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SPECIAL LECTURE

What is Validation and Why are We so Concerned about It

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The scientific community has been discussing the issue of the validation of new *in vitro* methods for several years, and he question arises as to where we now stand. There are several components of the issue which should be addressed: (1) definition of validation, (2) procedure, (3) criteria for evaluation, and (4) status. Each of these points will be discussed in some detail.

A practical definition of validation has been proposed in the report of the CAAT/ ERGATT Amden workshop (Balls, 1990): validation is the process by hich the reliability and relevance of a new method is established for a specific purpose. The choice of words in this definition was carefully debated by the workshop participants and should be elaborated upon. First, validation of a method is a **process** which, in all likelihood, evolves continuously. Validation never really stops since any new information concerning the reliability or relevance of the method merely adds to our understanding of its range and liminations (its domain of validity). Secondly, the process consists of two components: **reliability** and **relevance**. The former component refers to establishing how reproducible the results are over space and time. The latter refers to understanding the usefulness of the data produced by the method for the particular purpose for which the test is proposed. This definition suggests what is conceptually needed for validation but certainly does not define a unique procedure to attain the goal, or more importantly how to decide when he process is complete. As mentioned above, validation is never finished; however, there must be some key landmarks which can indicate our progress down the validation trail.

How validation is accomplished—the procedure—cannot be given as an unique prescription. Several reports have discussed important components of the process which must be taken into consideration (Frazier, 1990; Balls, et al., 1990; Ekwall, 1992). The issues of reliability (ruggedness) can be established by intra- and inter-laboratory comparative studies. The question of relevance is much more difficult to establish in the realm of toxicity testing and risk assessment. Consider a simpler case: suppose a new method for measuring distance was developed in a research laboratory. The validation of the new method would be relatively straightforward. The new method would be used to measure a reference standard under a range of conditions which would be expected under the normal use of the method, e.g. at different temperatures, under different conditions of humidity and vibration, with different technicians running the test, etc. The reference standard used for the validation process is not usually the "gold" standard, which is kept in a controlled environment under lock and key, but a secondary standard which is an accurate approximation of the "gold" standard and can be taken out into the field. The criterion used

to evaluate the validity of the method is not an absolute yes or no, but in fact consists of establishing the limitations of the method, i.e., what is the precision of hte method, under what conditions does the method work and not work, within what range of accuracy does the method agree with the secondary standard. Someone who wished to make a measurement of a length then can decide whether the new method is sufficiently precise and accurate for their intended purposes.

The analogy with validation of new *in vitro* methods is useful for ellucidating the problems we face. The first major problem is that we do no have a "gold" standard, much less a secondary standard, to evaluate the validity of new methods. The "gold" standard would be a set of chemicals with: (1) known mechanisms of action at the molecular/cellular level in humans, and (2) adequate toicokinetic data (concentration-time profile) to establish an *in vivo* concentration response relationship at the target cells. Unfortunately, we do not have a selection of chemicals which meet these criteria to investigate the predictive power of new *in vitro* methods. The seocndary standard in this situation, by default, is *in vivo* data from animal models (mostly from rodents). In general, this database is also inadequate since mechanisms of action are still unknown and many toxicity studies do not collect the data required for validation: early molecular/cellular markers of toxic responses *in vivo* and plasma concentration—time profiles. The second major problem is that we generally do not know exactly what the test method under evaluation actually measures in a mechanistic sense. When we run an MTT assay we know that the endpoint measurement (the optical density of the extracted blue dye) is related to the mitochondrial reducing capacity of the test cells during the course of the assay incubation. If the endpoint response is inhibited by exposure to the test chemical, does this mean that the chemical directly poisons the mitochondrial energy pathways or does it mean that the chemical killed the cell by some other mechanism and inhibition of mitochondrial function is a secondary effect. In most cases using single endpoint assays it is impossible to discriminate between the two possibilities. Thus, we are faced with the situation that if we want to validate a new method we must establish some measure of relevance and to do this we must compare two ill-defined sets of data. This is exactly why we worry about validation.

There have been numerous "validation" projects over the last few years (CAAT Technical Report No. 5) which have attempted to slove the practical and theoretical problems which arise. Basically, all of these projects have use individual *in vitro* methods to generate concentration response data for a selection of test chemicals and then compared these data sets to some measure of *in vivo* toxicity using statistical techniques. Little consideration has been given to the mechanisms by which the chemicals selected cause toxicity. Thus, from a mechanistic point of view, the selection of chemicals has been essentially random (selection of chemicals belonging to a similar chemical class may minimize this problem). Given the discussion above about the importance of mechanistic knowledge in the validation process, it is to be expected that most good mechanistic tests will give poor correlations under these conditions since many of the chemicals selected blindly will elicit their toxic effects by mechanisms other than those which the test can detect. The traditional evaluation techniques of determining false positives and false negatives or simple rank correlations are not adequate for our purposes. It is imperative to develop new techniques to appropriately evaluate new tests.

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In Vitro-In Vivo Extrapolation: Essential Tools for Risk Assessment

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If *in vitro* testing methods are ever to become more than just adjunct methods in the risk assessment process, then it is necessary to develop new techniques to extrapolate data generated in *in vitro* models to the *in vivo* situation. The scientific basis for this extrapolation is the focus of this presentation.

In vitro test systems (consisting of a biological component, an endpoint measurement and a test protocol) are useful models to investigate the central events in the toxicological process, which occur at the molecular level in the environment of the cell. The concentration—response relationship (CRR) developed in an *in vitro* model provide quantitative toxicological data representing the probability that a particular cellular response will result given a known concentration of the chemical in the extracellular fluid space. Usually, CCRs are determined at a fixed time of exposure, however, it should be kept in mind that the concentration-time exposure profile determines the type of toxicity being evaluated. High concentrations for short exposure times are more relevant to acute toxicity considerations while low concentrations over long exposure times are important for the evaluation of chronic toxicity. The determination of CRRs for several endpoints over time provides the maximum toxicological information.

At the present time, *in vitro* toxicity test systems can provide concentrarion-time-response relationships for multiple endpoint measurements as input data to the risk assessment process. Without additional experimental input, what can this information tell us? Appropriately designed test batteries can tell us much about the basic mechanisms of toxicity at the cellular level (intrinsic cellular toxicity of the chemical). If we want to extend the usefullness of *in vitro* toxicity data in a meaningful way it will be necessary to develop new quantitative techniques in two additional areas: (1) prediction of chemical toxicokinetics which relates chemical exposure to the concentration of the chemical and its metabolites at the cellular targets within the organism, and (2) prediction of toxicodynamics which describe the relationship between molecular/cellular perturbations and the ultimate expression of toxicological alterations at higher levels of biological organization. The first problem is being addresses by *in vitro* models of xenobiotic metabolism and toxicokinetic. Data produced by these models can be integrated by physiologically-based toxicokinetic modelling to simulate *in vivo* kinetics of the chemical and its metabolites. The second area, toxicodynamics, is more difficult to address for two reasons. First, this area is poorly understood *in vivo*, we have little mechanistic knowlege about the sequence of events connecting cellular events to organ pathology, particularly in the case of chronic toxicity. Secondly, these events most likely involve interations between various cell types which are difficult to reconstruct *in vitro*. The solution to the toxicodynamic problem is not simple, however complex models, such as the multistage cancer models, are one approach. A second approach lies in

the identification of early *in vivo* biomarkers of chronic pathologies which can be detected *in vitro* allowing for the prediction of chronic pathologies.

Toxicity and Irritation Prediction Using a Three-dimensional Human Skin Mouse

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A three-dimensional, human skin model has been developed, characterized, and used as a substrate for topical toxicity testing of undiluted or high concentrations of raw materials. The model consists of a cultured dermis and stratified epidermis grown on nylon mesh. Fibroblasts are seeded onto nylon mesh and grown into a dermis comprised of metabolically active fibroblasts which deposit a network of natural extracellular matrix components, including collagen (Types I and III) and glycosaminoglycans. This dermis provides a support structure for the growth and differentiation of a functional epidermis. Keratinocytes are seeded and grown on this dermal model until an epidermis with distinct basal, spinous, granular and corneal layers is formed.

Several applications have been developed to utilize this model in cytotoxicity and irritation tests that would normally be performed on humans or animals. For cytotoxicity and irritancy testing, test materials are applied to the epidermal surface of the tissue for up to 24 hours. The model itself can then be assayed for cytotoxicity using the MTT assay. The release of cellular enzymes (LDH) or soluble mediators (PGE_2, TNF-α, IL-1α, IL-6 and IL-8) into the culture medium can also be measured in response to test agents. A new protocol has been developed to accommodate the testing of raw materials. Eleven chemicals, designated by the Commission of the European Community (CEC), have been assayed using this model and an excellent correlation (R=.888) has been obtained between the *in vivo* data and the *in vitro* studies.

Comparative Studies of the Toxicity of Biomaterials in Cytotoxicity Test and *In Vivo* Tissue Irritancy Test

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Polyurethane film which contained various amounts of zinc diethyl dithiocarbamate (ZDEC) were prepared as the standard reference materials (SRM). We used SRM films and SRM coated lenses as test materials for *in vivo* muscle implantation, eye- and skin-irritation test. As a result, the cytotoxic potencies correlated well with muscle tissue inflammatory layers, and also well with Draize scores in the eye irritation test. Further, we

can determine the differences in the sensitivity of each tissue quantitatively. The order of sensitiveness is eye>muscle>>skin.

An Alternative to the Hepatotoxicity Testing Using Multicellular Spheroids Composed of Rat Liver Cells

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The massive liver cell necrosis can be induced by *Corynebacterium parvum* and lipopolysaccharide (LPS) in rats. In this model, serum LDH, GOT and GPT activities are significantly increased *in vivo* within several hours after LPS injection. In *in vitro* experiments, the animal model of acute liver injury was produced using multicellular spheroids composed of rat parenchymal and non-parenchymal liver cells. These multicellular spheroids were prepared by detaching the confluent monolayer on the collagen-conjugated thermoresponsive polymer coated culture dish at a temperature below lower critical solution temperature and further culturing it on the non-adhesive substratum. Clear elevations of GOT, GPT and LDH activities from these spheroids into the medium caused by LPS were observed. However, the increase of LDH activity was only observed in the monolayer culture system. These results suggest that the spheroids of liver cells use here are useful models of an alternative to animal testing of hepatotoxicity.

