Annual Meeting Report

The14th Annual Meeting of the Japanese Society for Alternatives Animal Experiments (JSAAE)

November 15-17, 2000, Chiba

Plenary Lecture

Current Validation Studies on Alternatives to Animal Experiments in Europe
Horst Spielmann and Manfred Liebsch

Abstract

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Utilization and Some Problems with Skin Irritation Alternative Test Kit

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1. International harmonisation of animal test in regulatory toxicology

1989, ZEBET was established at the Federal Health Office (BGA) in Berlin as the National German Centre for Documentation and Evaluation of Alternatives to Animal Testing. ZEBET's mission is to reduce animal testing for regulatory purposes. The only concept available in 1989 to reduce testing in animals was the Three Rs principles of Russell and Burch [1].

It is the goal of regulatory toxicology in the field of chemicals to ensure the occupational safety of workers in the process of producing chemicals, to ensure the safety of food and beverages, to protect patients against possible hazards represented by drugs and medical devices, and to protect humans and the environment against possible hazards posed by residues of chemicals, e.g. pesticides. The standard approach in regulatory toxicology to assess the toxicity of chemicals is the determination of toxic properties in standardised animal tests, as described in the *OECD Guidelines for Testing of Chemicals* [2]. This information is then used by regulators to classify each chemical according to internationally harmonised guidelines in the first step, e.g. as harmful, toxic, irritant, then to label them in the second step according to EU risk (R) phrases, e.g. "R-41: risk of serious damage to the eye". The consequences of classification and labelling are the restricted use of the tested chemical in finished products (depending on exposure), and safety and labelling recommendations.

The international harmonisation of toxicity tests by the OECD in 1982 was the first, and so far, the most effective step in reducing duplication of testing in animals for regulatory purposes, since a toxicity test conducted according to the OECD guidelines will be accepted by regulatory agencies in all OECD Member States. These Member States are the world's major industrial nations. A similar approach has thereafter been used for the safety and efficacy testing of drugs by the International Conference on Harmonisation (ICH), which represents the three major economic regions namely, Europe, Japan and the USA. Since 1990, the ICH has accepted harmonised guidelines for efficacy and safety testing of drugs and medicines, including animal tests. Again, the harmonisation of test guidelines has led to significant reduction of testing in animals, since regulatory agencies around the world now accept the results of a test conducted according to ICH guidelines.

2. Evolution of the principles of scientific validation I: 1st Amden workshop on validation

Regulators will only accept alternatives to animal tests in toxicology, if the new tests will allow them to classify and label chemicals in the same way as the results of current animal tests allow them to do. The OECD has therefore indicated that *in vitro* toxicity tests can be accepted for regulatory purposes only after a successful experimental validation study. This procedure is essential to prove that the new *in vitro* toxicity tests will provide the same level of protection as the animal tests are currently providing.

To approach this problem scientifically, European and American scientists interested in the validation of toxicity tests met in Amden, Switzerland, to agree on a definition of experimental validation and to define the essential steps in this process. In the workshop report of the 1st Amden validation workshop, validation was defined as the process by which reproducibility and relevance of a toxicity testing procedure are established for a particular purpose [3], regardless of whether the method is an *in vitro* or *in vivo* test. In addition, at this workshop, the essential steps of the experimental validation process were defined in the following manner:

1. test development in a single laboratory;
2. experimental validation under blind conditions in several laboratories in a ring trial;
3. independent assessment of the results of the validation trial; and
4. regulatory acceptance.

Steps 2 and 3 were identified as the core part of a formal validation study conducted for regulatory purposes. The report of the 1st Amden workshop on validation encouraged scientists to start formal validation studies. Since the Draize eye test has been the most widely criticised toxicity tests, several international validation studies on alternatives to the Draize eye test were initiated:

1. BGA/BMBF study: national validation study in Germany 1988-1995 [4].
2. IRAG study: retrospective international study, organised by US regulatory agencies 1991- 1994 [5].
3. EC/HO study: international validation study organised by the UK, sponsored by EU 1992-1995 [6].
4. Japanese study: national validation study 1991-1995 [7].
5. COLIPA study: international validation study 1994-997 [8].

The management team of the EC/HO validation study, in which nine alternatives to the Draize eye test were tested under blind conditions with 60 carefully selected test chemcials in 36 laboratories, concluded at the end of the study in 1995 that none of the in vitro alternatives was able to completely replace the Draize eye test, and that the validation process had to be improved [6].

3. Evolution of the principles of scientific validation II: 2nd Amden workshop on validation

Thus, despite the joint efforts of many scientists around the world, the first attempt at validation failed, and the leading scientists involved met for a 2nd validation workshop in Amden in 1994, to learn from the unsuccessful attempts and to improve the validation procedure. Taking this experience into account, the participants in the 2nd Amden validation workshop recommended the inclusion of new elements into the validation process [9], which had not sufficiently been identified in the 1st Amden validation workshop. The three essential elements recommended were the definition of a biostatistically based *prediction model*, the inclusion of a *prevalidation stage* between test development and formal validation under blind conditions, and a well-defined *management structure*.

A *prediction model* should allow the prediction *in vivo* endpoints in animals or humans from the endpoints determined in the *in vitro* test. The prediction model must be defined mathematically in the standard operation procedure of the test that will undergo experimental validation under blind conditions with coded chemicals [9]. In order to assess the limitations of a new test before it will be evaluated in a validation study, the test should be standardised in a *prevalidation study* with a few test chemicals in a few laboratories [10]. This will ensure that the *in vitro* test method, including the prediction model, is robust and that the formal validation study with coded chemicals is likely to be successful. Finally, the goal of a validation study has to be defined clearly, and the *management structure* has to ensure that within the study the scientists who are responsible for essential tasks can conduct their duties independently from the sponsors and the managers of the study, e.g. biostatistical analysis, and the selection, coding and shipment of the test chemicals.

The improved concept of experimental validation for regulatory purposes defined in the 2nd validation workshop in Amden was accepted by the EU validation centre, ECVAM, in 1995, and in 1996 it was accepted by US regulatory agencies [11] and also by the OECD [12]. After this agreement at the international level, scientists have tried to follow

the ECVAM/US/OECD principles for validation in new validation trials. The improved validation concept was immediately introduced into ongoing validation studies, such as the ECVAM/COLIPA validation study on *in vitro* phototoxicity tests.

4. Successful validation and regulatory acceptance of *in vitro* toxicity tests

4.1 Validation of the 3T3 NRU in vitro phototoxicity test

Phototoxicity is an acute reaction, which can be induced by a single treatment with a chemical and UV or visible radiation. Since no standard guideline for the testing of photoirritation potential, either *in vivo* or *in vitro*, had been accepted for regulatory purposes at the international level by the OECD, in 1991, the European Commission (EC) and the European Cosmetics, Toiletry and Perfumery Association (COLIPA) established a joint programme on developing and validating *in vitro* photoirritation tests. In the first phase of the study, which was funded by DG XI of the EC and co-ordinated by ZEBET, *in vitro* phototoxicity tests established in laboratories of the cosmetics industry were evaluated, and a new assay, the 3T3 NRU PT test, which is a photocytotoxicity test using the mouse fibroblast cell line 3T3 and neutral red uptake (NRU) as the endpoint for cytotoxicity.

In the prevalidation study, which was conducted with 20 test chemicals (11 phototoxic and 9 non-phototoxic ones) quite unexpectedly, the 3T3 NRU PT *in vitro* phototoxicity test was the only *in vitro* test in which all of the test chemicals were correctly identified as phototoxic or non-phototoxic [13]. Independently of this prevalidation exercise, a laboratory in Japan subsequently obtained the same correct results in the 3T3 NRU PT, when testing the same set of 20 test chemcials.

