vivo* data were each satisfactory to justify further study. In the neutral red uptake phototoxicity method, the sensitivity of correspondence using BALB/3T3 was better than NB1RGB. The ratio of false negative obtained by both hemoglobin photooxidation method and the measurement of singlet oxygen were lower than other methods . These results suggest that the four methods evaluated in this study may be useful as an alternative to phototoxicity test, but we need more investigation considering the mechanisms of each method in order to conclude.

P12. Alternative to Primary Draize Skin Irritation Test using Three-Dimensional Cultured Human Skin Model

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In this study, we evaluated an alternative to the primary Draize skin irritation test using three-dimensional cultured human skin model. Toxicity of about 15 materials included hydrophobes were assessed. As a result, severe irritants (more than 4 points Draize score) showed about 100% of cytotoxity, and weak irritants (less than 2 points Draize score) showed lass than 20% of cytotoxity. We could distinguish between severe and weak irritants.

P13. Evaluation of Alternative to Primary Draize Skin Irritation Test using Skin Model(EpiDerm)

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EpiDerm consists of normal human-derived epidermal kerati- nocytes (NHEK) which have been cultured to form a multilayererd, highly differentiated model of the human epidermis. Because of this human skin-like structure, it is expected to get better results for *in vitro* skin irritancy test. Especially, in primary Draize skin irritation tests, multilayered cornified layers are very important to block the invasion of test materials. In general, even if the toxicity of only one test material is evaluated, we have to assay at various concentrations or exposuring times. It takes much labor and cost. But it would be better to simplify them. So, in this presentation, we show the easier method and the result by conducting tests at the single point of the concentration and exposuring time of each test material. We conducted two experiments by using EpiDerm as follows; 1)Typical 10 kinds of surfactants were evaluated at some points of exposuring time to get the MTT50 values. 2) 20 kinds of cosmetic products were evaluated at the single point(1000/0 concentration and 24 hrs exposure). These results were compared with the primary Draize skin irritation test using rabbits. In the experiment 1), obvious false-negative materials and a false-positive one were observed. On the whole, we could discriminate between strong irritants and weak or non-irritants, but could not discriminate between weak or non irritants and moderate ones. In the experiment 2),

the coefficiency of correlation for 18 materials was relatively low (r = -0.608), but the coefficiency of correlation for 16 materials excepted 2 materials, which were hair liquids evaluated as false-positive, became good (r = - 0.869). As a result, the method conducting at a single point of exposure condition can give us a more convenient application for alternative methods.

P14. An Attempt at Cell Recovery Test Utilizing *In VItro* Reconstructed Cell System

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We compared cell recovery test utilizing *in vitro* reconstructed cell culture system (MATREX) and a test without cell recovery. The cytotoxicity of denture base resins to a tissue model which is human dermal cells within a collagen gel, was tested and evaluated by MTT and adenosine triphosphate (ATP) assay. Four heat- curing type resins were hardly cytotoxic to the tissue model. But, seven cold- curing type resins yielded strong cytotoxicity. The tissue model system with cell recovery seems to allow to show different aspect of materials cytotoxicity. Further improvement of the *in vitro* reconstructed system is necessary.

P15. Simultaneous evaluation of Cytotoxicity in the Neutral Red, MTT, and Crystal Violet Staining Assays in the Same Microplate

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An improved technique was established in the cytotoxicity method to simultaneously evaluate cell viability in three toxic markers,the neutral red assay (NR), the MTT assay (MTT), and the crystal violet staining (CVS)assay, in the same microplate. We have combined these assays by doing the NR assay followed sequentially by the MTT assay, and then by the CVS assay. When this method (NMC method) is compared with each original method, a good correlation is found with regard to the absorbance at different viable cell concentrations. In the evaluation of cytotoxicity of sodium lauryl sulfate (SLS) ,the dose-response curves in

the NMC method coincide with those of each original method. On the other hand,some test chemicals,which have effects on each toxic marker,give the different dose-response curve patterns depending upon their specific mechanism of action in the NMC method. These results suggest that the NMC method is capable of demonstrating the same results of original NR, MTT and CVS methods. Furthermore,this method is a promising technique to investigate the toxic mechanisms of test chemicals.

P16. English abstruct was not submitted.