Prediction of Drug-induced Adverse Reactions to CNS Based on *In Vitro* Studies-Convulsant Interaction Between New Quinolone Antibacterial Agents and Non-steroidal Anti-inflammatory

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Based on *in vitro* studies, we tried to predict the convulsion induced by synergistic interaction between new quinolone antibacterial agents (NQs) and non-steroidal anti-inflammatory drugs (NSAIDs), which resulted in serious problems in clinical field. As a possible mechanism of this toxicological interaction, the inhibitory effect of NQs on the $GABA_A$-receptor binding and its potentiation in the presence of NSAIDs were suggested. Therefore we compared the data from *in vitro* studies; inhibitory effect of

NQs-NSAIDs on the direct response of $GABA_A$ receptor-Cl^- channel complex expressed by injection of mouse-brain mRNA into *Xenopus* oocytes (EC_{50}) and on the specific ^3H-GABA binding (IC_{50}) with the result from *in vivo* experiments; threshold brain concentration of NQs (TC) for clonic convulsion induced by administration of both drugs in mice. The IC_{50} values based on *in vitro* studies had significant correlation with TC values for induction of convulsion among several NQs. And EC_{50} value for the inhibition of GABA-receptor response corresponded to predicted brain concentration in human after administration of therapeutic dose. From the above, these *in vitro* were found to be useful for the prediction of drug concentration for the induction of convulsion *in vivo*. Moreover, it was possible to evaluate the risk of adverse reactions by using a safety index among NQs in case of clinical use based on comparison between therapeutic dose determined from essential pharmacological effects and toxic dose for the occurrence of adverse reactions.

Embryo Culture-Can It be Used as a Predictor of Teratogenicity?

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With ever increasing costs of development of new chemical compounds, it is essential to obtain, at an early stage, any indications of potential adverse effects which may curtail further development. Reliable short term *in vivo* and *in vitro* studies can play important roles and acute and sub-acute toxicity and mutagenicity studies are frequently used in this context. With regard to obtaining data on the potential teratogenicity of a new compound, conventional *in vivo* teratogenicity or developmental toxicity studies are available. However, such studies are relavtively expensive in terms of cost, use of animals, amount of compound required and time taken. The rat whole embryo culture system can fulfill an important role in overcoming some of these problems, for example, it is relatively inexpensive, less than 100 mg of test compound is required, it is rapid and the results are reproducible. However, as with any other biological screening system disadvantages exist, for example specialized equipment and expertise are required, it is currently only suitable for use with rodents, only a small window of development is studied and it only represents one part of what is normally a very complex interaction between the mother/placenta and embryo/foetus.

More than 8 years ago we commenced a programme of research to *in vitro* systems that were considered to be applicable to reproduction and development, and which included whole embryo culture. Our experimental approach is based upon the method described by New (1978) and employs rat embryos explanted on day 9.5 of gestation. Following harvesting of embryos, the parietal yolk sacs are removed, but leaving the visceral yolk sacs and placental cones intact. Embryos are incubated for a period of 48 hour at 38°C±1°C in a roller incubator. A culture medium consisting of equal volumes of heat inactivated rat serum and buffered Eagles MEM is used. In order to supplement any embryonic metabolism of a test compound, a metabolic activating system consisting of S-9 plus co-factors NADP and glucose-6-phosphate can be used. Following the incubation period embryos are evaluated in terms of morphological development, using a modification of the method of Brown and Fabro (1981). All abnormalities are recorded.

To-date we have investigated more than 700 compounds from a wide range of different chemical and pharmacological classes.

One major question which naturally arises with any *in vitro* assay is, how well do the results correlate with those obtained from *in vivo* assays?

Currently we have performed *in vivo/in vitro* comparison of 72 compounds. The compounds are mainly pharmaceutical agents of a wide range of different pharmacological classes, but other compounds including agrochemicals, heavy metals etc. are represented. Positive and negative teratogens are included.

The teratogenic potential of 67 out of 72 compounds (93%) has been correctly predicted from the embryo culture studies. Therefore, on the basis of these results it may be concluded that whole embryo culture represents a very attractive test system for the early screening of new compounds for potential teratogenicity.

Acknowledgements

I wish to thank my colleagues, Miss H.C. Bowden and Mr. F.W. Ross for their contributions to these investigations.

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Symposium II Standadization of In Vitro Toxicity Testing

Factors Affecting the Results of *In Vitro* Toxicological Tests

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There are many factors which may affect the results of toxicological tests using cultured mammalian cells. In this paper, I would like to introduce several factors affecting the results of *in vitro* chromosomal aberration tests, such as serum, cell type, ion balance, pH, solvent and experimental protocol. These factors are thought to be important to perform other *in vitro* toxicological tests, and I would like to emphasize that to control the experimental condition is the most important to have reliable results in the toxicological tests using cultured mammalian cells.

Effect of Cell Type and Serum on Cytotoxicity Test of Chemicals

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We studied the effect of cell type and serum concentration on cytotoxicity of 20 chemicals, including detergents, glycerol and oil. We used three different cells, NRCE (normal rabbit corneal epithelial cells), SIRC(established rabbit corneal cells) and RCE-91 (established rabbit corneal epithelial cells) cells which were cultured in serum free medium (RCGM), DMEM medium with 10% FBS and DMEM+F12 medium with 15% FBS, respectively. Cells were inoculated into 96 well culture plate and for 3 days in each medium. Then cells were treated with various concentration of chemicals. Cytotoxicity was estimated with the uptake of neutral red by living cells. NRCE cells was the most sensitive to all chemicals than the other cell lines. However, if NRCE cells were cultured in medium containing serum (RCGM with 10% FBS), their sensitivity was clearly reduced and was almost same level as those of SIRC and RCE-91 cells. Because the extent of interaction between chemicals and albumin (a major component of serum) were closed related with the level of sensitivity reduction, some protein components in serum may play a important role in reduction of chemical sensitivity of cultured cells. In summary, our results indicate that normal primary NRCE cells cultured in serum-free medium is more useful for cytotoxicity test than established SIRC and RCE-91 cultured in serum containing medium.

Evaluation of Combined Toxic Effects of Two Chemicals

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The procedure for evaluation on the combined effects of two chemicals on survival and 6TG-resistant mutations in Chinese hamster V79 cells were examined by the general mathematical technique. The combination of EMS ad MMS, 4NQO, MNNG, Vitamin C, vanillin, Chouji extract or Gennoshoko extract were evaluated by this techniques. Results showed that some combinations of two chemicals gave additional effects and others gave synergistic or antagonistic effects.

Kinetic Analysis of Cell-Killing Action of Antitumor Agents and Its Application to Screening of Antitumor Agents

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First of all, present status of *in vitro* screening of antitumor agents using human cancer cell lines will be introduced. Second, results of our kinetic analysis of cell killing action of antitumor agents will be presented. In this analysis, we could conclude that cell-killing effect of cell cycle phase nonspecific drugs depends on AUC (area under the time-concentration curve). Finally, applicability of this result into *in vitro* screening will be discussed.

Standardization of Cytotoxicity Assay for Medical Devices and Cosmetic Products

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In vitro cytotoxicity assays are useful tools for predicting *in vivo* toxicity with less time and at lower costs. Over 30 *in vitro* tests have been currently validated for alternatives to eye and skin irritations, and it will provide valuable data for standardization of *in vitro* test methods.

The draft of updated OECD guidline "404: Acute Dermal Irritation/Corrosion" and the proposal of guideline "405: Eye Irritation" have recommended that *in vivo* test may not be needed for materials which toxic properties are predicted on the basis of results from *in vitro* test. At present, standardization of test methods including cytotoxicity assay is in progress in the field of medical and dental devices under the ISO (the International Organization for Standardization), and the

Ministry of Health and Welfare as a national standard of Japan.

In this presentation, we will discuss several items for the development of a standardization of *in vitro* cytotoxicity assays.

First Phase Validation Plan of the *In Vitro* Eye Irritation Tests for Cosmetic Ingredients

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We studied *in vitro* eye irritation tests during these two years by literary works for the purpose to examine the possibility to introduce those methods for safety evaluation of cosmetic ingredients, and selected 16 methods for the candidates for experimental evaluation. We established a committee to proceed this validation and asked the members of Japan Cosm. Industry Assoc. to participate the program. We have got applications from 16 organizations. In this validation we are going to apply 9 surfactants. Surfactants are only one group of cosmetic ingredients. Thus, we consider this validation as a preliminary study. The test methods, which will be validated, were as follows: ① CAM-trypan blue staining method, ② erythrocytes and hemoglobin methods, ③ artificial skin model (TESTSKIN, SKIN[2]), ④cytotoxicity test in SIRC cells, ⑤ cytotoxicity test in HeLa and CHL cells, ⑥ EYTEX method, ⑦ cytotoxicity test in primary cultured rabbit corneal cells (Corne Pack). Principally, those methods are going to be examined by several organization and participants are requested to examine several methods by using blind samples to enable us to get objective informations about the methods and those about inter-laboratory variance.

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Standardization of Cytotoxicity Test for Biomaterials

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The concepts and rationale of cytotoxicity test in the draft Guidelines of Preclinical Toxicity Tests for Medical Devices and Biomaterials and described.

Usefulness and Standardization of Cell Culture System
—At Present State and in Future—

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We'd like to talk about the present state of cell supply system and the problem of cell bank function in private industries according to our latest data in our department at this annual meeting of Japanese Society for Alternatives to Animal Experiments. Our department has supplied various kinds of established cell lines inported from ICN Biomedicals Inc. (formerly Flow Laboratories Inc.) in USA since 1972. Most cells we transact are derived from American Type Culture Collection (ATCC) through ICN Biomedicals. We supply these cells in cultured state after we test the quality of cells by checking the contamination of microorganisms and the viability of these cells cultured under the optimum conditions and give them the adequate treatment for their states. We also supply the cells established by ICN Biomedicals, Searle and Japanese research laboratories. We think that the duty of cell bank is to collect and supply standard reference cell lines with good quality and much information about them smoothly. We think that the following four matters are very important in our cell-supplying system. the first is as follows; the cells we supply can be used as reference cell lines because the passage number of these cells is kept under 15 even if they of which origin is ATCC, have experienced passage and freezing-thawing in the process of their maintenance in ICN Biomedicals and our department. The second is as follows; the character and function of the cells we supply change in various conditions. But we don't characterize the function and property of each cell line because it needs a lot of technical knowledge and work and on top of that a lot of time and cost. Therefore we would like to check whether the properties of cells they use are suitable for their research work. The third is as follows; the information about each cell line we serve to users is derived from ATCC and original references. The user's manual of each cell line we also serve with the cell line is produced by our department. Thte culture conditions are run in our general catalogue in order to let users know them in advance of culturing. The forth is as follows; we never fail to make a phone call to users in order to avoid the standing of cells under inadequate conditions and ask users to give us the information concerning the state of the cell line we supplied by the post card enclosed. In brief, the cell-supplying system in our department is very small in size. We don't collect and serve a lot of information concerning each cell line. In this sense the function of our system may be far from that of what people call cell bank. But we think that it's very important for us to develop the system for supplying cells cultured under the same conditions for everyone's research work. We'll make the greatest effort to get cells with good quality in response to the needs and information of researchers.