In the second phase of the study, which was funded by ECVAM and co-ordinated by ZEBET, the 3T3 NRU PT test was validated with 30 carefully selected test chemicals in 11 laboratories in a blind trial on the 3T3 NRU PT test. A special ECVAM workshop was held to independently select a representative set of test chemcials covering all major classes of phototoxins was selected according to results from standardised photopatch testing in humans [14]. The results obtained in this *in vitro* test under blind conditions were reproducible, and the correlation between *in vitro* and *in vivo* data was almost perfect [15]. Therefore, the ECVAM Scientific Advisory Committee (ESAC) concluded, that the 3T3 NRU PT is a scientifically validated test which is ready to be considered for regulatory acceptance [16].

However, the EU expert committee on the safety of cosmetics, the Scientific Committee on Cosmetology and Non-Food-Products (SCCNFP), criticised the fact that there was an insufficient number of UV-filter chemicals (widely used as sunblockers) tested in the formal validation study. In the blind trial on UV filter chemicals, which was again funded by ECVAM and co-ordinated by ZEBET, the phototoxic potential of all of the 20 test chemicals (10 UV-filter chemicals, which were non-phototoxic, and 10 phototoxic test chemicals) was predicted correctly in the 3T3 NRU PT *in vitro* phototoxicity test [17].

Therefore, in 1998, the EU, having accepted the 3T3 NRU PT test as the first experimentally validated *in vitro* toxicity test for regulatory purposes, officially applied to the OECD for world-wide acceptance of this *in vitro* toxicity test. Early in 2000 the European Commission has officially accepted and published the 3T3 NRU PT phototoxicity test in *Annex V* of *Directive 67/548 EEC on the Classification, Packaging and Labelling of Dangerous Substances* [18]. Thus, this *in vitro* test is the first formally validated *in vitro* toxicity test that has been accepted into Annex V, and it is the only phototoxicity that is accepted for regulatory purposes in Europe. However, the OECD has

so far not taken the acceptance of this in vitro toxicity test on their agenda during the past two years.

4.2 Validation of two in vitro skin corrosivity tests

Two *in vitro* test for skin corrosivity testing applying a human skin model EPISKIN™ and excised rat skin, were successfully validated in an ECVAM validation study from 1996-1998 [19]. The ECVAM Scientific Advisory Committee ESAC concluded in 1998 [20]: The results obtained with the EPISKIN™ test involving the use of a reconstructed human skin model and the rat skin transcutaneous electrical resistance (TER) test in the international ECVAM validation study on *in vitro* tests for skin corrosivity were reproducible, both within and among laboratories that performed the test. The tests were able to distinguish between corrosive and non-corrosive chemicals for all of the chemical types studied. ESAC therefore agrees with the conclusions from the fornmal validation study that the EPISKIN™ test and the TER test are scientifically validated to be used as replacement for the animal test for distinguishing between corrosive and non-corrosive test chemicals, and that the tests are ready to be considered for regulatory acceptance. As a result, the two *in vitro* corrosivity test have been accepted by the European Commission for regulatory purposes in the year 2000 [21].

4.3 Validation of the Epiderm™ human skin model for corrosivity testing

Since the EPISKIN™ human skin model was not commercially available any more after it had been experimentally validated, a second human skin model the EpiDerm™ was validated in an ECVAM study from 1998-2000. This short study, which was co-ordinated by ZEBET and conducted in three laboratories with chemicals from the previous validation study, proved that the EPISKIN™ human skin model met the acceptance criteria of the TER and EPISKIN™ *in vitro* corrosivity tests. Therefore, ESAC concluded at its last meting in March of 2000 that the EpiDerm™ human skin model can be used for distinguishing between corrosive and non-corrosive chemicals within the context of the EU test guideline for skin corrosion [22].

4.4 ECVAM validation study of three in vitro embryotoxicity tests

In an ECVAM validation study, three *in vitro* embryotoxicity test were validated under blind conditions from the years 1997-2000. In the EU, there is a strong demand for validated *in vitro* tests in developmental toxicity testing using mammalian embryos as well as primary cultures of embryonic cells and permanent cell lines. As the most important result of the present validation study, for the first time, three *in vitro* embryotoxicity tests have been established that are backed by validated test protocols, which will be available through ECVAM as INVITTOX protocols 1. the whole embryo culture (WEC) test using cultures of whole rat embryos, 2. the micro mass (MM) test employing primary cultures of dissociated limb bud cells of rat embryos and 3. the embryonic stem cell test (EST), which is using two established mouse embryonic cell lines and which does not require to sacrifice pregnant animals.

In the ECVAM validation study 20 test chemicals were tested that are backed by high quality *in vivo* data in humans and animals. Each *in vitro* test was experimentally validated evaluated under blind conditions in four laboratories. All of the *in vitro* embryotoxicity tests met three essential criteria of validated alternative toxicity tests. Firstly, standard operation procedures (SOPs) were established, which are now available to public. Secondly, sound biostatistical prediction models (PMs) have been established and validated [23] The PMs for all of the three tests provide an accuracy of close to 80% and, more importantly, 100% predictivity for strong embryotoxic chemicals. Thus,

they can routinely be used to identify strongly embryotoxic chemicals, e.g. when screening new substances. Thirdly, the three *in vitro* tests were experimentally validated in a blind ring trial according to the validation scheme recommended by the EU, the OECD and the US NIEHS [2, 3, 11, 12]

Thus, this study clearly demonstrated, that the ECVAM strategy for prevalidation and validation of *in vitro* toxicity tests is sound. This conclusion based on the final report of the study was were accepted by ECVAM in June of the year 2000.

4.5 Validation of the Local Lymph Node Assay (LLNA) for sensitising properties

The Local Lymph Node Assay (LLNA) for the evaluation of sensitising properties, which was developed an validated in laboratories of the chemical industry in the UK (England), was accepted for regulatory purposes in 1999 by the federal regulatory authorities of the USA under the chairmanship of the US Validation Centre ICCVAM at the NIEHS [24]. In the year 2000, ESAC, the ECVAM Scientific Advisory Committee, concluded from reviewing this report that the LLNA is a scientifically validated test which can be used to assess the skin sensitisation potential of chemicals. Therefore, ESAC recommended that the LLNA should be the preferred method for sensitisation testing since it used fewer animals and causes less distress than the conventional guinea-pig methods [25]. However, in some instances and for scientific reasons ESAC accepted the use of the conventional methods.

4.6 Ban of the ascites method for the production of monoclonal antibodies

Taking into account studies conducted in several EU member states, ESAC has also recommended banning the production of monoclonal antibodies using the *in vivo* ascites mouse technique. Several companies have developed bioreactors, which allow to culture mouse hybridoma cells *in vitro*, which are producing monoclonal antibodies. A few EU member states are strictly enforcing the ban of the ascites mouse method, which causes pain, suffering and death of the mice, e.g. Germany, the Netherlands, Sweden and the UK, while other EU member states have not yet implemented the ban. Taking into account the progress in Europe, meanwhile the US NIH has stated that any research institute that continues to approve the routine use of ascites in producing monoclonal antibodies will no longer be eligible to receive a US Government research grant. is also recommending to use bioreactors rather than ascites mice for the production of monoclonal antibodies [25]..

4.7 Regulatory acceptance of four alternatives to the Draize eye irritation test

Several validation studies of *in vitro* alternatives to the Draize eye test have been conducted in Europe during the past decade. As a result, four *in vitro* alternatives have been accepted for regulatory purposes to identify severely eye irritating materials according to EU Directive 86/906/EEC for the classification and labelling of hazardous chemicals: the HET-CAM assay on the embryonated chicken egg, the BCOP test on the isolated bovine cornea from slaughterhouse, and two *in vitro* test on isolated chicken and rabbit eyes from animals that have been sacrificed for other purposes. Chemicals, which provide a negative reaction in any of the four *in vitro* tests still have to be tested in the Draize eye test in 1-3 rabbits in order to confirm the absence of exe irritation potential. In several EU member states, e.g. France an Germany, the HET-CAM test is accepted by the national authorities for the safety testing of cosmetics.