P17.A New Fluorometric Assay for Cell Proliferation and Viability Using Nucleic Acid Staining Agents

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A new fluorometric assay for cell proliferation and viability was established using a nucleic acid staining agent, PO-PRO-1 which passed through membranes of dead cells but not those of viable cells. The combination of the agent and a detergent, Triton X-100 was demonstrated to be suitable for the fluorescence assay of cell viability and cytotoxicity. The remarkable feature is to be able to measure rapidly (about 5 min), conveniently and multiply on microplates without incubation time, washing procedures nor using radioactive materials nor organic solvents. This assay could be widely applicable to cytotoxic evaluations of compounds and bioactive materials in many of fields such as medicine, pharmacy and agriculture.

P18.Histlogical Changes of Chorioallantoic Membrane (CAM) Caused by Surfactants

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In this study, histological changes of CAM caused by surfactants were investigated by light and scanning electron microscopy. Anionic surfactants

treatment caused cellular desquamation and dilation of capillary,while cationic surfactants treatment resulted in cell death and tissue destruction. Nonionic surfactants treatment did not cause any effect except for slight dilation of capillary.

As a result, various kinds of histological changes such as cellular desquamation, dilation of capillary and cell death caused by some surfactants on CAM were observed by light and scanning electron microscopical technique.

P19. An Assay for Developmental Toxicity with Cultured Cladoceran Eggs *in vitro*

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An *in vitro* assay using *Daphnia magna* eggs was endeavored for the detection of developmental toxicity. Parthenogenetic eggs removed from the brood chambers of female adults were cultured individually in 96-well microtiter plate with Elendt M7 medium at 23°C. Embryonic development proceeded completely *in vitro* with more than 95% hatchability. Egg development time *in vitro* was 2 days which was almost equal to that in the brood chamber of the mother. Ethylenethiourea (ETU), a teratogenic compound, was investigated for the toxicity on development of the eggs. Isolated eggs were cultured in the presence of ETU during the period of embryonic development for 3 days. Treatment with ETU at 20-40 μg/ml induced morphological abnormalities in the cladoceran carapace. Partheno-genetic eggs of Cladocera are genotypically identical so that they are considered to be suitable biological materials for a toxicity test on aquatic pollutants.

P20.Experimental Study Using Turkey as Alternative Animals

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Furazolidone (FZ) is well known as an antibacterial agent which induces dilated

cardiomyopathy (round heart disease) in duck), chicken2) and turkey3}. In this study, we used turkey poults in order to push on the alternative method to mammals and observed biochemical, physiological and pathological changes of the hear t and liver . From these results we discussed whether turkey can be used as alternative animals, especially focused on the genesis of cardiomyopathy.

Materials and Methods:

FZ was given to turkey poults from 2 weeks posthatched through stomach tube twice a day at 8:00 and 16:00, 60 mg/kg/day. The turkeys were weighed every morning and the doses of FZ were calculated from the body weight. They were sacrificed at 3 weeks (5 weeks after birth) and 5 weeks (7 weeks) after treatment of FZ. Electrocardiograms (ECGs) were recorded, and serum LDH, GOT, GPT and ALP values were measured in this time. Then, the heart and liver were removed, and were observed pathologically. Control group, treated with 0.5% CMC-Na was used in the same time course.

Results and Discussion:

In the 3 weeks-FZ treated group, relative heart and liver weights were heavier than the control . Regressive changes such as fatty degeneration and necrosis in the hepatic cells were observed pathologically. Although left ventricular free wall(LV wall) was significantly thin to the control, but no pathological changes were observed in the myocardial cells. Only serum GOT revealed high value. In the 5 weeks-FZ treated group, the whole body weight decreased, but the relative heart weight did not show any difference to the control. However, the thickness of LV wall and ventricular septum were significantly thin due to degenerative change in myocardial cells. These results suggest that the dilated cardiomyopathy in FZ-treated turkey probably due to some metabolites created in the liver by FZ. Furthermore, FZ treated turkey may be used as an alternative animals for cardiomyopathy models.

P21.Pharmacological and Toxicological Studies Using Chick Embryos(10) Study on Hypothyroid Models

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It is well known that many patients with functional abnormalities of thyroid

gland show unexpected responses against cardiovascular drugs, especially cardiotonics. In order to predict the unexpected responses to cardiovascular drugs, some experimental animals with peculiar sensitivity of the heart such as hypothyroidism are necessary. In the present study, we tried to produce hypothyroid model in chick embryos. Furthermore,the effects of digoxin to this model were studied to evaluate whether this model can be used as alternative method.