Establishment of Cell Lines Which Express Cytochrome P-450

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It is generally accepted that established mammalian cells do not express cytochrome P-450. Because of the lack of the enzyme, these cell lines are rather insensitive to chemicals including carcinogens which show toxicities only after undergoing metabolic activation by cytochrome P-450. Because of the lack of the enzyme, these cell lines are rather insensitive to chemicals including carcinogens wihch show toxicities only after undergoing metabolic activation by cytochrome P-450. Thus, this study was undertaken to establish cell lines by introduction of cytochrome P-450 cDNA to CHL cells. Firstly, we could establish a cell line highly sensitive to aflatoxin B_1 by introduction of monkey P-450 1A1 and mouse NADPH-cytochrome P-450 reductase. This new cell line showed sensitivity approx. 300-fold greater than the parental CHL cells. Secondly, we could establish a cell line by introduction of human 1A2 and human N-acetyltransferase in addition to the reductase. This cell line was highly sensitive to some heterocyclic amines including IQ.

Immortalization of Human Cells

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In 1961, Hayflick and Moorhead reported that human diploid fibroblasts are unable to be cultured for more than a period of about 10 months or 60 population doublings and undergo cellular senescence. This observation has been confirmed by many investigators during these three decades. When overcome the crisis of cellular senescence, cells are immortalized and grow *in vitro* permanently. Length of life span of diploid cells and frequency of immortalization and largely dependent on species from which cells are derived. Spontaneous immortalization of human cells and chicken cells is an extraordinary rare event, while mouse cells and Chinese hamster cells are relatively easily immortalized.

Immortalization of human cells is achieved by oncoproteins of DNA tumor viruses such as SV40 large T antigen, adenovirus EIA, human papilloma virus (HPV) E6 and E7 and EB virus. Human epithelial cells are relatively easily immortalized by these oncoproteins, while fibroblasts are resistant, being immortaized by SV40 at a frequency of 3×10^{-7}. Infection of EB virus immortalizes B lymphocytes. However, RNA tumor viruses or activated oncogenes only rarely immortalized human cells. Repeated treatments are required for immortalization by chemical carcinogens or X-ray irradiation.

Hybrids between diploid cells and immortalized cells show a limited life span, indicat-

ing recessive nature of immortalization. Recently fibroblasts of Li-fraumeni syndrome patients are reported to be easily immortalized without any treatment. DNA tumor virus oncogenes bind to tumor suppressor gene products. Taken together these observations, involvement of tumor suppressor genes in immortalization is strongly suggested.

A Cytotoxicity Test Using a Composite Cultured Skin

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Various antibacterial drugs have been successfully applied onto the wound surface so as to promote the wound healing. The cytotoxicity caused by antibacterial drugs should be examined on a skin defect inflicted in animals. The alternative approach would be to use a human skin substitute as a wound surface model.

The composite cultured skin was created through successive cultivation of human fibroblasts and human keratinocytes, and these cells were combined within a collagen matrix, respectively. The collagen matrix was composed of a honeycomb collagen spongy sheet combined with a collgen gel. Fibroblasts were inoculated into a collagen matrix, and cultured for 7 days in Dulbecco's modified Eagle's medium supplemented with 10% FBS so as to prepare a composite cultured dermis (CCD). Keratinocytes were plated on a collagen matrix, and cultured for 7 days in a keratinocytic growth medium so as to prepare a composite cultured epidermis (CCE).

Cytotoxicity tests of the antibacterial drugs, released from ointment or wound dressing were conducted using CCD and CCE. A piece of CCD or CCE was sited on a stainless steel net in a cultured medium to create an air and a medium interface. Then, a piece of gauze with antibacterial drug-impregnated ointment was placed onto CCD and CCE. After a given period of cultivation, the gauze was removed, and a portion of CCD and CCE was fixed in 10% formalin, embedded in paraffin, sectioned, and treated with hematosylin eosin, and the cross section of CCD and CCE were observed histologically. Another portion of CCD and CCE were treated by 0.5% collagenase in Hank's solution for 30 min at 37°C to obtain a cell suspension, and the survived cells were counted. The cytotoxicity caused by antibacterial drug-impregnated wound dressing was examined in a similar manner.

In previous studies, the *in vitro* cellular response of cells was examined by adding different amount of drug into a culture medium, in which cells proliferated to confluence, followed by counting survived cells after a given period of cultivation. For this study, we employed an improved cytotoxicity test that used composite cultured skin (CCD) and CCE) which was fixed to create an air and a medium interface. This interface system provides a wound surface model, so that the resulting *in vitro* cellular response is helpful in predicting the *in vivo* cellular response.

Testosterone Metabolism In an *In Vitro* Skin Model

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The metabolic activity of skin is important in penetration of topically applied compounds. Currently, animal or cadaver skin is used to evaluate the relationship between metabolism and penetration. Here, testosterone metabolism and penetration in a three-dimensional human skin model consisting of keratinocytes and fibroblasts derived from neonatal foreskins, was characterized. The model was laser cut into 2.25 cm^2 pieces and placed on 35 mm tissue culture inserts with 1 ml of HEPES buffered medium on the dermal side. Penetration of [^{3}H]-testosterone was faster at 32°C than at 4°C suggesting that metabolism affects penetration. To evaluate this metabolism, [^{3}H]-testosterone was applied to the stratum corneum side of the skin model. Radiolabeled metabolites released into the medium after incubation were separated by HPTLC and analyzed by autoradiography. This skin model metabolized [^{3}H]-testosterone to both polar and non-polar compounds which were similar to metabolites of neonatal foreskins. The appearance of nonpolar compounds was earlier than the appearance of polar compounds. Both dermal fibroblasts and differentiated epidermal keratinocytes contributed to the metabolism of testosterone. Two testosterone metabolites, dihydrotestosterone and androstane-3, 17-diol, were sensitive to the cytochrome P450 inhibitor metyrapone and were only produced by the keratinocytes. In conclusion, this model is a reproducible source of metabolically active skin and therefore a good alternative to animal or cadaver skin for evaluation of the contribution of metabolism to penetration.

A Human Skin Model

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An organotypic human skin model has been developed for research and testing needs. The skin model consists of a dermal component, reconstituted with collagen and human dermal fibroblasts that are mitotically and metabolically active, and a differentiated epidermis that arises from cultured keratinocytes that have been seeded onto the surface of the dermal equivalent. The human skin model is configured such that the dermal component is in contact with a nutrient source while the stratum corneum is exposed to the atmosphere. The air exposed surface of this human skin model allows for the application of test samples in various forms for example: liquids, powders, gels and emulsions. Samples applied in this fashion allow for testing protocols that closely paralle *in vivo* testing protocols. The model has been used to monitor the metabolism and percutaneous absorption of topically applied chemicals. In addition, the thershold doses for damage caused by various human skin irritants in this model are similar to those for damage to skin.

Nephrotoxicity Testing Using Cell Culture System

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Recently, cell culture systems are generally accepted as a useful model for elucidating the interaction between toxicants and tissues at the cellular or subcellular levels. In order to clarify the mechanisms of action of toxicants, it is necesary to apply the cell culture systems using cells that are derived from the target organ. Its usefulness as compared with the other *in vitro* methods can be served as one of attractive methods for elucidating the mechanisms of action of toxicants at the cellular or subcellular levels and the better reproducibility among the expriments especially when using established cell lines. Since large numbers of chemical substances including drugs are excreted through kidneys, there is a high possibility of risks that the kidney are exposed to much larger quantities of chemical substances than the other organs. Considering these problems and that renal injuries may be a serious menace to the body, it comes up as an important problem that the development of the nephrotoxicity testing using renal cell culture systems is an essential step.

We investigated the cytotoxic effects of gentamicin, which is known to elicit renal injuries in while animals, and formaldehyde using various biochemical parameters in JTC-12 cell, an established cell line from the kidney of the normalcynomolgus female monkey. Gentamicin caused concentration-dependent increases in intracellular calcium ion. Transepithelial resistance was decreased by gentamicin in a concentration- and time-dependent manner accompanied by leakage of fluorescin, suggesting that the membrane integrity was disrupted by the treatment with gentamicin. Within the range of concentrations examined, gentamicin had no stimulating effects on leakage of lactate dehydrogenase and γ-glutamyl transpeptidase from cells and there were no differences between gentamicin-treated cells and controls from results of investigating the double staining with calcein AM and propidiu iodide, suggesting that a primary site of action in the early renal injuries by gentamicin is a cellular membrane. Formaldehyde also induced concentration- and time-dependent increases in intracellular calcium ion and this effect was more dependent on extemal calcium ion. Alkaline phosphatase, $(Na^+ + K^+)$-ATPase and $(H^+ + K^+)$-ATPase were concentration-dependently inhibited by formaldehyde. These results suggest that JTC-12 cell is considered to be a useful established cell line in case of utilizing nephrotoxicity testing as early indices of renal injuries.

Cytotoxicity Assay Using Human Liver Cells

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Our purpose of this experiment is to investigate cytotoxic effects of various drugs using human liver cells in culture and to know whether or not animal experiments can be replaced by in vitro experiments.

Material and Methods

Six hepatoma cell lines, Hep G2, Hep 3B, HLE, HuH6, HuH-7, and PLC/PRF/5, were used. Culture medium was RPMI-1640 supplemented with 10% FBS and 100 μg/ml kanamycin. Effects of drugs on cells were determined by measuring colony formation.

Results

1) Cytotoxic effects of ethanol: When the cells were inoculated into plastic dishes and incubated in a CO_2 incubator (open system), no cytotoxicity was observed with concentrations of up to 1%. However, when flasks were used and their caps were tightly closed (closed system), cytotoxicity was demonstrated, the lethal dose of ethanol for 50% cell death being around 0.5 to 0.7%.

2) No significant difference in cytotoxicity of ethanol and methanol was observed among the hepatoma cell lines.

3) The hepatoma cell lines, Hep 3B, HLE, and PLC/PRF/5, were suitable for cytotoxicity assay, being easy to culture and to be prepared as single cells by trypsinization. In addition, they formed large flat colonies.

4) Ethanol showed stronger cytotoxicity than methanol in cultured cells.

Discussion

Because of the difficulties inherent in culturing normal human liver cells, we used human hepatoma cells, which have various features specific to liver cells. However, we should attempt to determine culture conditions of normal human liver cells and find out cytotoxicity assay specific to liver cells.

Development of a Prediction System for Eye Irritation Considering Membrane Destruction Factor and Protein Denaturation Factor

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At the last meeting, we reported the alternative method to predict eye irritation using hemoglobin's denaturation properties. The method provides the practical alternative to the Draize test when applied to the surfactant. At this meeting, we propose the new method that the accuracy is further improved by combining the membrane destruction evaluation method (liposome examination and RBC examination) and the hemoglobin denaturation examination (HDR examination).

The 12 surfactants were evaluated by HDR, EYTEX, SIRC cells, HeLa cells, CAM, liposome, and RBC (human, rabbit, guinea pig, and rat). These results and Draize scores previously obtained were analyzed by the factor analysis. As a result, the membrane destruction factor, the protein denaturation factor, the cytotoxicity factor, etc, were extracted. At the same time, the factor analysis suggested that the Draize test would be estimated by appropriately combining the evaluation of the membrane destruction and the protein denauration (HDR examination). In order to find out the optimum combination, multiple linear regression analysis was applied.