5. Conclusions and recommendations

The successful validation and regulatory acceptance of several *in vitro* toxicity test in the European Union proves that the validation

procedure recommended by ECVAM and the OECD [2, 3, 11, 12] is most appropriate for the validation of *in vitro* toxicity tests. However, taking into account both time frame and costs, e.g. of the ECVAM/COLIPA validation study of *in vitro* embryotoxicity tests, the formal validation procedure must be improved in order to reduce both costs and duration of the studies. To illustrate the problem, this particular study required funding of more than 1 million ECU (≅ US $) and the ECVAM validation study of three *in vitro* embryotoxicity was funded with a budget of 1.6 million ECU. The two examples illustrate that validation studies are very expensive and time-consuming, since it appears to take, on an average, 10 years from test development to regulatory acceptance.

Until today under the chairmanship of Professor Michael Balls and ECVAM the European Union has taken the lead in the experimental validation of *in vitro* test methods for regulatory purposes. In Japan several validation studies have been conducted on alternatives to the Draize eye test and on acute local irritation testing on the skin. In contrast, the US validation centre ICCVAM has focused its activity on reviewing the results of validation studies that were funded by other institutions, since money of the National Toxicology Program has so far not been set aside for the validation of *in vitro* methods but the situation may improve in the very near future.

Taking into account the lessons learned during the past decade, progress in the acceptance of *in vitro* toxicity tests at an international level will be achieved only, if Europe, Japan and the USA would share the burden of funding validation studies in a co-ordinated manner. The OECD might provide an appropriate forum for this important international activity although, due to other priorities, the OECD has not yet accepted the in vitro toxicity test that are used for regulatory purposes in Europe after successful validation.

Acknowledgements

The progress in the validation and acceptance of *in vitro* methods for toxicity testing could only be achieved with the continuous support of the staff of ZEBET, and the high priority that our work has been given both by ECVAM and COLIPA. I am particularly indebted to my colleagues in the management team of the ECVAM/COLIPA validation project on *in vitro* phototoxicity tests, namely Michael Balls (ECVAM, Ispra, Italy), Wolfgang Pape (Beiersdorf, Hamburg, Germany), Odile de Silva (L'Oréal, Paris, France) and Jack Dupuis (COLIPA, Brussels, Belgium), who passed away quite unexpectedly one month ago and to whom we want to dedicate the present manuscript.

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Abstract

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Utilization and Some Problems with a Skin Irritation Alternative Test Kit

Chairpersons:
Hajime Kojima, *Nippon Menard Cosmetic, Co. Ltd.,*
Toyozo Kaneko, *National Institute of Health Science*

SS. How to Use a Skin Irritation Alternative Test Kit (Technical Transfer Course)

Hajime Kojima[1] and Toyozo Kaneko[2]
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In Japan, some skin irritation alternative test kits are now on sale. Among these kits, EpiDerm™ has been evaluated in the ECVAM(European Center for Validation of Alternative Methods) validation study, and the results showed that it could be used for corrosivity testing for a wide range of chemicals. Corrositex™ was also evaluated by the Peer Review Panel in ICCVAM(Interagency Coordinating Committee on the Validation of Alternative Methods) . But no other kits, e.g.VitroLife™ or Testskin™ have been validated.

As a first trial of pre-validation on alternatives to skin irritation testing, we will hold a technical transfer course examining three kits(EpiDerm™, VitroLife™, and Testskin™) as a satellite session at the 14th annual meeting of Alternative to Animal Experiment and Testing in Japan. In this session, a researcher from three kit suppliers, Kurabo Industries Ltd., Gunze Co. Ltd., and Toyobo Co. Ltd., will explain the kit characteristics and method of use to more than 30 participants. Participants will use the kits, and grasp their main features.
After this session, we will supply a few sample kits, and furthermore have a plan to start a pre-validation study of these kits for each participant. Data of this pre-validation will be analysed by a biostatistician.

Symposium 1

Current Status of Alternative to Animal Experiments in Safety Assessment

Chairpersons:
Hiroshi Itagaki, *Shiseido Life Science Research Center*
Toyozo Kaneko, *National Institute of Health Science*

S1-1. Mutagenicity Tests as Alternatives to Animal Carcinogenicity Bio-Assays

Motoi Ishidate, Jr.
Former Director, Dept. of Mutagenesis and Genetic Toxicology, Natl Inst. of Health Sci., Tokyo, Japan

Introduction

Mutagens are those which induce gene mutations, chromosomal aberrations and/or DNA damage in prokaryotes or eukaryotes. The tests are principally to find genetic hazards of environmental chemicals, as well as radiation. Since the Ames test was established in 1975[1], it was considered to be a useful tool for the screening of environmental carcinogens. However, during the past 20 years, such a concept has been criticized and reevaluated by many investigators (*e.g.* Bartsch & Tomatis, 1983[2], Ashby, 1990[3]). According to the US-NTP report, mutagenicity tests may not be very predictive for carcinogenicity, even when the test systems with different genetic endpoints are used.[4] If this is the case, a question will arise as to why we should apply these mutagenicity tests to the current regulatory guidelines for chemical safety evaluation. It is assumed, however, that cancer

cells develop by a somatic mutation, in the process of a multi-step of gene mutation and/or chromosomal rearrangement during a quite long period. If we expect better predictive values in mutagenicity tests for alternatives to carcinogenicity bio-assays, we should consider the limitations of the mutagenicity tests and reevaluate more carefully the data accumulated during past years.

Recently Dr. S. C. Gad (1999)[5] proposed the 4th R, 'responsibility', in addition to the current 3R (Replacement, Reduction and Refinement; proposed by Russel & Burch, 1959)[6] which form the conceptual basis for reconsideration of humane animal use[5]. This concept should also be applied to the mutagenicity tests. At the present time, it is an urgent problem that possible carcinogens should be identified from the many chemicals in our environment. Only a limited number of chemicals have been tested for their carcinogenicity, but a great number of chemicals have not been properly validated so far. Our responsibility is to screen out the best candidates for carcinogens as quickly as possible and to eliminate them from our environment. It has been known that the majority of carcinogens are mutagenic, but some kind of carcinogens cannot be detected by the current mutagenicity tests (non-genotoxic carcinogens). On the other hand, it is plausible that mutagens found in *in vitro* systems are not always positive either in *in vivo* mutagenicity systems or in carcinogenicity bio-assays. Our responsibility is to reduce such false negatives as well as false positives in the screening tests.

In this paper, I would like to review the problems to be solved when we use the mutagenicity tests as alternatives to animal carinogenicity tests, and to propose a need for quantitative rather than qualitative evaluation on the mutagenicity data obtained.

National project (MHW in Japan) of short-term tests for detecting carcinogens

The collaborative studies conducted by the Ministry of Health Welfare (1973-1983) indicated that the mutagenicity tests can still be useful when a battery system with different genetic endpoints was used.[7] The results can be summarized as follows:

1) The majority of carcinogens (more than 80%) were positive for either the Ames test or chromosomal aberration test in cultured mammalian cells.
2) The chromosome test is one of the complementary tests to the Ames test, since some carcinogens negative in the Ames test were positive in the chromosome test. A similar conclusion has been obtained also in the collaborative studies conducted by the IPCS/WHO.[8]
3) Of the mutagens and/or clastogens, only approximately 25% were detected as carcinogens in animal experiments. Carcinogens such as phenacetin, hydrogen peroxide, potassium bromate and a number of heterocyclic amines were prelimenary detected as mutagens.

A battery system of the mutagenicity tests

In Japan, the Ministry of Labor requests first the Ames test for new industrial chemicals, and second, the chromosomal aberration test with cultured mammalian cells when the potency was relatively high in the Ames test. The Ministry of Health and Welfare, on the other hand, requests both the Ames test and chromosome test as a screen for industrial chemicals, and requests additionally the micronucleus test *in vivo* for new medical drugs.[9] Such a concept of the battery test system has been accepted in the genotoxicity guidelines introduced by other countries and the OECD. Sometimes, the unscheduled DNA synthesis (UDS) assay using hepatocytes is also proposed when the micronucleus test is negative.