Materials and Methods:

Exp. 1

Thiamazole (INN, 0.6 - 6mg/egg) was injected into the albumen of fertile eggs of White Leghoms on the 9th day of incubation. On the 16th day of incubation, electrocardiograms(ECGs) of chick embryos were recorded under anesthesia(urethane+ α-chloralose) up to 60 min every 5-10 min Heart rate(HR) was calculated from RR intervals, as reported previously). Then, thyroids, heart and liver were removed, weighed and fixed in 10 % buffered forrnaline for pathological observations.

Exp.2

After 25 μg/egg of digoxin was injected into the air sac of INN-treated eggs on the 1 6th day of incubation, ECGs were recorded up to 60 min. Results: Survival rate and body weight in the INN-treated embryos decreased in the dose dependent manner. Relative heart and liver weight did not show any difference to those of control. In spite of retardation of development in follicles of the thyroid glands in INN-treated embryos, they were heavier and larger than the control. HR of the INN-treated 16th day embryos showed tendency to bradycardia and it was emphasized after additional digoxin injection. In conclusion, physiological and pathological responses in chick embryos treated with INN showed similar changes in those of mammals. Accordingly, INN-treated chick embryos may be applicable as an alternative animal to predict unexpected effects of cardiovascular drugs.

P22. Pharmacological and Toxicological Studies Using Chick Embryos(11) Effect of Temperature in Hemodynamic Changes of chick Embryos

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Echocardiograms including color Doppler systems as well as electrocardiogram has been widely used as the most fundamental diagnostic tools for heart diseases in human. We have been tried to the chick embryonic heart and have been repor ted usefulness of this technique as alternative methods to mammals. In this study, we investigated effects of temperature to hemodynamics of chick embryos.

Materials and methods :

Fertile eggs of White Leghorns were used on the 18th day of incubation. After injection of anesthetics (urethane+α-chloralose), egg shell round air sac was removed and the air space was filled with warm jelly in order to get good coupling to probe. Color Doppler system(YHP77520A) with 5 MHZ probe were used in this study. After confirmation of vertebrae and heart in chick embryos, FFT patterns in descending aorta which runs parallel to vertebrae were recorded. Maximum velocity (MV), acceleration time (AT), flow integral (FI) and RR intervals were calculated from FFT patterns. Bipolar leads of electrocardiograms were recorded simultaneously.

Results and discussion:

FFT patterns of chick embryos were recorded at 33, 37 or 39°C. HR became slower and MV became lower at 33°C than those of 37°C. Myocardial contractility reduced in this low temperature. However, no remarkable changes are noted on FI values which reflect changes of blood flow volume. On the other hand, the chick embryos exposed to high temperature at 39°C led to rapid heart rate, and MV increased, reflecting high contractility of myocardium.

In conclusion, in order to observe hemodynamic changes in chick embryos, experimental circumstances are important. Color Doppler echocardiography may be applicable to estimate the cardiac functions as alternative methods.

P23. Detection of Environmental Pollutants Using Cultured Cells

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Environmental pollutants have generally been monitored by chemical analysis. To assess the toxic effects of those pollutants, however, there are some limitations for the chemical analysis on the specific indicators. Therefore, biological assays

including experimental animal are needed. In this study, we tried to assess by the cytotoxicity test for the toxic pollutants on the river water, water from cooling towers and ashes from waste incinerators. Colony formation assay was performed because of its sensitivity. Five cell lines were preliminary screened, finally BALB 3T3 and VERO cells were used. Test water was filtrated, then prepared for medium. Other samples were extracted with solvent.

Colony formation assay using BALB 3T3 and VERO cells could be used to distinguish clearly between polluted and clean ones. Colony formations were decreased depending on the extent of water pollution from the upper- to the lower-stream of Sakawagawa, Kanagawa. The water from cooling tower collected from the top of building in the urban area also showed low colony formations suggesting air pollutant. This tendency was agreed with chemical indicators such as COD, ammonium nitrogen and total organic carbon.

P24. Evaluation of an Alternative to Skin Irritation Test (2): Determination of Pro-inflammatory Cytokine Gene Expression

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The effect of cosmetic surfactants on pro-inflammatory cytokines interlenkin (IL) - Icc and IL-8 release from human keratinocyte cell culture has been studied to investigate the feasibility of using this effect for the prediction of the irritation potential of chemicals. The cytokine gene expression was determined by a new system, ABI PRISM 7700 sequence detector (Perkin Elmer).