Higher accuracy was obtained by the combination of the HDR examination and the

liposome examination (corneal and total: r=0.922, r=0.880) compared with the HDR single method. The combination of the HDR examination and RBC (rat) examination also showed higher correlations (corneal and total: r=0.878, r=0.941).

The system is not the only means to predict eye irritation, but we believe the system provides the practical and reliable method to evaluate the eye irritancy as far as the surfactant is concerned.

Symposium IV Non-biological Alternatives

Molecular Design of Artificial Cytochrome P-450 and its Application to Drug Metabosism

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Cytochrome P-450 plays an important role in metabolizing biomelecules and xenobiotics. Recently many attempts have been made to reproduce the reactivity in chemical systems with a simple metalloporphyrin. We have achieved the synthesis of many effective P-450 mimics to clarify their functions. To develope artificial metabolic systems having high similarity to the native P-450 system is important because of the usefulness as a tool for the investigation of drug metabolism and organic synthesis. We therefore forcused on more complex compounds like actual drugs as substrates to compare microsomal oxidation with chemical oxidation systems.

Drug Metabolism Mediated by Artificial Enzymes

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Active reaction sites of holoenzymes, which require a relevant coenzyme, are well shielded from the bulk aqueous phase and provide waterlacking and hydrophobic microenvironments for coenzymes and substrates. We constructed artificial enzymes composed of synthetic bilayer vesicles, as substitutes for appoproteins, and hydrophobic vitamin B_6 or vitamin B_{12}. These artificial enzymes successfully simulated catalytic functions mediated by the corresponding natural enzymes, and are expected to be utilized for investigations of drug metabolism.

An artificial enzyme composed of single walled bilayer vesicles and a hydrophobic vitamin B_6 mediated the formation of tryptophan derivatives from L serine and indoles in a manner as observed with the naturally occurring tryptophan synthase. An attack of an indole to the Schiffbase of aminoacrylic acid was confirmed to be the rate-determining step.

A hydrophobic vitamin B_{12}, which was derived from cobalamin by converting all the peripheral amide moieties to carboxylic esters, was non-covalently fixed in single-walled bilayer vesicles to form an artificial enzyme. The artificial enzyme. The artificial enzyme successfully mediated varous carbon-skeleton rearrangements, such as those catalyzed by methylmalonyl-CoA mutase and glutamate mutase.

Toxicity Prediction of Chemicals Using Structure-Activity Relationships

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In the risk assessment of chemicals, the risks to humans are estimated from the results of animal experiments.

However, studies of alternative test methods are carried out to recognize the toxicity profile or reaction mechanism. The purpose of development for the alternative methods can be classified as 1) screening, 2) adjunct test, and 3) replacement to the animal experiments. On the other hand, researches on toxicity prediction using structure-activity relationships are developed actively. These are include acute toxicity, repeated dose toxicity, carcinogenicity, mutagenicity, teratogenicity, irritancy, allergenicity and metabolism.

In this sympsium, numerous existing reports concerned with structure-activity relationships for toxicity of chemicals will be reviewed. In addition, their availability in risk assessment of chemicals will be discussed.

Poster Presentation

Effects of Thalidomide on Cultured Rabbit and Rat Embryos

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It is reported that a thalidomide commonly produces malformation of extremities in human. The type of malformation is amelia (fore limb), phocomelia (fore limb) and radius defect. In order to examine these malformations, rabbit and monkey could be used in animal experiments, but the same type of abnormality could not be examined in rat and mouse. In the present study, in order to confirm whether the difference between these reactions is due to the species difference in the sensitivity of embryonic cells among in various animals previously described or to the drug metabolic activity of dams, we examined the effects of thalidomide on cultured rabbit and rat embryos.

Rabbit embryos on the 12th day of gestation (plug day =0) were cultured in 100% rat serum for 24 hours. Rat embryos on the 12th day of gestation (plug day=0) were cultured for 48 hours. 500 and 700 μg/ml of thalidomide were addd to the medium at 2 hours after preincubation, 200 μmol of S-9mix was added to the rat serum at 30 minutes before thalidmice tretment. The embryos at the end of culture were compared using a computer graphic analyzer.

The cultured rabbit embryos: At the end of incubation, the blood circulation of cultured embryos decreased in the 700 μg/ml thalidomide treatment group, compared with the control group. The area of fore limb was $66\times10^{-4}mm^2$ (control), 64×10^{-4} (500 μg/ml) and $57\times10^{-4}mm^2$ (700 μg/ml). The value to each crown-rump length was 8.5 (control), 8.5 (500 μg/ml) and 7.8 (700 μg/ml). and 9.2% decrease was observed in embryos. No other abnormality was observed in the morphology of external surface. In te S-9mix pretreatment group, the value of "area of fore limb/crown-rump length" was 8.4 (control) 8.4 (500 μg/ml) and 7.8 (700 μg/ml).

The cultured rat embryos: In the embryos on the 12th day of gestation, the crown-rump length, the total somite and the area of fore limb were 5.8 mm, 41 and $44\times10^{-4}mm^2$, respectively. The area of fore limb after 24-hour culture was $44\times10^{-4}mm^2$ (control), $59\times10^{-4}mm$ (500 μg/ml and $60\times10^{-4}mm^2$ (700 μg/ml). The morphology of external surface was not different among control and both the thalidomide treated embryos at 24-hour culture. AFfter 48 hours, however, although no change was observed in embryos treated with 500 μg/ml compared with control embryos, 14.5% decrease in the area of fore limb and hematomas in the head, tail and extremities wree identified in embryos treated with 700 μg/ml, and a decrease in the embryonic blood circulation was also observed. Additionally, there was 9.3% decrease in the "area of fore limb/crown-rump length".

From the above results, the effect of thalidomide was observed in the fore limb of cultured rabbit and rat embryos, and it was suggested that the same phenomenon may occur also by the metabolites possiblely produced by the S-9mix treatment.

The Teratogenicity of Methylazoxymethanol in Cultured Rat Embryos

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The purpose of the present experiment was to examine the effect of methylazoxymethanol (MAM) on cultured rat embryos. Since its effect was assessed in a narrow dose range, MAM were tested at concentrations at 50, 100, 250, 500 and 1000 μg/ml. The culture conditions of whole embryos were based on the method described by New (1978). Each one rat embryo on the 11th day gestation (plug day=0) was cultured in 25 ml culture bottle containing 5 ml of heat-inactivated rat serum for 48 hr at 37°C. As growth markers of rat embryos, crown-rump length was measured and the number of somites was counted. The protein content of embryos was also determined by the Lowry's method.

The total protein contents of embryos treated with 250, 500 or 1000 μg/ml MAM were 30~40% lower than that in control embryos. The crown-rump length of embryos treated with 250 μg/ml or higher consentrations of MAM was 25% shorter than that in control embryos. Furthermore, the body width examined preliminarily was also found to be decreased. The anomaly seen in the external morphology of the cultured rat embryos was microcephaly, with an incidence of about 90% in embryos treated with 250, 500 and 1000 μg/ml MAM. In particular, treatment with 250 μg/ml inhibited the growth of prosencephalon of the antiniad and caused a dysgenesis of the mesencephalon and occipitalis.

The results suggested that MAM acted directly ont he embryos rather than via the dam, and that its effect was related to the growth of prosencephalon and the morphogenetic development of the head.

The Study of the Mechanism of Embryonal Resorption Using Cultured Rat Embryos —The Comparison of Effects of Sodium Salicylate and Egta—

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In studying the reproductive toxicity of chemicals in rats, a cesarean section is usually performed on the day 20 of pregnancy to examine for embryonal resorption (embryo death). However, it is difficult to analze the cause of embryonal resorption from the observation on the day 20 of pregnancy. Therefore, we recently studied the course of embryo until death by treating incubated rat embryos (in the stage of organogenesis) with sodium salicylate (SSA) or EGTA.

A dose of SSA or EGTA, high enough to induce resorption (death) of 505 embryos *in vivo* (1000 ug/ml for SSA and 100 μg/ml for EGTA), was added to the medium. Using this medium, rat embryos were cultured in a rotating incubator for 48 hours, starting at the day 11 of gestation.

In the EGTA-treated group, the heart rate began to decrease 15 minutes after treatment, and the heart stopped beating 27 hours after the start of incubation. When the external appearance of these embryos was observed under a stereoscopic microscope, no change

was observed in the placenta, but the *yolk sac* showed severe vascular injury and whitish degeneration. At 16 hours, the *yolk sac* membrane and amnion in all EGTA-treated cultures showed atrophy. These cultures also showed embryo abnormalities (curly or short tail, and suppressed elevation of forehead). in the SSA-treated group, the *yolk sac* became white 24 hours after the start of incubation, thus making it impossible to observe blood circulation under the microscope. Atrophy of the embryo membranes, which was observed n the EGTA-treated group was not observed in the SSA-treated group. However, all SSA-treated cultured rat embryos showed detachment of the *yolk sac* membrance at 26 hours. The heart rate was lower in SSA-treated embryos when compared to control embryos. However, the heart did not stop beating in any SSA-treated embryos. Thus, EGTA treatment first abolished the cardiac function of embryos, followed by atrophy of the embryomembranes and then death. On the other hand, SSA treatment first induced fetal membrane degeneration, leading to whitening of embryos and then to death.

These results from experimental induction of featal death with chemicals on the day 20 of pregnancy suggested that caused of intrauterine embryo death can be effectively analyzed by using cultured embryos.

Oxygen Contents in Medium in Which Rat Whole Embryos were Cultured

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The culture of rat embryos on the day 9.5 of gestation for 48 hours is used as a standard model. On the other hand, many of the current commercially available incubators are the type into which large amount of gas is continuously introduced. This system may easily induce edema, if the contents and amounts of gas are changed. In order to minimize the amount of gas dissolved in the culture medium, we successfully developed a characteristic culture vial (mini-vial), and inhibited the occurrence of edema, fellowed by the 100% normal culture. In the rat embryos ont he day 9.5 after gestation, total somite was 7±1, and the major axis and minor axis of *yolk sac* were 2.0±0.3 mm and 1.0±01. mm, respectively. When embryos were cultured for 48 hours, total somite, major and axis of *yolk sac* and crown-rump length developed to 32±1, 3.7±0.2 mm, 3.4±0.2 mm and 3.3±0.2 mm, respectively. No abnormality such as edema was observed. There was no difference in the morphology of external surface compared with the *in vivo* embryos on the same day of gestation. The average oxygen contents in culture medium was shown to be the value of 180 mmHg in this point. These results, indicated that rat embryo on the day 9.5 day of gestation is normally cultured in the special coated mini-vial in the rotary incubator into which the air is continuously delivered.