Establishment of new *Salmonella* strains sensitive to particular compounds

For the Ames test, *S. typhimurium* TA100, TA1535 or TA92 and TA98, TA1537, TA1538 or TA94 are generally used to detect base-change and frame-shift type mutagens, respectively. In its experimental protocol, either the plate incorporation method or the liquid preincubation method can be applied when the cells are treated with the test agents. It has been known, however, that long-chain nitrosamines are more readily detected in the former, while short-chain nitrosamines are more readily detected in the latter method. Metal compounds, azo-dyes or hydrazine derivatives were weakly mutaenic in *Salmonella,* but strongly mutagenic *in E.coli* WP2*uvrA* or WP2*hcr*. According to the data obtained by the Ministry of Labor in Japan, approximately 7% of mutagens were detected only in the *E. coli* strains.[10] In addition, the OECD guideline has recommended *S. typhimurium* TA 102 or *E. coli* WP2 pkM which may be useful for detecting some oxidizing mutagens or cross-linking agents [11].

More recently, new sub-strains of *S. typhimurium*, YG-series, were established by M. Watanabe *et al.*. These were transformed by an engineering technique introducing *O*-acetyl-transferase or nitroreductase gene and

showed more sensitivity to specific classes of mutagens; *i.e.*, aromatic nitro, amino and hydroxylamino or nitroaromatic mutagens[12].

Problems in the qualitative evaluation of the results

If the data were evaluated only qualitatively, the predictive values for carcinogenicity are much lower than we expected. For example, some food additives such as caramel or Fast Green FCF were weakly positive only at high dose levels in both the Ames and chromosome test *in vitro*, but they were negative either in the micronucleus test or in the rodent carcinogenicity bio-assays. As indicated by D. Scott *et al.*, under some physico-chemically extreme conditions, *e.g.* changes in pH or osmolarity in the culture medium, false positives could be obtained at the dose close to cytotoxicity.[13]

In the chromosome aberration tests, some chemicals showed a quite different cytogenetic response with vs. without metabolic activation; *e.g.* a heterocyclic amine, MeAα C, induced only polyploid cells (70% at 0.062 mg/ml) in the absence of rat-S9, but induced structural aberrations (34% at 0.25 mg/ml) in the presence of the S9. It indicates that the parent compound may attack microtubular-associated protein, while its metabolite may directly attack the chromosomal DNA.[14]

Structural aberrations induced in chromosomes are generally classified into two types, breaks and exchanges. From our data, it was suggested that well-known carcinogens induce many exchanges in addition to break-type aberrations.[15] Ultraviolet radiation (UV) is one agent which typically induces predominantly the exchange-type.

The experimental outcomes vary by different protocols used even when the same substance was tested. Sofuni *et al.* tested 25 compounds which had been tested under the US-NTP program for their clastogenic responses. They found different results when these compound were tested according to the Japanese protocol using CHL/IU cells, instead of the US-protocol using CHO cells. It was concluded that the differences did depend on the experimental conditions, *e.g.* treating time or the S9 concentration *etc.*, but not on the difference between cell lines used. [16]

Quantitative evaluation on the results

For the Ames test, the mutagenic potency of a chemical can be quantitatively estimated from the maximum number of induced revertants/mg in a particular test strain. It was found that the mutagenic potencies among different mutagens differed very widely; for example, from 2-4 revertants/mg for caramel to 6×10^8 for a heterocyclic amine, MeIQ, in the Ames test, *i.e.* the mutagenic potency range is more than 100 million.

On the other hand, for the chromosomal aberration test, potency can be estimated from the minimum effective dose (D_{20} value in mg/ml) at which aberrations (structural or numerical) were detected in about 20% of metaphases.

It was found that potent mutagens in the Ames test are also potent clastogens (except the compounds being positive in only one of the two test systems) and only potent mutagens and/or clastogens were also positive in the *in vivo* micronucleus test.

Relative Values Calculated from the Mutagenic Potency

The quantitative values calculated from each mutagenicity test were compared with the TD_{50} which represents the approximate dose (mg/kg/body weight) at which any types of malignant tumors were found in 50% of animals in the carcinogenicity bio-assay conducted by the NTP (either mice or rats). The mutagenic potency was considered from the following factors:

1) The maximum numbers of revertants/mg for the Ames test
2) The minimum D20 values for the chromosome test
3) The response increased after metabolic activation
4) Exchange type exceeded break type aberrations in the chromosome test
5) Positives at more than 10mM were eliminated.
6) The minimum dose effective in the bone marrow cytogenetic test in mice
7) Positive in more than 2 test systems with different genetic end points

Taking into consideration the above factors and on the assumption that the total mutagenic potency of each chemical can be represented by a multiple regression analysis, the predictive TD_{50} (PTD_{50}) was calculated. The

result indicated there is a good correlation between the TD_{50} and the PTD_{50} , indicating that carcinogenic response in animals can be predicted by the mutagenic responses detected in a battery system with different genetic endpoints. Such quantitative estimation is however, the first trial, and needs further validation before concluding whether or not the mutagenicity tests can be applied as alternatives for animal bio-assays.

It must be borne in mind that there are some carcinogens which could not be detected by any of the current mutagenicity tests. They do not react with DNA, but show a chronic stimulation of cell proliferation or of UDS in DNA of damaged cells, or inhibition of apoptosis or of gap-junctional intercellular communications, *etc.* Such chemicals include certain oestrogenic hormones, H-2 blockers, peroxisome proliferators and immunosuppressive agents. We should develop appropriate *in vitro* screening tools for so-called non-genotoxic (tumor-enhancing) carcinogens or possible promotors which can play a role in the carcinogenic process. In addition, before we evaluate any positive correlation between mutagens and carcinogens, we have to clarify whether or not the carcinogenicity bio-assay in rodents can predict with confidence future human carcinogens. For this purpose, we need to focus research in molecular biology to analyze the mechanisms concerned. Newly developed techniques using transgenic animals are very promising and useful for the reduction of experimental animals. Cell transformation assays, especially those using cultured human cells, are also attractive as alternatives to the *in vivo* cancer bio-assay. This research should be also promoted in the future.

Conclusion

1) Mutagenicity tests have been applied to the primary screening for chemical carcinogens.
2) The majority of carcinogens showed potent mutagenic response in test systems with different genetic endpoints.
3) Mutagens detected in *in vitro* systems are not always carcinogenic in rodents (false positives).
4) Some carcinogens may not be detected in current mutagenicity tests (false negatives).
5) The new sublines genetically modified are useful for detecting particular chemicals and for reducing the number of false negatives .
6) Predictive values for carcinogens can be calculated if the mutagenic responses are comprehensively evaluated quantitatively rather than qualitatively.
7) Development of new biological techniques to identify unknown non-genotoxic carcinogens is expected.

We as scientists are reponsible for diligent mutagenic data evaluation. We should identify mutagens first using the current test systems with different genetic endpoints, and then estimate their mutagenic potency quantitatively. If a mutagen is potent, we confirm its mode of action from the viewpoint of chemical structure. If it is negative and is not suspected to have any promoting activity, additional carcinogenicity bioassays may not be required. We know that any short-term test is incomplete as an alternative to animal experiments. The urgent problem is not the argument of the relationship between mutagenicity and carcinogenicity of chemicals, but rather the need for data which provide mechanistic information in the applied test systems.

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S1-2. The Bacterial Endotoxin Test

Toshimi Murai
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Bacterial endotoxin, a characteristic component of the cell wall of Gram-negative bacteria, is known as the most powerful pyrogen present in nature. Chemically, endotoxin is composed of a complex heteropolysaccharide and a lipid moiety. In the blood stream, endotoxin levels as small as nanogram quantities can cause fever, whereas larger amounts may produce irreversible shock and death. The release of endotoxin from bacteria takes place after death and lysis of cells, and the released endotoxin is found almost everywhere in nature and is especially plentiful in water supplies. Thus, and because of its strong heat resistance, endotoxin is the most frequently occurring pyrogenic contaminant of pharmaceutical products intended for parenteral administration. The pyrogenic contamination of parenteral pharmaceuticals has been detected by the rabbit pyrogen test. The bacterial endotoxin test is an *in-vitro* alternative to the rabbit pyrogen test and is now being accepted generally because of its specificity, simplicity and remarkable sensitivity.