After exposed to surfactants, cultured human keratinocytes were solubilized with isogen (Nippon Gene). The cell extract was subjected to chloroform extraction, isopropanol, precipitation and ethanol precipitation. Obtained total RNA was transformed in cDNA strand using oligo(dT) primer and MMLV reverse transcriptase. Copy numbers of IL- Icc and IL-8 cDNA were determined by ABI PRISM 7700 sequence detector.

IL-8 gene expression was dramatically up-regulated by sodium dodecylsulfate and polyethylene glycol fatty acid ester compared with non-treated control. IL-1a expression was also stimulated, but the increment of the expression was less than that of IL-8 expression. The sensitivity and accuracy of these results were better than the data obtained from agarose-gel electrophoresis.

This system is a good method for determination of amount of gene expression than Northern blotting or image analysis in points of experimental period and accuracy of the data. The combination of this system and cytotoxicity tests may apply to alternative to skin irritation test.

P25. First Validation Study of Cyototoxicity Tests under JSAAE Project(Correction)

Tadao OHNO[1] and 98 co-workers[2]

[1]RIKEN Cell Bank, The Institute of Physical and Chemical Research (RIKEN) and [2]The list is available on request.

Since a new violation of previous agreements were found in the data files on the first step inter-laboratory validation study on 5 cytotoxicity assays organized by JSAAE, we correct Table I which has once been given in the last annual meeting of

Assay	Cells	Number of data files a*	b	c	d	e	Performance rate of runnings$ b/a	candidate files$$ d/a	finally accepted e/a
CF	HeLa.S3(sc)	168	130	14	116	100	77%	69%	60%
	BALB 3T3 A31.1.1	168	149	0	149	126	89	89	75
CV	HeLa.S3(sc)	112	98	14	84	75	88	75	67
	CHL	112	97	14	83	78	87	74	70
LDH-1#	HeLa.S3(sc)	126	70	13	57	39	56	45	31
	SQ-5	126	70	20	50	25	56	40	20
LDH-2A	HeLa.S3(sc)	126	68	14	54	26	54	43	21
	SQ-5	126	67	28	39	28	53	31	22
LDH-2B	HeLa.S3(sc)	126	62	21	41	18	49	33	14
	SQ-5	126	62	28	34	20	49	27	16
LDH-2C	HeLa.S3(sc)	126	68	14	54	26	54	43	21
	SQ-5	126	61	21	40	25	48	32	20
MTT	HeLa.S3(sc)	140	123	28	95	89	88	68	64
	SQ-5	140	117	28	89	83	84	64	59
NR	HeLa.S3(sc)	168	154	28	126	108	92	75	64
	NRCE	168	139	7	132	103	83	79	61
Total number of files		2184	1535	292	1243	969			

* a, expected; b, submitted; c, unacceptable; d, acceptable before ED50 calculation; e, finally accepted by the logistic analysis program, LAP-JSAAE, and the intra-laboratory variation analysis.
$ runnings = b/a x 100
$$ candidate files = d/a x 100, accepted by The Working Group before ED50 calculation.
Different series in the LDH release assay, see Materials and Methods.

Table I. Data files of final definitive tests submitted to The Working Group

JSAAE. However, conclusion was not essentially changed. Considering time consuming, simplicity, and precision in each assay, we recommend that CV assay is the most practical as the cytotoxicity assay.

The figures in the table were updated and amended after our presentation in the meeting INVITOX'94 (Zurich, Switzerland, 1994) to which data were submitted and published in the proceedings (Ohno et al., 1995). Definition of performance rates were changed in order to reflect reality from that described in the proceedings. Other than these final test data files, cumulative 157 laboratories submitted 2275 data files of preliminary tests.

P26. Inter-laboratory Validation of Alternative Methods to Eye Irritation Test for Safety Evaluation of Cosmetic Ingredients (1) Evaluation of Chorioallantoic Membrane (CAM) Test

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A chorioallantoic membrane (CAM) assay evaluates the blood vessel reaction and the damage to the CAM of fertile hen's egg. Two types of CAM assays, the macroscopic observation method (HET-CAM) and trypan blue staining method (CAM-TB) were evaluated as alternative methods to Draize eye irritation test by using 38 cosmetic ingredients and physiological saline. Test procedures were controlled under the same SOP among 5 laboratories. The inter-laboratory variations of both methods were not small though the rank correlation was relatively high among the values obtained by 5 laboratories. Their average values were compared with the maximum average score (MAS) of *in vivo* eye irritation test. The correlation coefficient (r) between HET-CAM scores and the maximum average scores (MAS) was 0.688. Though the results indicate that the simple linear regression may not be appropriate for HET-CAM, the Spearman's rank correlation coefficient (rs) turned out to be rather high (rs=0.802). On the other hand, the results obtained by CAM-TB showed a good correlation with MAS when the test chemicals were classified by their physical property (liquid or powder) [All samples: r=0.718, Liquid: p0.801, Powder: r=0.926]. Other characteristics of these methods, also, have become apparent through this project. These are (1) both methods are applicable to