Culture of Rat Whole Embryo at the Stage of Egg Cylinder

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Some of the nitrosourea derivative antineoplastic agents, such as nimustine and ranimus-

tine, induce gross structural abnormalities in fetuses when administered *i.p.* to rats on day 6 to pregnancy. However, it is impossible to detect the abnormalities induced after treatment of postimplantation embryos with these chemicals when evaluating using the 9.5-day embryo culture technique. keeping these in mind, we attempted the culture of rat embryos at the stage of egg cylinder (6.5-day). Rat I.C. serum plus DMEM (1: 1) supplemented with glucose and methionine were used as medium. 7.5-Day embryos developed normally for 48 hrs in comparison with *in vivo* embryos, whereas those developed abnormally after next 48 hrs in culture. 6.5-Day embryos failed to develop normally. However, developed yolk-sack and blood circulation in yolk-sack wre observed. These results suggest the possibility to culture embryo from the egg cylinder stage with development of the culture medium suitable to embryos at this stage.

In Vitro Developmental Toxicity Assay System: Search of Embryogenesis Promoter for Whole Embryo Culture Medium

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Whole embryo culture techniques have been applied as an *in vitro* test system of developmental toxicity. However, there are some difficulties and problems to be improved or modified, including the requirement of 100% rat I.C. serum as culture medium. It is necessary to develop serum-free medium, in order to decrease the number of experimental animals to be sacrificed. Thus embryogenesis promoter (EGP) present in rat serum was searched. Slc: SD rat embryos on day 9.5 of gestation were cultured according to New et al.[1] I.C. serum was differentially supplemented with Dulbecco's modified Eagle medium (DMEM), or replaced by delayed centrifuged (D.C.) rat serum. After 48 hrs of incubation, embryos were examined and scored according to Brown & Fabro. The results from experiments for supplementation of I.C. serum with DMEM and for replacement by D.C. serum showed that EGP is still effectively active in 30% rat I.C. serum-70% DMEM or 50% D.C. serum-50% DMEM. When the amount of DMEM content was increased to more than 75%, embryos were deviated from normal development, and then had verious abnormalities in 90% DMEM. Preliminary experiments on isolation and identification of EGP suggested that hte major element of EGP was contain in a fraction isolated by anion exchanger and was of a molecular weight within 100,000 150,000.

In Vitro Tests for Evaluating Teratogenic Potentials of 1-Nitropyrene and its Derivatives Using Micromass Culture Systems

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National Institute of Hygienic Sciences

1-Nitropyrene (1-NP) and its derivatives were assayed using rat embryonic cell dif-

ferentiation systems. In the midbrain cell differentiation assay, the inhibitory activities of the compounds were in the following order: 1-nitropyrene-3, 6-quinone>1-nitrosoquinone > 1-nitropyrene-3, 8-quinone > 1-nitro-6-hydroxypyrene >1 -nitro-8-hydroxypyrene > 1-NP>1-aminopyrene>1-acetamidopyrene>1-nitro-3-hydroxypyrene. 1-Nitrosopyrene and 1-acetamidopyrene showed the stimulative effects on the differentiation of limb bud cells. Thequinone and nitroso derivatives of 1-NP, therefore, showed greater teratogenic potential than 1-NP itself.

In Vitro Toxicity Test for Biomaterials: Inhibition of Metabolic Cooperation in V79 Cells by Metal Ions and Antioxidants Including Tumor Promotors

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Thte effects of various metal ions and phenolic antioxidants on me tabolic cooperation in the hypoxanthine-guanine phosphoribosyl transferase system were surveyed. Among 11 kinds of metal ions, 5 metal ions, such as Cd, Cu, Zn, Pb and Li inhibited metabolic cooperation. We found a correlation between the teratogenicity and the inhibitory activity on metabolic cooperation by metal ions.

In the case of 6 antioxidants tested, 5 chemicals inhibited metabolic cooperation. BHT, a tumor promoter, showed the inhibitory activity at higher concentration than the other four antioxidants.

Pharmacological and Toxicological Studies Using Chick Embryos (1) Cardiovascular Anomalies in Chick Embryos Induced by Fertilysin Injected at the Different Routes

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In order to evaluate the biological effects of some drugs, using chick embryos, the routes of administration is very important factors as well as the time of injection. In this study we describe the difference in expression of toxicity of fertilysin (Fer), an inhibitor of polyamine synthesis, through the different injection routes. Materials and methods: 25 or 50 μg/egg of Fer was injected once into the air sac (AS) or yolk sac (YS) of fertile eggs of White Leghorns on the 0, 1, 2, 3, 5 or 7 days of incubation. Chick embryos were sacrificed on the 14th to 18th day of incubation and mortality, external anomalies, the frequence of cardiovascular anomalies were estimated. Mortality induced by Fer administered *via* AS route reduced getting longer and longer the period of incubation. The mortality of chick embryos induced by Fer administered via a AS route was stronger than that of via a YS. Chick embryos injected with Fer did not show any external anomalies, but cardiovascular

anomalies, such as double aortic arch and the single or complex retardation of pulmonary artery, branchial artery or common carotid artery, were observed. Injection with Fer within the 3rd-day incubation resulted in higher incidence (50–100%) of cardiovascular anomalies. But any anomalies ware not seen in te 7th day-treated embryos of two groups. There is no difference between two groups in frequency of cardiovascular anomalies. In conclusion, The lethal toxicity of fertilysin showed a significant difference between two injection routes, i.e. air sac and yolk sac. But the frequency of cardiovascular anomalies showed no influence between to injection route.

Pharmacological and Toxicological Studies Using Chick Embryos (2) Estimation of Drugs Induced Abnormalities in Cardiovascular Systems of Chick Embryos Using Color Doppler Echocardiography

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In order to develop the alternative method to mammalian, we used chick embryos and got many information. In this study, we tried to evaluate the effects of some drugs on cardiovascular systems in chick embryos, using color Dopplar echocardiography.

Experimental procedures: The egg shell round the air sac of fertile eggs was removed by scissors and filled with jelly on the outer memmbrane. Then a small tube was inserted to the air sac for drug injection. Then 2D echocardiograms and FFT patterns of the ascending and descending aorta of chick embryos were recorded using 5MHZ transducer. Volume of Max velosity (MV), accelation time (AT), flow integration (FI) and R-R inteval (HR) were calculated from fFT patterns of the descending aorta. All chick embryos were kept about 37°C during mesurement. Exp. 1. Epinephrine (Epi, 0.01 mg/egg) or acethylchorine (Ach, 0.0001 mg/egg) was injected through the tube on the 18th day of incubation. HR of the chick embryos was increased Epi, but volume of MV and FI decreased at the Epi injection. On the contrary, Ach showed the opposite reaction to cardiovascular system. Exp. 2 Based on te data as above described, the 18th days-chick embryos treated with fertilysin (Fer. 25 or 50 μg/egg) on the 2nd day of incubation were examined. HR of Fer-treated embryos was more rapid than that of control, but volume of MV, AT and FI showed low value to the control. This facts may suggeste that physiological function of cardiovascular systems of Fer-treated embryos may be disturbed by the drugs. In conclusion, color Dopplar echocardiography may be applicable to evaluate the cardiac functions and physiological abnormalities of the chick embryos heart.)

Assessment of Developmental Toxicity of 2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD) Using *Xenopus laevis*. —A Morphological Study of the Liver Lesion—

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We previously reported that TCDD-exposed embryos and larvae of *Xenopus laevis* had edemas in the head, thorax and abdomen, and most of them then died within one or two days. In this study, liver of the larvae with edema was examined histologically. LM observation showed that liver lesions were more extensive as the edema became more marked. Normal liver architecture was lost and many degenerative cells appeared. TEM observation of viable parenchymal cells showed an increase in the sER with a decrease in the rER. these morphological changes suggest that the synthesis of albumin in the liver decreased which may be one of the causes on te development of edema.

Induction of Drug-Metabolizing Enzymes in Primary Culture of Dog Hepatocytes Treated with Various Inducers

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We have cultured hepatocytes isolated from dog liver using William's Emedium and found that the cells actively catalyzed the dealkylation of alkoxycoumarins and hydroxylation of progesterones though these activities wre moderately decreased during 96 h of the culture. When phenobarbital (PB) was added to the primary culture, 7-alkoxycoumarin O-dealkylase progesterone hydroxylase activities and total cytochrome P-450 content markedly increased. Western blot analysis of microsomes isolated from the primary culture of hepatocytes with anti-cytochrome P-450 antibodies showed tht PB-treatment increased P-450 IIB and IIIA immunoreactive proteins. Omeprazole and β-naphthoflavone elevated the level of 7-ethoxyresorufin O-deethylase activity, while rifampicin elevated that of progesterone 6 β-hydroxylase. Our results indicate that the primary culture of dog hepatocytes responds wit appreciably high activities to various inducers of hepatic cytochrome P-450 as observed *in vivo*.

In Vitro Toxicity Test Using Isolated rat Liver Mitochondria

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It is well known tha variety sort of natural toxins and drugs are tosic both to mitochon-

dria *in vivo* and to isolated mitochondria *in vitro*. Isolated mitochondria are, however, not used in general for toxicity test of chemicals, because it is, as a reason, very difficult to isolate the tightly coupled mitochondria, loosely coupled mitochondria must be carefully removed from precipitate laid over tightly coupled (heavy) mitochondria by the well-trained hand work.

Recently we have greatly developed the preparation method, by which loosely coupled mitochondria are easily removed to supernatant fraction remaining the tightly coupled mitochondria as precipitate. The method is characterized by the very simple centrifugation procedure and the isolated mitochondria show very high and constant level of RCR every time.

Nephrotoxicity Using Cultured Monolayers of JTC-12 Cells Drived from Monkey Kidney

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In general, toxicity tests have been based upon the lethal dose of toxicants. But it is important to establish the experimental system to detect early changes of toxic injury of cultured cells. It should provied useful information on mechanisms of action and expect to reduce some side effects of toxicants.

In this study we used the monolayer of JTC-12 and the decrease of transepithelial resistance (TER) was measured as changed by nephrotoxicicants; amikacin, kanamycin, neomycin, sisomicin, gentamycin. Leakage of sodium fluorescein was also measured as a integrity test of the monolayer after treatment with nephrotoxicicants. Differential staining of live and dead cells staining wre also performed.

Nephrotoxicicants caused decrease of TER after 10–15 min. And TER reached to stationary phase at 30 min. Dependence of concentration for antibiotics were observed at each one. And using leakage test of sodium fluorescein, disrupt of tight junctions were observed on each treated cell monolayer. These result indicated that the monolayer of JTC-12 would be usefully for toxic assay as same as MDCK.

Inui et al. reported toxicity index of aminoglicoside antibiodics on LLC-pK_1 cells using suppression of enzyme. Their result almost was same as ours.

Using measurement of TER of JTC-12 monolayers some initial changes of cell injury are able to detect.