The bacterial endotoxin test, also called the *Limulus* test, is based on a clotting reaction elicited in lysates of amebocytes from the horseshoe crab, *Limulus polyphemus*, by small amounts of endotoxin. The *in-vitro* test is very simple and more sensitive than the rabbit pyrogen test for detecting and quantifying endotoxin. Further, the test has been demonstrated to be extremely specific and useful for detecting endotoxin contamination in a wide variety of drugs. Thus, the bacterial endotoxin test has been rated highly for attaining 3R -Replacement, Reduction and Refinement- and is now being adopted widely in the world as an official test method which can replace the rabbit pyrogen test.

In this presentation, I review the principle and practice of the bacterial endotoxin test and also on its distinguishing features and applications. Furthermore, I refer to some inherent limitations of the test as an alternative. In addition, I will introduce our recent work on the development of a novel *in-vitro* alternative which can compensate for the limitations of the bacterial endotoxin test.

S1-3. Biological Tests of Medical Devices and Materials

Masaaki Nakamura
Department of Biomaterials, Osaka Dental University

Medical devices and materials, which are used in medical or dental treatment, are mostly made of synthetic substances. Similarly many drugs are synthetic, though their mode of application contrasts with devices and materials. Biological safety tests consist of similar principles, but are different in detail. Animal alternatives have been extensively discussed in establishing new testing methods. A status quo in biological tests will be presented. Also future perspective for achieving animal alternatives in the arena will be given.

S1-4-a. Alternatives to Animal Testing for the Safety Evaluation of Cosmetics : Example a

Hiroshi Itagaki
Shiseido Life Science Research Center

Safety assessment is one of the most important requirements for cosmetics. In general, safety assurance of cosmetics or ingredients formulated is performed based on accumulated data, QSAR (quantitative structure-activity relationship), alternatives to animal testing, human patch test or product use testing.

We have actively investigated the development of alternatives to animal testing since 1981. The development of an alternative method can be divided principally into three stages, viz., research and development, validation, and practical application. We have proposed some new *in vitro* methods at the annual meeting of JSAAE. In order to obtain social or regulatory acceptance, we have positively participated in domestic or foreign validation projects including the project supported by the Japanese Ministry of Health and Welfare and JSAAE. Now, our results of the studies for alternatives have been routinely used at our institute for safety evaluation including eye irritation, skin irritation, and phototoxicity, etc.

The case studies of safety evaluation of cosmetics or cosmetic ingredients using alternative methods will be presented.

S1-4-b. Safety Evaluation of Cosmetics Using Alternatives to Animal Experiments : Example b

Atsushi Sato
Research Laboratories, Nippon Menard Cosmetic Co., Ltd..

Introduction

Our company has already adopted alternative methods to conduct the first screening of safety evaluation in terms of eye irritation test, primary skin irritation test and phototoxicity test, etc. In addition to these efforts, we have overseen a reduction in the number of experimental animals used and have pursued development of new evaluating methods.

Our efforts toward alternative methods include:

1) Eye irritation test

By referring to the evaluation scheme set by the scientific research group of MHW, we have a battery of Balb/c 3T3 Neutral Red Uptake Test and animal testing to evaluate eye irritation.

2) Primary skin irritation test

We have evaluated the skin irritation of an ingredient with a battery of cytotoxicity test using cultured monolayer cells and 3-dimensional cultured skin model, and animal testing.

3) Phototoxicity test

For compounds which show absorption peaks in the ultraviolet areas, we have utilized the Balb/c 3T3 Neutral Red Uptake Test (*in vitro* 3T3 NRU PT assay) for the first screening of phototoxicity.

4) Acute oral toxicity test

We have evaluated toxicity with an acute oral toxicity test on a unit of 2,000 mg/kg oral intake. If compound-related mortality is produced, a full study may need to be considered.

5) Skin sensitization test

We have principally used guinea pigs for assaying skin sensitization of cosmetic ingredients. We have developed tests such as the Mouse Ear Swelling Test (MEST) and Local Lymph Node Assay (LLNA) without radioactive isotopes.

S1-4-c. The Current Status of Animal Alternatives in Safety Assessment : Example c

Dana Laurie
The Procter & Gamble Company

This presentation will provide perspective about how alternative test methods (those that incorporate some aspect of replacement, reduction, or refinement of animal use) can be used to assess product safety. Judicious use of alternative methods in the hazard identification component of risk assessment combined with other components of risk assessment can support product safety assessment while eliminating or reducing animal use.

In addition to increasing use for safety assessments within Procter & Gamble, the use of alternative tests in safety assessment is increasingly being accepted by industry and regulatory agencies in many parts of the world. A current challenge for a wider use of alternative tests and further reduction of animal usage is to gain world wide regulatory acceptance of alternative test methods.

There are several reasons for the increased acceptance of alternative test systems as an integral component of product safety evaluation.

First, changes in the science of toxicology are being driven by the increased sophistication of tools available in biology including increasingly sensitive analytical methods; increased understanding of basic biological processes leading to much better understanding of the mechanisms of toxicological injury, sometimes even at a subcellular level; improved methods for *in vitro* preparation and manipulation of cells and tissues; large databases containing information on the toxicological properties of many substances; and major advances in computer tools and computing power. Together these tools are pushing all biological sciences more and more to *in vitro* and *in silico* methods, including toxicology.

Second, the increased understanding that most current alternative test methods are improvements over older alternative methods. Validation of new alternative methods has been extremely helpful in providing reassurance that the most current methods are predictive for the human experience.

Third, the recognition by companies and regulatory agencies that the amount of information available on most commonly used ingredients is now sufficient, and continually re-testing similar ingredients and formulas is not necessary or useful for assessing product safety.

Fourth, the international acceptance of criteria by which to validate new toxicology tests and the development of recognized processes to gain acceptance of new test methods by regulatory bodies throughout the world.

Finally, the continued concern of the public, including many research scientists, that animals should only be used in research and testing when no other method is available. Recent examples of the increased acceptance of these methods are the acceptance of three *in vitro* tests for skin corrosion by the European Union, the acceptance of the local lymph node assay by regulatory agencies in the United States, and the changes in the High Production Volume Chemicals program in the United States that will significantly reduce the amount of animal testing necessary for this program. Many companies are reducing or eliminating finished product testing, particularly for products made of common ingredients. Future progress will depend upon the development of processes which allow assessment of new ingredients using alternative methods.

Examples of test methods currently being used or being evaluated for use by Procter & Gamble include: *in vitro* and *ex vivo* methods for eye irritation, skin irritation, skin corrosion, phototoxicity, Type IV hypersensitivity, and carcinogenicity, some of which will be discussed during this presentation.

Plenary Lecture

Chairperson: Yuji Kurokawa, *National Institute of Health Science*

PL. Current Validation Studies on Alternatives to Animal Experiments in Europe

Horst Spielmann and Manfred Liebsch

ZEBET, National German Centre for Documentation and Evaluation of Alternative Methods to Animal Experiments, at the BgVV, D-12277 Berlin, Germany

The mission of the European Centre for the Validation of Alternative Methods (ECVAM) is the evaluation of the reliability and relevance of toxicity tests for specific purposes, in particular through prevalidation and validation studies, so that chemicals and products of various kinds can be manufactured, transported, and used more economically and safely, whilst the current reliance on animal test procedures is progressively reduced. Thus, apart from many other duties, it is ECVAM's main duty to initiate, fund, co-ordinate, and finally evaluate validation studies according to internationally harmonised validation and acceptance criteria, as agreed in the international OECD Workshop held 1996 in Solna (OECD: ENV/MC/CHEM/TG (96) 9).