most of the test chemicals under the same conditions as *in vivo* test, (2)colored substances should be evaluated carefully, 3) the HET-CAM requires experimental skill, 4) powder and liquid should be separately evaluated by different regression equations in the CAM-TB. This study was partly supported by a Research Grant for Health Sciences, MHW.

P27. Eye Irritation Test for Safety Evaluation of Cosmetic Ingredients (2) Evaluation of Red Blood Cell Hemolysis Test

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A hemolysis test evaluates membrane damage by measuring hemoglobin leaking from red blood cells. This method was evaluated as an alternative method to Draize eye irritation test Thirty-nine test substances were used and test procedure was controlled under the same SOP among six laboratories. The concentration of test substance showing 50% hemolysis(HC50) was determined and HC50 values were compared with *in vivo* results. Good inter-laboratory reproducibility was obtained for water-soluble substances. The correlation coefficient and Spearman's rank correlation between HC50 and the maximum average scores(MAS) was 0.631 and 0.641 respectively. The HC50 values were not obtained for water-insoluble or colored substances. The acid caused denaturation of leaked hemoglobin and interfered the determination of HC50. Also, several other characteristic of this method have become apparent through this study. These are 1) this method is easy handling and inexpensive, 2)this method is notappiicable to water-insoluble substance, 3)variances of HC50 become large when selection of solvent differ among laboratories, 4) colored substance should be evaluated carefully. This study was partly supported by a Research Grant for Health Sciences. MHW.

P28. Inter-laboratories Validation of Alternative Methods to Eye Irritation Test for Safety Evaluation of Cosmetic Ingredients (3)Evaluation of Hemoglobin Denaturation Test

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The hemoglobin denaturation (HD) test was evaluated as an alternative method to the eye irritation test by using three indices of protein denaturation such as a test chemical concentration inducing a 50% HD of positive control (RDC50), a relative HD ratio at 1% of the chemical (1%RDR) and a relative change of maximum absorption wavelength (1%λmax). Thirty-nine test chemicals were commonly used in this project. The inter-laboratory variation of HD test among participating laboratories (6 to 8) was within a practically tolerable range. The *in vitro* test results were compared with the *in vivo* eye irritation test results and showed a reasonably good correlation. The correlation coefficients (r) between the *in vivo* maximum average score and log (RDC_{50}), l%RDR or 1%λmax were -0.91, 0.67 or 0.79, respectively. Also, some limitations have become apparent, i.e.(1) HD test cannot be applied to colored chemicals with a strong absorption around 418nm, (2) water-insoluble chemicals cannot be evaluated by RDC_{50} and 1%RDR, (3) HD test cannot be applied to strong acids which exceeds the buffering potential of a phosphate buffer solution, 4) HD test cannot detect the eye irritation potential caused by factors other than protein denaturation. As the HD test evaluates only the potential of protein denaturation by chemicals, it is not appropriate to predict eye irritation potential solely by the HD test. Nevertheless, HD test results matched the *in vivo* irritation scores fairly by itself. In addition, this method is easy to handle, and results can be obtained in a short time. This study was partly supported by a Research Grant for Health Sciences, MHW.

P29. Inter-laboratory Validation of Alternative Methods to Eye Irritation Test for Safety Evaluation of Cosmetic Ingredients (4) Evaluation of Skin^{2TM}ZK1100 and ZK1200 Models

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Yoriko KADOTA[1,8], Norikazu IKEDA[1,8], Naoki HAYASHI[9], Minoru KATAGIRI[9], Akihiko HIROSE[10], Toyozo KANEKO[10], Yasuo OHNO[11]