An *in Vitro* Model for Assessing Muscle Irritation of Antibiotics Using Rat Primary Cultured Skeletal Muscle Fibers

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This study examined the possibility of using rat primary cultured skeletal muscle fiber to estimate the muscle irritation of antibiotics. In order to determine the adequate concentra-

tions of antibiotics (cefaloridine, cefazolin sodium, flomoxef sodium, cefamandole sodium, latamoxef sodium or defalotin sodium) in culture medium at which the primary cultured muscle fibers are exposed to them, the injuring effects of antibiotics were preliminary examined by exposing L6 cells to them at concentrations of 31.25, 62.5, 125, and 250 mg/ml (common rate=2) for 1 hour. The cell injury was determined by measuring residual cellular creatine kinase (CK) activity. From the results in L6 cells, primary cultured muscle fibers were exposed to antibiotics at various concentrations (common rate=1.2–1.4) for 1 hour. on day 7 of culture. The concentration fo the antibiotic, at which CK activity decreased to 50% of the control (depletion concentration 50%, DC_{50}), was utilized as an index of cytotoxicity. There was a good correlation between DC_{50} obtained in the rat primary cultured skeletal muscle fibers and the irritation volume in the *in vivo* test. These results suggested that the present *in vitro* system using L6 cells and rat primary cultured skeletal present *in vitro* system using L6 cells and rat primary cultured skeletal muscle fibers is a useful alternative model for *in vivo* rabbit study to evaluate muscle irritation.

An *In Vitro* Test System for Assessing the Phototoxic Potential of Test Materials

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Photoirritation occurs *in vivo* when the dermal irritation response elicited by a test substance increases in the presence of excitating radiation due to such factors as redox reactions, free radical formation, direct interaction with cell membranes or the production of toxic photoproducts. The *in vitro* alternative SOLATEX ®—PI test system was developed in our laboratory to predict the photoirritation potential of test compounds. Using this test system, a panel of 13 test materials containing nonphotoirritants, UV absorbers (nonphotoirritants) and known *in vivo* photoirritants, was diluted in several solvents, then added to membrane discs coated with keratin, collagen and a red dye (biomembrane barrier, BB). Thtese discs were placed into a highly ordered, biomacromolecular matrix consisting of several proteins in solution. Duplicate sets were prepared; one set was exposed to UV_A radiation; 320–400 nm; 800–950 $\mu J/cm^2$ for a period of 23 hours, the other maintained under identical coonditions except in the absence of UV_A. The background irritation response is quantified as the test sample-induced alteration(s) in the BB and/or in the protein matrix. The red dye is released when the integrity of this barrier is altered. Chemical irritants traverse the BB and perturb the underlying matrix to produce turbidity which is measured spectrophotometrically at 400 nm. The nonirradiated readings, as well as the contribution of the appropriate solvent vehicle, are subtracted from the irradiated readings and the UV_A-induced, enhanced response is quantified and categorized. Chloropromazine, promethazine, 67-methylcoumarin, 3-nitrobenzoic acid, acridine, doxycycline, salicylic acid and sulfanilamide were found to be photoirritating as expected. Chlorhexidine and p-aminobenzoic acid (PABA), UV absorbers, were found to be nonphotoirritants, also as expected. These findings agree well with reported *in vivo* phototoxicity data and demonstrate the utility of the *in vitro* SOLATEX-IP test system to correctly predict *in vivo* photoirritation potential.

Prediction of Phototoxicity by Red Blood Cells Hemolysis Test and Yeast Growth Inhibition Test

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Yeast growth inhibition, a phenomenon based on photodynamic effect of chemicals, was validated as a possible alternative to predict phototoxicity of chemicals.

After a paper disk with a test chemical was placed on the surface of yeast inoculated agar in a plate, the plate was exposed to UVA radiation (50 J/cm). Following incubation for 72 hr, diameter of J/cm). Following incubation for 72 hr, diameter of growth inhibition zone around the paper disk was measured.

The yeast growth inhibition test had a sensitivity of 89%, a specificity of 80%, a positive predictive value of 73%, negative predictive value of 92% and an equivalence of 81%, when the data for 24 chemicals wre compared with these of phototoxicity test in guinea pigs.

The combination of the photohemolysis test, which we already reported at the 5th Annual Meeting of Japanese Society for Alternatives to Animal Experiments, and the yeast test was used as a battery system. Sensitivity, specificity, positive predictive value, negative predictive value and equivalence were 100%, 67%, 64%, 100% and 77%, respectively.

Because of the sensitivity of 100%, this battery system, which is composed to these methods based on different mechanisms has shown to be useful as a screening tool for predicting phototoxic potential of new chemicals as a animal test alternatives.

Differences of Lymph Node Activation Among Three Species and Effect of Serum on Lymphocyte Proliferation in the Local Lymph Node Assay

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Contact sensitivities of metal allergens (nickel sulfate, potassium dichromate and cobalt chloride) were examined using the local lymph node assay in the CBA/N mice, F344 rats and Hartley guinea pigs. The effect of variation of serum on lymph node cells proliferation was also investigated. Mice exhibited the highest response to cobalt chloride among the three species. The responses to potassium dichromate in rats were higher than those in mice and guinea pigs. Nickel sulfate did not induce a marked proliferation in every species. The thymidine incorporation into the lymph node cells of each species when cultured in the presence of the homologous serum was lower than those in the presence or absence of fetal calf serum. However, there was no significant difference of the stimulation indices among these different culture conditions.

Evaluation of Contact Sensitivity from DNCB using *In Vitro* IL-2 Production

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Purpose

The present study was aimed to evaluate contact sensitivity from DNCB using hapten-stimulated *in vitro* interleukin-2 (IL-2) production by the lymph node cells from mice contact sensitized with DNCB. The correlationship between *in vitro* IL-2 production and *in vivo* hypersensitivity evaluated by mouse ear swelling test was also analyzed.

Materials & Methods

Six mice per group were skin-painted by topical application of 0.1 ml of the indicated concentration of DNCB in acetone/olive oil (4:1) to the trimed abdomen. On day 6 of the topical application, the mice were sacrificed to prepare politeal lymph node cell suspensions. Lymph node cells were cultured with the indicated concentrations of DNCB or medium in 0.2 ml of RPMI-FCS medium with the antibotics at a density of 5×10^5 cells/well in U-shaped microwells for 48 h. Supernatant was removed, frozen and assayed for IL-2 production. IL-2 activity was measured by its ability to sustained the growth of IL-2-dependent CTLL-2 cells (obtained from RCB, Tsukuba) as assessed by colorimetric assay and its activity was represented as stimulation index (optical density in CTLL-2 cell culture containing supernatants/optical density in CTLL-2 cell culture containing control medium).

Results

All results were shown in Fig 1 and Fig. 2.

Discussion

As seen in Fig 1, IL-2 productions in mice

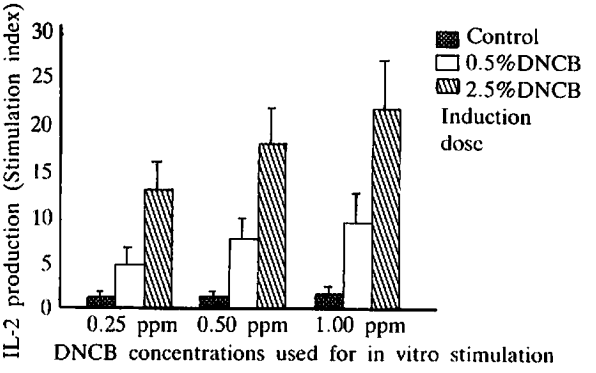

Fig. 1. In vitro interleukin-2 productions stimulated by DNCB in the lymph node cells from DNCB sensitized mice and non-sensitized mice.

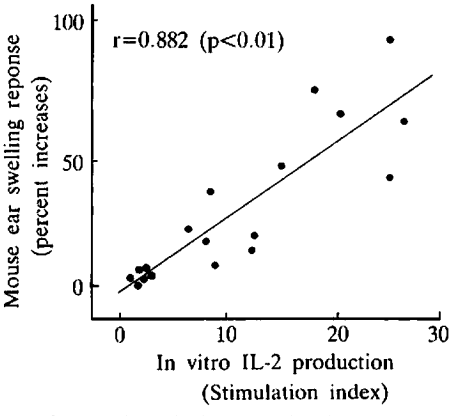

Fig. 2. Correlationship between in vitro IL-2 production and mouse ear swelling response.

sensitized with 0.5% and 2.5% DNCB were statistically higher than that in control group in the three doses of DNCB employed for the *in vitro* stimulation. Moreover, IL-2 productions in both DNCB sensitized groups wer increasing with te increased concentration of DNCB used for *in vitro* stimulation. As for the correlationship between *in vitro* IL-2 production and mouse ear swelling response, the former was closely correlated to the latter (Fig. 2). The correlation coefficient was 0.882 ($p<0.01$). Finally, *in vitro* IL-2 production was hapten-specific, and there was cross reac-

tion between dNCB and DNBS (Data not shown).

In conclusion the present study suggests that in vitro IL-2 production by lymph node cells can be used for evaluation of contact sensitivity from chemicals.

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Effective Extraction of Endotoxins with Albumin Solution for the Limulus Test of Sterile Disposable Devices

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We developed a new method for extraction of bacterial endotoxins with human serum albumin (HSA) from sterile disposable devices. Water for injection, saline, phosphate buffer, sodium dodecyl sulfate (SDS), triethylamine (TEA), and HSA were used for extraction from the sterile disposable polypropylene tubes which were charged with endotoxins. The extracts were quantitatively assayed by using the kinetic turbidimetric method with Limulus amebocyte lysate. The endotoxin recovery with HSA or TEA was the highest.

In order to confirm the efficiency of extraction of endotoxins with HSA, we compared HSA with TEA in extraction from commercially available sterile disposable devices. HSA was more effective to extract endotoxins than TEA.

Application of a Micro-Culture Method Using the Whole Blood to the Prediction of Lipopolysaccharide (LPS)-Induced Lethal Toxicity in Squirrel Monkeys

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LPS and its main active portion, lipid A, have various biological responses including antitumor activities and induction of some cytokines. However, there are extremely potent specificities not only among animal species but also among individuals concerning the toxicity. If a procedure capable of predicting these toxicity can be found out, it may be estimated at a higher probability in a smaller number of animals. Threfore, the present study was designed to examine whether *in vitro* mitogen response of the whole blood to LPS was available to prediction of LPS-induced lethal toxicity in monkeys. LPS (E. coli: 055) was administered subcutaneously at 0.3 mg/kg to female common squirrel monkeys for 14 consecutive days. Prior to the injection, the mitogen response to LPS was assessed *in vitro* using a small amount of the whole blood (25 μl) with a micro-culture method. As the result, the monkeys having a dose-dependent response to LPS *in vitro* elicited higher mortality as compared with those showing no response. The probability of

predicting potential was more than 80%. These results suggest that discrimination between a responder and non-responder to LPS can be performed by the micro-culture method prior to the investigation commences.