The easiest way to reduce regulatory testing in animals is the international harmonisation of toxicological guidelines. A further reduction can be accomplished according to the 3-Rs-concept of Russel and Burch (1959). Very promising replacement and reduction alternatives have been developed and validated in Europe since 1992. In a few instances not only reduction alternatives but even replacement alternatives have been accepted at the international level.

So far, ECVAM has examined five alternative toxicological methods for hazard assessment in a series of subsequent prevalidation studies, validation studies, and additional special studies to achieve scientific evaluation of their validity for specific purposes. In one case (*in vitro* phototoxicity testing), the whole procedure needed seven years, and in the other case (*in vitro* skin corrosivity testing) the procedure needed four years. In all stages of these long-lasting processes a variety of independent experts and expert groups were involved e.g. in designing the studies, in the evaluation of the *in vivo* data, in the selection of test chemicals, and in the biostatistical planning and evaluation of the outcome of the studies. This almost permanent involvement of independent experts, as well as the fact that the outcomes of all stages of these studies were published in peer-reviewed journals, significantly contributed to the credibility of these studies and speeded up both the process of scientific acceptance by the ECVAM Scientific Advisory Committee (ESAC) as well as the process of regulatory acceptance of these methods by the responsible services of the European Commission.

An overview will be given on the principles of experimental validation developed in Europe during the past decade. In more detail the following validation studies will be presented, which were conducted in Europe since ECVAM was established in 1992:

1. The successful validation of an *in vitro* phototoxicity test applying the mouse fibroblast cell line 3T3 was finished in 1998. As a result, the 3T3 NRU *in vitro* phototoxicity test has been accepted for regulatory purposes in the year 2000. The validation study was managed by ZEBET, the German Centre for Documentation and Evaluation of Alternative Methods.
2. The experimental validation of two *in vitro* tests for skin corrosivity, applying a human skin model (Episkin) and excised rat skin, was successfully finished in 1999. As a result, the two *in vitro* corrosivity tests have been accepted for regulatory purposes in the year 2000.
3. A second human skin model, which is commercially available, met the acceptance criteria of the two accepted *in vitro* corrosivity tests. This skin corrosivity test applying the EpiDerm skin model has been recommended to be used for regulatory purposes by the ECVAM Scientific Advisory Committee ESAC in the year 2000.
4. In an ECVAM validation study, three *in vitro* embryotoxicity tests were validated under blind conditions from the years 1997-2000. The three tests are the whole embryo culture (WEC) test using organogenesis-stage rat embryos, the micro mass (MM) test using limb buds of organogenesis-stage mouse embryos, and the embryonic stem cell test (EST) using embryonic stem (ES) cells of the permanent mouse cell line D3. The 20 test chemicals used in the ECVAM validation study were backed by high quality *in vivo* embryotoxicity data. Under blind conditions of the validation study, the three *in vitro* embryotoxicity tests discriminated

quite well between strong embryotoxic and non-embryotoxic test chemicals. The validation study was successfully finished in the year 2000.

5. The Local Lymph Node Assay (LLNA) for the evaluation of sensitising properties, which was developed and validated in laboratories of the chemical industry in the UK (England), was accepted for regulatory purposes in 1999 by the US regulatory authorities under the chairmanship of the US Validation Centre ICCVAM at the NIEHS. In the year 2000, ESAC, the ECVAM Scientific Advisory Committee, recommended to accept the LLNA *in vitro* sensitisation test also in EU member states for regulatory purposes.
6. Taking into account studies conducted in several EU member states, ESAC has also recommended to ban the production of monoclonal antibodies using the *in vivo* ascites mouse technique. Several companies have developed bioreactors, which allow culture of mouse hybridoma cells *in vitro*, which are producing monoclonal antibodies. A few EU member states are strictly enforcing the ban of the ascites mouse method, which causes pain, suffering and death of the mice, e.g. Germany, the Netherlands, Sweden and the UK, while other EU member states have not yet implemented the ban. Taking into account the progress in Europe, meanwhile the US NIH is also recommending to use bioreactors rather than ascites mice for the production of monoclonal antibodies.
7. Several validation studies of *in vitro* alternatives to the Draize eye test have been conducted in Europe during the past decade. As a result, four *in vitro* alternatives have been accepted for regulatory purposes to identify severely eye irritating materials; the HET-CAM assay on the embryonated chicken egg, the BCOP test on isolated bovine corneas obtained from slaughterhouses, and two *in vitro* tests on isolated chicken and rabbit eyes from animals that have been sacrificed for other purposes.

AATEX Excellent Paper Award Lecture

Chairperson: Tadao Ohno, *Institute of Physical and Chemical Research*

AW. Utilization of Chick Embryonic Electrocardiograms to Detect the Pro-arrhythmic Actions by Antiarrhythmic Drugs

Takashi Sugiyama, Hiroyuki Miyazaki and Hideyo Shimada
Division of Pathophysiology, Center for Clinical Pharmacy and Clinical Sciences, School of Pharmaceutical Sciences, Kitasato University

It is our great honor that our paper was nominated the best paper in AATEX 1999. In addition, we are glad that it was recognized that chick embryo could replace mammals in experimentation and that our investigations might be able to contribute to the field of "refinement".

Since the Cardiac Arrhythmia Suppresion Trial (CAST, 1989) was reported, proarrhythmic actions induced by antiarrhythmic drugs have been widely examined. In this study, we investigated the pharmacological effects of antiarrhythmic drugs, *i.e.* Ia (procainamide), Ib (lidocaine), Ic (flecainide), II (propranolol) and IV (verapamil) types, which were classified by Vaughan Williams. After injection of anesthetic, a single dose of drugs was performed into the air sac of White Leghorns-fertile eggs on the 16th day of incubation and ECG waves were recorded using our newly devised recording systems. Heart rate (HR) was calculated from RR intervals and PQ, QRS and QT intervals measured from ECG waves. All drugs decreased HR in a dose-dependent manner. When higher dosages of drugs were injected, these drugs induced various kinds of arrhythmias, A-V block etc., almost the same patterns as observed in mammals. Also, the ECG waves responded in the same manner as in mammals, with a few exceptions(Table 1). In conclusion, our ECG recording system in chick embryos may be applicable as a screening test for proarrhythmic actions of antiarrhythmic drugs without any stimulation to the chick embryos.

Table 1. Changes in ECG parameters in chick embryos, rat and man treated with antiarrhythmic drugs

Drugs	Class	Qualitative changes in ECG parameters			
		RR	PQ	QRS	QT
Procainamide	**Ia**	↑ ↑ (↑, ⇑)	↑ ↑ (↑, ⇑)	↑ ↑ (↑, ⇑)	↑ ↑ (↑, ⇑)
Lidocaine	**Ib**	↑ ↑ (↑, ⇒ / ⇑)	↑ (→, ⇒)	↑ ↑ (↑, ⇑)	↑ ↑ (↑, ⇓)
Flecainide	**Ic**	↑ ↑ (⇑)	↑ ↑ (⇑)	↑ ↑ (⇑)	↑ ↑ (⇑)
Propranolol	**II**	↑ ↑ (↑, ⇑)	↑ (↑, ⇒ / ⇑)	→ (↑, ⇑)	↑ (↑, ⇓)
Verapamil	**IV**	↑ (↓, ⇑)	↑ (↑, ⇑)	→ (→, ⇒)	→ (↑, ⇒)

↑↑ : significant prolongation, ↑ : prolongation, ↓ : shortening, → : no change
→ : chick embryo
(→) : rat
(⇒) : human

Symposium 2

Possibilities of High -throughput Screening in Alternative Animal Experiments

Chairpersons:
Tetsuya Kamataki, *Hokkaido University*
Ikuo Horii, *Nippon Roche Research Center*

S2-1. Present Stage of Rational Drug Design

Akiko Itai
Institute of Medicinal Molecular Design, Inc.