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Skin[2TM] ZK1100 and ZK1200 models are human dermal tissue cultures supplied by Advanced Tissue Sciences (ATS). These models were evaluated as alternatives to the Draize eye irritation test in rabbits. The thirty-nine chemicals used in this project. ZK1100 model testing was conducted according to the original kit protocol provided by ATS. ZK1200 model testing followed the Tissue Equivalent Assay (TEA) protocol developed by Procter & Gamble. The endpoints of these methods were determined as 50010 cell viability in a MTT assay. ZK 1100 model results showed an average coefficient of variation (C.V.) of 0.117 - 1.328. Pearson's correlation with maximum average score (MAS) from the Draize tests was r=-0.70. ZK1200 model results showed an average C.V. of 0.318-1.192, and Pearson's correlation with the Draize MAS values was r=-0.63. When a score of fifteen on the Draize scale (0.110) was set as the breakpoint for classification of eye irritancy on Cooper's plots comparing the *in vitro* and Draize data, the ZK1100 model results showed five false positives and four false negatives; the ZK1200 model results showed four false positives and no false negatives. These studies were partly supported by a Research Grant for Health Sciences, MHW.

P30. Eye Irritation Test for Safety Evaluation of Cosmetic Ingredients (5) Evaluation of Matrex[TM]

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The living dermal model (LDM) used for the system is consisted of human fibroblasts mounted in collagen gel, and shows a 3-dimensional structure. The method was evaluated by three phases inter-laboratory validation project for cosmetic ingredients. Both the EC50 value measurement and scoring method (improved method for EC50 value measurement at the third phase of validation) were applied. Both test procedures were controlled under the same SOP among all the laboratories participated. We used 39 test chemicals (including physiological saline solution) in this project . As results (1) all substances (either soluble or insoluble to water) were able to be evaluated by MATREX. (2) MATREX score was nearly equal to the EC50 value, the correlation coefficient between both data was 0.983. 3) variances of the data obtained were very small for both procedures. 4) The correlation between *in vitro* data and Draize maximum total score was examined, the correlation coefficient was 0.67. This study was partly supported by a Research Grant for Health Sciences, MHW.

P31. Eye Irritation Test for Safety Evaluation of Cosmetic Ingredients (6) Evaluation of Cytotoxicity Test on CornePack®

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The cytotoxicity test (CornePack ®) using neutral red uptake assay on normal rabbit corneal epithelium cells and serum-free medium was evaluated as an alternative method to Draize eye irritation test. Concentrations of the lest substances that caused 50% inhibition of cell growth (NR_{50}) was calculated in this assay. The procedures were controlled under the same SOP among 5 to 7 laboratories. Thirty nine test chemicals used in this project. All test samples were able to be evaluated by CornePack ® . Good inter-laboratory reproducibility was obtained for the chemicals in this test method. The *in vitro* test results were compared with *in vivo* eye irritation test results. The correlation coefficient(r) between log(NR_{50}) values and maximum average scores (MAS) was -0.583. Several other characteristics in this method have become apparent through this project. These are (1) NR_{50}s obtained by the most of the substances are smaller than those obtained by the other tests using established cell lines (HeLa, SIRC, CHL), (2)

CornePack ®) is applicable to dyes and lipids, 3>response to anionic detergents is different in this test from the other cell culture methods mentioned above . This study was partly supported by a Research Grant for Health Sciences, MHW.

P32.Inter-laboratory Validation of Alternative Methods to Eye Irritation Test for Safety Evaluation of Cosmetic Ingredients (7)Evaluation of Cytotoxicity Tests on SIRC Cells

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We evaluated cytotoxicity tests on SIRC cells using two types of common assay methods, the neutral red uptake assay (SIRC-NR) and the crystal violet staining assay (SIRC-CV) as alternatives to the Draize eye irritation test. Each of 39 cosmetic ingredients was tested at 5 to 7 laboratories. The test procedures for SIRC-NR and SIRC-CV were controlled under common SOPs. These methods were applicable to all substances used including water insoluble substances, a dye and lipids. EC_{50}, an effective concentration of the sample which results in 50% of cell viability rate, was determined for each ingredient. As confirmed EC_{50} value of SIRC-CV was quite similar to that of SIRC-NR, we mainly described the result of SIRC-NR. Inter- laboratory reproducibilities were good for both methods (SIRC-NR: cv = 0.315, SIRC-CV: cv = 0.328). There was a good correlation for 30 ingredients with a correlation coefficient of -0.8 1 6 between logEC_{50} values and Draize maximum total scores. If excluded strong acids, strong bases and primary alcohols, it increased a correlation to -0.916. A part of this study was supported by a Research Grant for Health Sciences, the Ministry of Health and Welfare.