Acute Lethal Toxicity in Man Predicted by Cytotoxicity in 52 Cellular Assays and by Oral LD50 Tests in Rodents for the First 30 MEIC Chemicals

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MEIC (Multicenter Evaluation of *In Vitro* Cytotoxicity Tests) is a five year international program to evaluate the relevance and reliability of *in vitro* tests of toxicity and toxicokinetics (1, 2). The program is coordinated by a committee of Swedish *in vitro* toxicologists, once elected for that purpose by the Scandinavian Society of Cell Toxicology. The study started in 1989 by the publication of a list of 50 non-selected chemicals, with relatively well-known data on human systemic toxicity and toxicokinetics (1). Interested international laboratories were invited to test the 50 chemicals in their various *in vitro* assays and to submit the results to the committee for evaluation. Presently, around 80 laboratories are participating—*new participants are still welcome.* The aim is to compare submitted results with data on acute systemic, chronic systemic, local skin and eye, and various target organ toxicity in man, plus toxicokinetic data, to be able to serutinize relevance of *in vitro* data as well as select test batteries for future alternative testing of the above-

mentioned toxicities (1, 3).

Just after th start of the programme a small, preliminary evaluation of acute systemic toxicity with the first 10 chemicals tested by 14 methods was made (2). This paper describes a *second preliminary evaluation of acute systemic lethal toxicity*, performed with results from bindfolded *in vitro* tests on the first 30 MEIC chemicals in a total of 53 different assays. First, the prediction of human acute lethal doses of hte 30 chemicals by rat and mouse LD50-values was calculated, showing a better prediction by the mouse data. Second, average cytotoxicc concentrations from the main subgroups of tests with use of similar (20–72 hrs) incubation times were compared with human, acutely lethal blood concentrations of chemicals, showing in general a reasonable prediction of human data, in party with the rodent LD50 prediction of human lethal doses. Third, a combination of cytotoxicity data and simple human toxicokinetic data (passage of chemicals through the blood-brain barrier, intestinal absorption, liver metabolism, distribution volumes) could be shown to predict human lethal toxicity (blood concentrations *and* doses) better than the rodent LD50 data. Thus, most of the cytotoxicity tests in the study were relevant for toxic tissue concentrations of most chemicals studied.

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Cytotoxicity Study of 32 MEIC Chemicals by Colony Formation and ATP Content Assays

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The cytotoxicity of the 32 chemicals listed in the Multicentre Evaluation of *In Vitro* Cytotoxicity (MEIC) programme was evaluated by colony formaton (BALB 3T3 cells) and ATP assays (HL-60 cells and mouse erythrocytes). Significant correlations, γ=0.92, 0.97 and 0.89, were obtained from ID_{50} values (50% inhibition dose in comparison with the control) of the 14–18 chemicals which could indicate ID_{50} values, in erythrocytes *vs.* HL-60 cells, BALB 3T3 *vs.* HL-60 cells and BALB 3T3 cells *vs.* erythrocytes, respectively. When ID_{50} values of six chemicals which could show ID_{50} values in BALB 3T3 cells were compared with acute lethal blood concentration, acute oral lethal dose (human) and oral LD_{50} (mouse), the regression coefficients indicated 0.56–0.67. These regression coefficients were increased to 0.83–0.98 by omitting the data of digoxin which did not correlate between *in vitro* and *in vivo*. These results suggest that colony formation and ATP assays were shown to be useful for screening of chemicals.

Determination of Cytotoxicity of MEIC Chemicals on a Human Lung Carcinoma Cell Line by Lactate Dehydrogenase (LDH) Release Assay

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Purpose

Lactate dehydrogenase (LDH) release assay was used to measure the cytotoxicity of MEIC chemicals to replace animal experiments. The method, which detected the LDH activity of injured and surviving cells simultaneously, was widely recognized to be sensitive (Babson and Philips, 1965; Ohno, 1991; Sasaki, et al, 1992)

Materials and Method

The MEIC Chemicals, which were recommended for the Multicenter Evaluation of in vitro Cytotoxicity (MEIC) by the Scandinavian Society of Cell Toxicology (Bondesson, I et al., 1989), were dissolved in 5% FBS-MEM directly or by means of appropriate solvents indirectly. SQ-5 cells (human lung squamous carcinoma cells) were treated for 48 hours with MEIC chemicals serially diluted in 5%FBS-MEM medium. LDH activity released from the injured cells and the surviving cells was quantified using LDH Test Kit (Kyokuto Pharmaceutical Industries Inc.). The cell growth inhibition and the cell killing index were calculated according to the following formula:

$$\text{Growth inhibition} = \left(1 - \frac{\text{OD450 of surviving cells after 48-hour treatment}}{\text{OD450 of untreated control cells}}\right) \times 100\%$$

$$\text{Cell surviving index} = \frac{\text{OD450 of surviving cells after 48-hour treatment}}{\text{OD450 of cells at the beginning of treatment}}$$

$$\text{Cell killing index} = \frac{\text{OD450 of killed cells after 48-hour treatment}}{\text{OD450 of cells at the beginning of treatment}}$$

Results

The cell growth inhibition of each MEIC chemical was measured at a dose range with 6 replicates for each point. Because of wide variety of their of their characteristics, inorganic or organic water-soluble or -insoluble and medical or industrial uses, their test ranges differed dramatically from 0.1 μM to 1,000,000 μM. ED10 and ED50 of the MEIC chemicals were calculated by a computer-assisted Probit method, and were listed in our paper to be published. Among all the MEIC chemicals tested here, the most toxic ones were digoxin (0.38 μM), then followed by arsenic trioxide (3.24 μM), mercuric chloride (13 μM), hexachlorophene (48 μM), cupric sulfate (50 μM), pentachlorophenol sodium (51 μM), ferrous sulfate (89 μM) and thallium sulfate (102 μM). The least toxic were ethylene glycol (1, 220 mM), methyl alcohol (716 mM), ethyl alcohol (448 mM), isopropyl alcohol (235 mM), sodium chloride (149 mM), xylene (131 mM) and dichloromethane (131

mM).

We also found that sodium fluoride, nicotine, sodium chloride and ethylene glycol could apparently stimulate cell growth at lower concentrations. Sodium fluoride promoted cell growth at the concentration range of 0.7–1.4 mM with the maximum of 23.54% at 1 mM, nicotine at 0.6–7.4 mM with a maximum of 11.19% at 4.9 mM, sodium chloride at 17–68 mM with the maximum of 15.17% at 34 mM, and ethylene glycol at 0–500 mM with a maximum of 45% at 242 mM.

Cell killing indexes (KI) were the ratios of OD450 values of damaged cells to OD450 of 24-hour precultured cells. The dose-response curves ascended to a peak with increasing chemical concentration, and then descended gradually in the media containing higher concentrations. KI0.5 and KI1 represented the ability of chemicals to damage cells equivalent to 1/2 or whole quantity of the initial cell population of this assay.

The correlation of LDH ED50 with the LD50 of animals and the lethal doses of human was analysed. The cytotoxicity correlated better with LD50 in dog to which chemicals were injected intravenously (r=0.914), but less with that in human who orally obtained hcemicals (r=0.626).

Discussion

It perhaps was a unique advantage of the LDH release assay to simultaneously measure surviving and damaged cells after treated with test chemicals. Although most researchers calculated cytotoxicity of chemicals using surviving cells in their tests (NR, MTT, colony formaton tests and so on), we found cell killing index was another important criterium for indicating chemical's cytotoxicity. The cell killing curves showed the mechanism of cytotoxicity. Some chemicals killed cells rapidly at lower concentrations of the effective dose range, while others inhibited cell growth at lower concentrations and only killed cells at higher concentrations. Thterefore, ED10 and ED50 represented totaly toxicity of chemicals, and KI0.5 and KI1 meant the ability to kill cells.

Cytotoxicity of MEIC chemicals determined by the LDH release assay showed relatively good correlation with toxicity in animal experiments, especially with the LD50 in dogs to which administrated intravenously, especially with the LD50 in dogs to which chemicals were administrated intravenously. It was believed that hte correlations of animal experiments with cytotoxicity tests relied upon species, sex and age of animal, drug's administration routes, and toxicological mechanism (Ekwall et al, 1989). It was essential to detect directly in target organs because some chemicals exerted toxic effects through highly specialized neuroreceptors or ion channels. Sometimes, cell lines wouldn't be appropriate models for these chemicals (Weiss and Sawyer, 1992).

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Comparison of Some *In Vitro* Assays as an Alternative to *In Vivo* Eye Irritation Test for Surfactants

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We comparerd the correlations between *in vivo* Draize eye irritation score and 4 *in vitro* cytotoxicity assays-1. neutral red assay, 2. MTT assay, 3. LDH assay, 4. crystal violet assay, on about 60 surfactants.

There were a good correlation between *in vivo* draize eye irritation score and each *in vitro* assays used in this study. These results suggested that the 4 assays used in this study may be useful as an alternative method of *in vivo* draize eye irritation test for surfactants. But the LDH assay may be affected by protein denaturation reagents as an anionic surfactants. So It must be careful to use the LDH assay as a screening of anionic surfactants. The natural red assay was better correlation to *in vivo* Draize eye irritation score than the other assays in this study.

Cytotoxicity of Eye Drops Detected by LDH Release Assay

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Using a sensitive lactate dehydrogenase (LDH) release assay with cultured human cell lines, we detected cytotoxicity of 8 commercially available eye drop preparations among 9 tested.

One of these preparations increased tear LDH activity 3-fold after application to human eyes.

Comparison of Four Alternative Methods to the Draize Eye Irritation Test Using Cytotoxicity Test, Red Blood Cell Test, Protein Degeneration Test and EYTEX

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Recently, many alternative methods for the Draize eye irritation test have been developed and reported. In this work, we compared four alternative methods for the Draize eye irritation test. Cytotoxicity Test, Red blood Cell Test, Protain Degeneration Test and EYTEX were performed with 25 chemicals. Our results suggested that four methods are respectively useful for alternative test. But, to make sure of prediction of eye irritation, it is necessary to attempt to synthesize the results.

Prediction of Draize Rabbit Eye Irritation Test on the Battery System of 7 Alternative to Animal Tests

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We compared *in vivo* Draize rabbit eye irritation tests and 7 alternative to animal tests [① MTT assays in Normal human skin fibroblast (NB1RGB), ② Neutral Red uptake assays in CHL-IU cells, ③ Huhner Embryonen Test-Chorioallantoic Membrane Test (HET-CAM), ④ Hemolysis of rat erythrocytes, ⑤ Protein denaturalization of hemoglobin from ⑥ Primary Draize skin irritation scores, and ⑦ values of pH] to 23 cosmetic ingredients.

There was a good correlation among Draize eye scores and alternative to animal tests except values of pH. We inquired further into the battery system of alternative to animal tests to replace the Draize rabbit eye irritation tests.

Alternative to Animal Test of Draize Primary Rabbit Skin Irritation Test Using Skin^{2TM} 1300 Model and MTT Cytotoxicity Assay Kit

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The Skin^{2TM} Topical Testing Model 1300 is a three-dimensional human skin tissue that has dermal, epidermal and corneal layers. We compared MTT cytotoxicity assay of the Skin^{2TM} Model 1300, primary Draize skin irritation scores (Maximal Average Draize skin Scores: M.A.D.S.), and the MTT cytotoxicity assay of cultured Normal human fibroblast (NB1RGB) to the 18 surfactants.