Goals of rational drug design are increasing successes of drug development by decreasing experimental trials and errors and hastening development processes. So far, we have developed new positive methods for constructing reliable working hypotheses, selecting more promising models or compounds from a vast number of possibilities, ranking them by rational measures and presenting promising structures and estimated binding constants, by deriving theoretical equations and parameters from experimental observations based on theories and experimental information. When the three-dimensional structure of the target protein is available, new ligands can be reliably searched by our virtual screening method from compound databases without performing high-throughput random screening. With our methods, a number of new ligands have been found by confirming the activities of a small number of promising compounds (~200), selected from a million compounds in commercially-available compound databases based on the stability and structural features of the most stable docking model estimated for each compound. The importance of *in silico* methods for handling molecules and molecular interactions is increasing.

S2-2. Pharmacogenomics and Drug Target Discovery

Toshio Tanaka, Yuhei Nishimura, Hiroshi Tsunoda and Michiko Naka
Department of Molecular and Cellular Pharmacology, Mie University School of Medicine

Gene sequencing and gene expression are of particular significance in pharmacogenomics. Sequencing is used to locate polymorphisms, and monitoring of gene expression can provide clues about the genomic response to disease and treatment. In addition, we have used gene expression profile analysis to identify therapeutic target genes by studying changes of gene expression in animal models of oxidative stress and hypoxia.; these conditions are important in the pathogenesis of various diseases. We found novel drug target candidates under oxidative stress and hypoxic conditions through this pharmacogenomic strategy. Our results suggest that pharmacogenomics have potential as a strategy for defining novel drug targets in various diseases.

S2-3. Application of DNA Chip (in Japanese)

Junichi Mineno
Bio Medical Center, Takara Shuzo Co., Ltd.

S2-4. Safety Studies and High Throughput Screening in Toxicology in Research and Development of New Drugs

Ikuo Horii
Dept. of Preclinical Science, Nippon Roche Research Center

Recent advances in molecular targeting techniques based on genomics have brought epoch-making progress in generating new drugs. In addition, combinatorial chemistry has facilitated the production of various kinds of new compounds. Toxicologists are now required to examine the toxicity of such new compounds, great in number but small in amount.

The concept of high throughput toxicity testing was developed as a result of these circumstances. New technologies of *in vitro*/*in vivo* screening systems and toxicogenomic approaches for evaluation of toxicity are drawing the attention of people engaged in the research and development of new drugs.

Given that "every adverse effect is caused by the expression of related genes or the change of genes affected by a compound", toxicogenomics in particular is expected to play an important role in elucidating the mechanisms of adverse effects, as well as in screening new compounds for candidate selection.

Taking this situation into account, the following need to be considered for improving new drug development: 1) toxicological assessment of new compounds in the early stage of development, 2) planning of toxicity studies and evaluation of the results for extrapolation to humans.

In the *in vivo* high throughput toxicity studies conducted in the early phase of development, it is necessary to obtain a toxicological profile using a small amount of a new compound in a short period of time. In addition, there are numerous parameters to be determined, such as target organs and their functions; moreover, the evaluation should be quickly done with the aid of highly sensitive and specific parameters (ex. urine NMR pattern recognition), newly-devised technique of rapid histopathological specimen preparation, telemetry, and so on. For prompt evaluation, an *in vitro* cell culture system using cells from target organs and gene chip technology are of significant utility.

We have to realize that the methods for investigating and evaluating toxicity are changing in every phase of drug development - from lead selection to candidate selection, entry-into-human, clinical research, NDA and post-marketing research.

S2-5. Development of High Throughput Screening (HTS) System of Drug Metabolism in Japan

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Introduction

In the development of new drugs, it is necessary to screen many chemicals with respect not only to pharmacological properties but also to properties of drug metabolism and toxicology. High throughput screening (HTS) of such chemicals in the early stage of drug development allows us to shorten the period and to lower the cost of drug development.

Cytochrome P450 (CYP) catalyzes the oxidation of a wide variety of drugs. CYP forms play roles in the detoxification and in the activation of chemicals. It is important to clarify whether or not model chemicals are metabolized or metabolically activated by CYP. If the properties of drug-metabolizing enzymes present in experimental animals are the same as those of human enzymes, the results obtained with experimental animals can be extrapolated to humans. Since the catalytic properties of CYP even in the same family vary among animal species, it is necessary to use human CYP to predict human drug metabolism. Large amounts of CYP enzymes are needed to perform HTS of model chemicals in the study of drug drug metabolism and toxicity. Human liver specimens have been used as a useful tool to predict human drug metabolism, while the use of these preparations was limited by several factors. The use of human livers has been limited for ethical reasons. In addition, the population of each form of CYP varies according to the medical background of donor patients.

Thus, the use of CYP preparations expressed in heterologous expression systems has become more popular as alternative methods for examining human drug metabolism. We have made efforts to establish expression systems of several CYP forms using bacteria, yeast, and cultured mammalian cells as host cells. In this symposium, I will talk about the establishment of the expression system for human CYP using bacterial cells and the properties of the human CYP forms expressed in these cells. I will also talk about the possibility of the application of the bacterial expression system to HTS system of drugs in metabolism and toxicity.

The establishment of bacterial cells expressing human drug-metabolizing enzymes and the evaluation of the properties of the system

1) The application of E. coli cells expressing human CYP to predict drug metabolism in humans.

We have successfully established eleven strains of *E. coli* expressing each form of human CYP together with human NADPH-CYP reductase (OR). For example, the expression level of CYP2C8 in the *E. coli* cells corresponded to that of 3.75 kg of human liver. The catalytic activity of CYP expressed in *E. coli* cells was examined with representative substrates for each form of CYP. CYP forms expressed in the *E. coli* cells efficiently metabolized the typical substrates with high *V*max values compared to the data obtained by using human liver microsomes or CYPs expressed in other host cells. *K*m values were similar to those obtained by human liver microsomes or CYPs expressed in other systems. It seems likely that the modification of N-terminal amino acid sequence does not affect the catalytic properties of CYP expressed in the *E. coli* cells. The substrate specificities of all CYP forms expressed in *E. coli* cells were examined. For example, testosterone, known as a substrate for CYP3A subfamily, was metabolized by CYP3A4, CYP3A5 and also CYP1A1. The metabolic clearance seen with CYP3A5 and CYP1A1 was about one eighth and one third that seen with CYP3A4, respectively. The substrate specificities of CYP forms expressed in the *E. coli* cells were consistent with the results previously obtained by using human liver microsomes or other heterologous expression systems. The established *E. coli* strains seem to be useful tools to predict metabolic pathways of drugs in humans.

The metabolic process often causes drug-drug interactions. It has been reported that the metabolism of terfenadine catalyzed by human CYP3A is inhibited by azole antifungal drugs, resulting in a remarkable increase of the drug concentration in plasma to induce severe side effects of terfenadine in humans. Thus, it is important to predict whether or not metabolism of a certain drug catalyzed by human CYP is inhibited by other drugs. We evaluated the inhibition of taxol 6α-hydroxylase activity of human CYP2C8 expressed in *E. coli* cells by drugs. Dihydropyridine derivatives of calcium antagonists strongly inhibited taxol 6α-hydroxylase activity. The *K*i

value of nicardipine was approximately 54.7 nM. We concluded that CYP forms expressed in the *E. coli* cells may be applicable to predict drug-drug interactions which occur in the process of drug metabolism.