P33. Inter-laboratory Validation of Alternative Methods to Eye Irritation Test for Safety Evaluation of Cosmetic Ingredients (8) Evaluation of Cytotoxicity Test on HeLa Cells

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The cytotoxicity test using 3- [4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) on HeLa cells (HeLa-MTT test) was evaluated as an alternative method to Draize eye irritation test. The effective concentration that caused 50% inhibition of cell growth (EC50) was calculated using the enzymatic reduction of MTT to formazan in HeLa cells. Thirty-nine test chemicals used in this project. All test samples including water insoluble substances,dyes and lipids were able to be evaluated by HeLa-MTT test. Good inter-laboratory reproducibility was obtained for chemicals in this test method. The *in vitro* test results were compared with *in vivo* eye irritation test results. The correlation coefficient (r) between log(EC50) values and maximum average scores (MAS) was -0.799. Exclusion of strong acids,alkalis and primary alcohols increased the correlation to -0.922, Special care must be needed for the substance which directly reduces MTT. This study was partly supported by a Research Grant for Health Sciences, MHW.

P34. Inter-laboratory Validation of Alternative Methods to Eye Irritation Test for Safety Evaluation of Cosmetic Ingredients (9) Evaluation of Cytotoxicity Test on CHL Cells

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A Chinese hamster lung cell lines - crystal violet staining method (CHL-CV) is common test to evaluate the cytotoxicity of chemicals. We evaluated CHL-CV as an alternative method to Draize eye irritation test using 38 cosmetic ingredients and physiological saline. Test procedures were controlled under the same SOP among 4 to 7 laboratories. We determined the concentration of test chemicals that decreased the viability of CHL cells to 50% of control level(EC_{50}). The results of *in vitro* were compared with these of *in vivo* eye irritation test.

The correlation coefficient (r) and the Spearman's rank correlation coefficient (rs) between log(EC_{50}) scores of CHL-CV and the maximum average scores (MAS) were -0.729 and 0.709, respectively. Exclusion of the data of strong acids, alkalines and

primary alcohols increased the correlation coefficient to -0.864. Several other characteristics of this method, also, have become apparent through this project. These are (1) CHL-CV could be applied to all chemicals included dyes and lipids in this study,(2) water-insoluble chemicals showed widely results among laboratories. This study was partly supported by a Research Grant for Health Sciences, MHW.

P35. Inter-laboratory Validation of Alternative Methods to Eye Irritation Test for Safety Evaluation of Cosmetic Ingredients (10)Evaluation of EYTEX

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EYTEX ® method is an *in vitro* method to predict the potential of ocular irritation of chemicals and formulations. The scientific hasis of this method is to determine changes in a highly organized macromolecular matrix which occur upon exposure of the matrix to chemical irritants and are similar to changes which occur in the cornea h viva Changes in the matrix result in turbidity which is quantitated spectrophotometrically to establish EYTEX ® /Draize equivalent and irritancy classification of test samples.

Test procedures were controlled under the same SOP among 5 to 7 laboratories. Thirty nine test chemicals used in this project Good inter-laboratory reproducibility was obtained (C.V. = 0.208). The correlation coefficient between EYTEX® scores and *in vivo* maximum average scores GMAS) was 0.313. However, taking the irritation-ranking estimation based on EYTEX ® Classification Method, 38 test results, among 54, corresponded to *in vivo* results, and corresponding ratio was 70.4%. Other characteristics of this method, also, have become apparent through this project These are (1) this method is applicable to most of test chemicals under the same conditions as *in vivo* test, (2) this method does not require high experimental skills and special facilities, (3) colored substances should he evaluated carefully. This study was partly supported by a Research Grant for Health Sciences, MHW.

P36. Inter-laboratory Validation of Alternative Methods to Eye Irritation Test for Safety Evaluation of Cosmetic Ingredients (11) Results of Draize Eye Irritation Test and Implication of Their Validation