There was a good correlation between cytotoxicities of Skin^2 Model 1300 and M.A.D.S., but correlation coefficient was low. There was no correlation between cytotoxicities of cultured cells and M.A.D.S..

Comparison Study of *In Vitro* Alternative Irritation Tests (Eytex^{TM}, Skintex^{TM}, CornePack and Skin^2) Using Some Antitumor Drugs

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Many *in vitro* irritation tests have been developed and are being developing and validated. However, there are, now, no complete methods as an alternative to the *in vivo* Draize test. If it is desirable to assess the irritancies of chemicals, it is recommended to

use one or more *in vitro* tests (if possible, different endopoints are preferable). Last meeting, we reported that one of alternative irritation tests, EYTEX/SKINTEX*[1], was useful for irritation assessment of chemicals including antitumor drugs. In the current report, we conducted the CornePack-IR*[2] and skin[2]*[3]-MTT assays to validate the predictability and usefulness among these three *in vitro* tests or between *in vitro* and *in vivo* tests. The results indicated that the two cytotoxicity assays had good correlation (70–80%) to *in vivo* data, and there were some chemicals which irritancies could be predicted by these assays and could not be by EYTEX/SKINTEX and vice versa. Using one or more of htese tests, we could get a better predictability of the *in vivo* results. Finally we also found some practical problems associated with these *in vitro* tests.

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Utilization of Target Macromolecular *In Vitro* Assay Systems in the Prediction of *In Vivo* Toxic Responses

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In vitro assay systems have been developed which utilize target macromolecules or molecular structures to predict *in vivo* toxic responses. Alternations in these macromolecules can be used as quantifiable endpoints. The EYTEX®, SKINTEX™, SOLATEX®—PI, Red Blood cell and Zein Methods are assay systems prototypic of this approach. The macromolecular structures used in these methods can be standardized and stabilized to provide reference databases. validation studies have established the relevance of these test methods in toxicity assessment of diverse chemical classes and formulations. For example, the relevance of the *in vitro* endpoint of turbidity of the EYTEX matrix to *in vivo* ocular irritation has been demonstrated for a large number of test agents. Integration of macromolecular-—based test methods with *in vitro* cytotoxicity or cellular response methods may provide complementary information about mechanisms of target-induced toxicity.

Nondestructive Structural Analysis of Rabbit Cornea and Structural Analysis of Protein Treated with Chemicals Using FT-1R Spectroscopy

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Nondestructive structural analysis of rabbit cornea and *in vitro* structural analysis f protein treated with chemicals were performed in order to investigate the mehanism of eye irritation using Fourier Transform Infrared (FT-1R) spectrometers.

Rabbit cornea and human serum γ-globulin (HSG) were used in this study. Surface of cornea and secondary structure of protein treated with chemicals were analyzed using a

fixed angle (45°) zinc selenide ATR prism and a Perkin Elmer FT-1R model 1720 spectrometer. The ATR/1R spectra of rabbit cornea and HSG were obtained at 4 cm^{-1} resolution with 30 scans. Difference spectrum between the spectrum of rabbit coenea and that of water was calculated by normalizing the intensity of a band near 2150 cm^{-1} due to water. difference spectrum between the spectrum of HSG and that of chemical solution was calculated in the same manner as for the rabbit cornea.

Decreases in the intensity of amide I and amide II bands were observed in the spectra of corneas treated with irritants and the dcrease was correlated with eye irritation score for 18 chemicals. The spectrum of HSG was close to that of rabbit cornea. Decreases in the intensity of amidc I and amide II bands were also observed in the spectra of HSG treated with irritants and a good correlation was obtained between the decrease of amide I band in the spectra of cornea and that in the spectra of HSG for 18 chemicals.

It was suggested that corneal opacity caused by chemicals mainly depends on the effect of chemicals on the structure of protein existing in the corneal surface and that *in vitro* study for assessing the effects of chemicals on protein can be used for the prediction of eye irritation potential of chemicals.

Comparison of the Responses of Human Skin to the Responses of the Living Skin Equivalent to Two Classes of Surfactant

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Living skin equivalents (LSE™) arc commercially available co-cultures of human dermal fibroblasts in a collagen lattice with overlying layers of stratified human epidermal keratinocytes. LSE were used to evaluate the relative dermal irritation potentials of selected anionic and cationic surfactants using a variety of endpoints. Time and dose-dependent changes in cellular viability, morphology, water permeation, and in the release of hte proinflammatory mediators, prostaglandin E2 and interleukin-1-alpha, were determined. For the five anionic surfactants tested the time required to inhibit mitochondrial function by 50%, as assessed by the thiazolyl blue (MTT) cytotoxicity assay, compared favorably to the rank ordering established by 24 hour occluded patch tests in humans. The capacity of these surfactants to disrupt the partial barrier function of the LSE's stratum corneum was assessed by measuring the rates of tritiated water penetration through the LSE. These results were compared to rates of transepidermal water loss on human volunteers exposed to these same surfactants. The variety of endpoints generated using the LSE illustrates the unique toxicological character of these classes of surfactant and emphasizes the advantages of using multiple endopoints for evaluating dermal irritation potential in this in vitro skin model.

Effect of Alkyl Chain Length on Skin Irritancy and Cytotoxicity of Sodium Alkyl Sulphate

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In this study we attempted to differentiate between *in vivo* and *in vitro* skin reactions to a homologous surfactant series (sodium alkyl sulphate) and to determine the usefulness of percutaneous absorption as an alternative system. Animal tests were performed: a primary skin irritation test in guinea pigs, primary eye irritation test in rabbits, and a human closed patch test. The peak skin irritation occurred with C_{10}-C_{14} sodium alkyl sulphate, which had lipophilic groups of different alkyl chain lengths. cell injury was also evaluated by neutral red dye uptake assay in rabbit corneal (RC) cells. C_4 and C_6 compounds had no effect; maximal effects occurred at c_{18}, the C_{18} compound causing more severe effects than C_{10}-C_{14} compounds. Percutaneous absorption in guinea pig skin was evaluated as an alternative to *in vivo* and *in vitro* testing. Permeation was low for the C_{18} compound and high for the C_4 compound. These results suggest that differences between cell injury and skin irritation resulted from skin permeation. It was found that the C_{18} compound caused cell injury, although membrane destruction and protein denaturation were more severe with C_{10}-C_{14} compounds due to their strong hemolytic and protein denaturation action.

An Alternative Metod of Darize Test Using Swine Cornea Implanted on the Chorioallantoic Membrane

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The present study has proposed an alternative *in vitro* method for the determnation of ocular irritancy using swine cornea implanted on the chick chorioallantoic membrane (CAM). The irritancy effects of five chemicals was investigated in the study. The CAM was exposed on day 14 of incubation and swine cornea was thereafter implanted on the CAM. The five chemicals were applied on the CAM on the next day. There was a close correlation in the toxicity of the five chemicals on CAM with swine cornea *in vitro* and the Draize score *in vivo*.

An Alternative to Rabbit Draize Test using Chorioallantoic Membrane (CAM) of Chick Embryo

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As alternative for draize test, a modified Luepke's CAM irritant test in the artificial air sac was evaluated. The fertiliged egg was carefully drilled and artificial air sac was mede on day 7 (the 7 days CAM test) or day 10 (the 10 days CAM test). In the 10 days CAM test, the chick embryo was almost alive before application of chemicals. In the 7 days CAM test, however, some embryos were inclined to be dead after the treatment of the shell equator.

As the conclusion, the modified CAM method, especially the 10 days CAM test, is recommended as alternative test for the rabbit Draize test.

Histopathologic and Biochemical Studies of Dermal Irritation —Histamine as a Possible Marker to Predict Skin Irritation *In Vitro*—

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Following application of dermal irritants, such as croton oil and oleic acid, to a guinea pig skin, both histopathological changes and hstamine release were examined. There seemed to be a relationship between the intensity of skin reactions and the appearance of certain types of cells responsible for the release of histamine. Also, there was a relatively good correlation between the magnitude of the release of histamine from rat mast cells and from TESTSKIN™ (LDM-MC: Organogenesis, U.S.A.) exposed to irritants and in vivo dermal irritation, suggesting that histamine he a possible marker to predict skin irritation in vitro. the peritoneal rat mast cells method and TESTSKIN™ will be further validated to see whether these models are promising alternatives to predict skin irritation.

Cytotoxicity Test for Various Diepoxy Compounds as a Crosslinker of Protein

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Cytotoxicity was studied for diepoxy compounds which have been used as a crosslinking agent of protein in the biomedical field. Neutral red assay[1)] was a crosslinking agent of protein in the biomedical field. Neutral red assay[1)] was carried out for this test using L929

and normal rabbit corneal epithelial cell (NRCE). The cells were first cultured for three days and then treated with the test compounds further for two days at 37°C and 5% CO_2. Viability of the cells was estimated from the uptake of neutral red.

It was found that there was no significant correlation between the *in vivo* primary irritation index and the *in vitro* test for both the cells (to NRCE R=0.50, to L929 R=0.53). This is probably because the test was performed over the very small range of toxicity. The correlation coefficient between NRCE and L929 was 0.77 and the cytotoxicity of diepoxy compounds to L929 cells was greater than that to NRCE.

In addition, as the chain length of the dicpoxy compounds became longer, the toxicity was less for the *in vivo* and the *in vitro* test. In this point, the *in vitro* test agreed with the *in vivo* test.

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On the Standardization of Cell Recovery Test

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The effect of repetitive 24-h treatments with cobalt and nickel ions followed by a 4 day recovery phase on the cell recovery of HEp-2 cells of human larynx origin was studied. in a 96 multi-well dish, containing MEM were plated 5×10^3 cells/ml, and kept in a CO_2 incubator (5%CO_2, 95%air, 37°C) for 24 hours, followed by a synchronous culture with a medium free of isoleucine. Then, either the cobalt or nickel ion was added to MEM at a concentration of 2.5, 5 and 10 ppm for treatment. Three cycles of treatmentrecovery phase were repeatedly carried out. The percent of cell recovery for nickel ion was higher than that for cobalt at a comparable concentration level of the respective cycle. Also, cell recovery values increased gradually with the advance in cycle. Complex aspects of cell reaction to various substances for medical and dental use should be evaluated by conventional cytotoxicity tests, together with a cell recovery test. Further validating approaches are needed for standardization.

Observation of Cultured Mouse Dorsal Root Ganglia (DRG) in Aging and in Chronic Ethanol Effect

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Living capsular cells in the mouse DRG were observed conforcally by laser scan microscorpe (Carl Zeiss). The cells were recognized in the 3rd day after explantation of DRG from the 17th day fetus. In some cases, the cells were scarcely found in all stages of the cultures.

Chronic effects of cthanol (0.47%, v/v, in medium) using young cultures proceeded on Schwann cells after 2 weeks and on neural cell safter about one month.