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We conducted Draize eye irritation test for the 39 compounds selected for the validation of *in vitro* alternatives for Draize test. The chemicals include 18 surfactants, 3 primary alcohols, one pigments, 3 acids, 3 animals, one esters, 5 water insoluble substances, one reducing agent, and one chemically unstable chemical. MAS values for most of the test chemicals were smaller in the present experiments than those expected from reported or in-house data used for the selection of the test chemicals. In the case of 10% solution, number of test substance classified into non irritants($0 \leqq MAS<0.5$), slight irritants($0.5 \leqq MAS<15$), mild irritants($15 \leqq MAS<25$), moderate irritants($25 \leqq MAS<50$), and severe irritants($50 \leqq MAS$) were 9, 9, 4,7, respectively. On the other hand, in the case of 0.1-100% solution, those were 12, 17, 4, 11 and 13, respectively. Those data including scores on cornea, iris and conjunctiva at each observation time until 2 weeks were conveyed to the Expert Committee of Validation for comparison after confirmation of the *in vitro* data. Means of coefficients of variation of non, slight, mild, moderate, and severe irritants were 0.0, 0.68. 0.30, 0.75 and 0.16, respectively. Data for the unstable chemical(m-phenylene diamine) were excluded when we compare the results to *in vitro* data. For the purpose to know the cause of the variation effects of 10% SLS on the eyes of Japanese White and New Zealand White rabbit were observed by several experienced researchers. Variation becomes large at 2-3 days after the application of SLS. Differences depending on the rabbit strain and the observes were smaller than those depending on individuals of rabbits in the response to irritants.

This study was partly supported by Research Grant for Health Sciences, MHW and The Japanese Society for Alternatives to Animal Experiments.

P37. Development of Drug Metabolism Simulator Using Three-Dimensional Culture of Hepatocyte - Studying of Induction on

Drug Metabolic Enzymes Using PUF/spheroid Cultured Hepatocyte

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The cultured hepatocytes converted 7-Ethoxycoumarin (7-EC) to 7-Hydroxycoumarine (7-HC) which was O-deethylation of 7-EC (phase I reaction). The 7-EC metabolic activities in monolayer and PUF/spheroid culture were almost same and maintained for 14 days. Treatment of hepatocytes with 3-Methylcholanthrene caused a induction of cytochrome P450 (CYP) and resulted increase 7-EC activity, The 7-HC appearance activity after 3-MC treatment was intensively induced (30-60 fold) in PUF/spheroid culture, while the activity was hardly enhanced in monolayer culture. These results indicate that PUF/spheroid cultured hepatocytes retain the sufficient capacity to respond to inducers for CYP, and PUF/spheroid culture system seems to be useful for alternatives of animal experimentation.

P38. Development of Drug Metabolism Simulator for Alternatives to Animal Experimentation Using 3-Dimensional Culture of Hepatocytes

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Chemical Engineering, Kyushu University

We developed a device for an *in vitro* liver model, namely drug metabolism simulator (DMS), using a PUF (polyurethane foam)/spheroid packed-bed module including about 0.4 g liver cells of rat, lidocaine (anesthetic) clearance and extraction ratio of hepatocytes in the DMS corresponded to 1.354 ± 0.318 ml/min/g-liver and 0.068 ± 0.016ml/min/g-liver, respectively (N=4). These values were comparable with *in vivo* values, 1.930 ml/min/g-liver and 0.0965ml/min/g-liver reported by G.Nyberg (1977), PUF/spheroid packed-bed module seems to provide a promising device as a DMS which will be used for prediction of liver pharmacokinetics.

P39. A Simple Method for Screening Assessment of Acute Toxicity of Chemicals

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We proposed a simple method for screening assessment of acute oral and dermal toxicity containing skin irritation. To distinguish strong acid and alkaline associated with skin irritation, pH of chemicals was measured in the first step. Animals were treated orally or dermally with chemicals at a dose of 2000mg/kg. If some of the animals died, toxicity tests at doses of 200 and 20mg/kg were performed. Skin irritation was observed in dermal toxicity test. We could obtain approximate oral and dermal LD_{50} values and classify chemicals poisonous or deleterious substances. Results obtained in four laboratories collaborated using this method were very similar except for chemicals to have LD_{50}s ranging near the fixed dose level. This simple method was suitable for the assessment of acute toxicity to classify chemicals by using minimal number of animals.

P40. Application of Dermal Papilla Cells for the Living Skin Equivalent

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In this study, we attempted to construct the living skin equivalent containing dermal follicles. Dermal papilla cells were isolated from rat vibrissae, and dermal papilla cell spheroids Were constructed after subculture. These spheroids were attached on the collagen-gel containing human dermal fibroblasts and then human epidermal keratinocytes seeded. After keratinocytes spread over the collagen-gel, the cultures were lifted to the air-liquid interface and kept further. At about 15th day, the cultures were fixed for histological analysis. In histological observation, dermal papilla cell spheroid localized between epidermis and dermis. Keratinized region was also observed in epidermis. It was suggested that dermal papilla cell could cause epidermal keratinization. This model may be applicable to *in vitro* evaluation system for hair growth products.