2) The mutation assay with Salmonella cells expressing human drug-metabolizing enzymes

Genetically engineered *Salmonella* cells co-expressing human CYP and OR can be applicable to mutation assays. The frame shift mutations are detectable with the *Salmonella typhimurium* TA1538 strain. The point mutations are detectable with the *Salmonella typhimurium* YG7108 cells with high sensitivity, since the strain lacks O^6-methylguanine DNA methyltransferase *ada* and *ogt* genes. We established *Salmonella* TA1538 and YG7108 strains expressing each of the eleven forms of human CYP together with OR. Mutagenic activation of aflatoxin B_1 and benzo[*a*]pyrene was examined with eleven strains of *Salmonella* TA1538 expressing each form of human CYP. The CYP forms responsible for the activation of these promutagens were consistent with those observed in previous studies. We established *Salmonella* TA1538 cells expressing human CYP1A2, OR and *Salmonella O*-acetyltransferase. The mutagenicity of mutagens formed from heterocyclic amines such as IQ and MeIQx was detectable with the *Salmonella* cells at a pM level of promutagens. We concluded that the established tester strains are useful to detect mutagenic activation of promutagens in humans.

Establishment of a system to produce a large amount of CYP with high efficiency using the established *E. coli*

Large amounts of CYP are necessary for the HTS system of examining metabolism and toxicity of drugs. However, there were problems in the classical culture methods of the genetically engineered *E. coli* cells expressing human CYP together with OR to obtain a large amount of enzyme. A hundred mL of medium in five hundred mL flasks was used to grow *E. coli* cells. Therefore, many flasks were needed to obtain a large amount of CYP. The other problem is that we could not optimize the culture condition of the genetically engineered *E. coli* cells, since the physicochemical conditions of culture medium affecting the expression of human CYP could not be controlled. We adopted the jarfermentor (JFM) to grow the *E. coli* cells. The maximal volume of culture medium is 1 L. Using the JFM, temperature, oxygen concentration and pH of the culture medium can be controlled. The expression level of CYP3A4 in the *E. coli* cells by using the JFM was three times higher than that obtained by the classical culture method. Two hundred and seventy nmol/L of expression was observed when culture was performed 18 h at 30℃. The optimum oxygen concentration of the medium was 0.1 ppm. Thus, we could establish a system producing a large amount of CYP by using JFM.

Conclusions

We concluded that CYP forms expressed in bacterial cells may be applicable to predict metabolism and toxicity of model chemicals. We could establish a method to produce a large amount of CYP by JFM. The bacterial expression system of human CYP can be applicable to an HTS system of studying metabolism and toxicity of drugs.

S2-6. High-Throughput Screening in Drug Metabolism : Significance and Problems

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Recent progress has been made in increasing the throughput of measurements of absorption, distribution, metabolism and excretion (ADME) parameters for drug candidates. This presentation focuses on three methods: (1) *in vitro* screens for metabolic stability with LC-MS detection, (2) *in vitro* screens for permeability with LC-MS detection and (3) screens for the inhibition of cytochrome P450-mediated metabolism (a mechanism for drug-drug interaction) using fluorometric assays. Methods are available that substantially increase the throughput and decrease the resources needed for conducting these routine preclinical measurements.

ADME measurements that are commonly measured in vitro and the in vivo significance of these parameters are listed below.

1. The overall rate of metabolism of the drug. The rate of metabolism influences pharmacokinetic properties such as oral bioavailability and clearance in humans and preclinical species.
2. The specific cytochrome P450 enzyme(s) involved in metabolism. The number and identity of the

enzymes involved in metabolism influences the interindividual variability in metabolism (i.e. metabolism exclusively by polymorphic enzymes will lead to high levels of population variability) and the potential for drug-drug interactions.

3. The profile of metabolites. The profile of metabolites is important for prodrugs, for pharmacologically active metabolites and for the selection of appropriate species for preclinical safety assessment (i.e. are the metabolites formed in humans also formed in the toxicology species?).
4. Aqueous solubility and membrane permeability influence the extent of oral drug absorption.

In the development of new chemical entities (NCEs) as human therapeutics, the rapid estimation of aspects of ADME parameters contributes useful information to the optimization process. While many of these parameters can be determined using the classical methods, the need to increase throughput has lead to certain assumptions and simplifications of experimental design. For example: (1) the number of replicates and assay volume may be reduced. (2) A low NCE concentration (1 μM) is used. It is assumed to be below the apparent K_m and the determined metabolic rates are related to *in vitro* intrinsic clearance (V_{max}/K_m). (3) A single time point may be used as a triage. These changes are justified because these methods are for decision making purposes. For any NCEs which are selected for further development, these (and many more) parameters will be further characterized using more rigorous, classical methods.

Higher throughput methods for measurement of permeability through cell monolayers, metabolic stability in human and animal liver microsomes and inhibition of cytochrome P450s will be presented. For all of these methods, critical experimental design parameter and the limitations to these assays will be discussed. One recent finding that is of particular interest is the probe substrate selective effects commonly observed for the inhibition of cytochrome P4503A4 - the most important human drug metabolizing enzyme.

In summary, substantial progress has been made in accelerating the acquisition of *in vitro* data permeability, metabolic stability and cytochrome P450 inhibition data. These data, in conjunction with other ADME data, provide useful information in the optimization process of drug development. Continued progress will likely be in areas of instrumentation, assay automation and the development of novel fluorometric probe substrates.

S2-7. Significance and Problems of High-Throughput Screening (HTS) of Enzyme Inducers

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As an approach to drug discovery, the screening of large numbers of new chemical entities (NCEs), such as those present in a combinatorial library, is a process of attrition; meaning that chemicals are largely eliminated on the basis of their unfavorable pharmacodynamic or pharmacokinetic properties. In the former case, chemicals might be eliminated for their failure to bind to the desired therapeutic target (*e.g.*, a receptor) or for their lack of specificity. In the latter case, chemicals might be eliminated because of potential ADME problems; namely problems with absorption (such as acid lability or poor gastrointestinal absorption), distribution (such as high protein binding or poor penetration of the blood-brain barrier), metabolism (such as metabolic instability) and elimination (such as active transport). The potential for drug interactions is another criterion that can be used to eliminate chemicals with unfavorable pharmacokinetic properties. It is desirable to eliminate chemicals that inhibit cytochrome P450 (CYP) and other drug-metabolizing enzymes (DMEs) because such drugs might inhibit their own metabolism (thereby giving rise to dose-dependent pharmacokinetics) or they might inhibit the metabolism of other drugs (and thereby increase their side effects). It is similarly desirable to eliminate drugs that have the opposite effect, *i.e*, those that induce (up-regulate) the expression of CYP enzymes and other DME's. Such drugs might induce their own metabolism or the metabolism of other drugs, which would diminish their therapeutic effect. This is especially a problem with drugs that have a narrow therapeutic index (such as warfarin and cyclosporin) and with drugs that do not exert a graded therapeutic effect (such as oral contraceptive steroids, which either do or do not block ovulation). Another concern is that many enzyme inducers are tumor promotors in rodents. Consequently, chronic administration of enzyme inducers to rats and mice often results in the devel-

opment of liver and/or thyroid tumors.

Problems with traditional approaches to identifying enzyme inducers

The traditional approach to evaluating the enzyme-inducing potential of NCEs involves treating laboratory animals *in vivo* with high doses of chemical for several days (or weeks), followed by an *in vitro* analysis of changes in CYP enzyme levels/activity in microsomes prepared from the livers of such treated animals. Although this *ex vivo* approach can readily be incorporated as part of 14-day toxicity study and offers several advantages over other approaches, it suffers four major disadvantages. First, an *ex vivo* study is time consuming. Second, it requires large quantities of chemical. Third, it requires the use of numerous animals. Fourth, it produces results in animals that may not be applicable to humans because of differences in dosage (enzyme induction in laboratory animals often occurs only at dosages that are not clinically relevant) or because of species differences in the ligand-binding properties of the receptors/ transcription factors that mediate enzyme induction. For example, omeprazole and rifampin are inducers of CYP1A and CYP3A in humans, respectively, but they are not inducers in rodents because of species differences in the aryl hydrocarbon (*Ah*) receptor, which mediates CYP1A induction, and the pregnane-X receptor (PXR), which mediates CYP3A induction. As a result of these limitations, alternative approaches have been sought to evaluate the enzyme-inducing potential of chemicals with the goal of developing a rapid, in vitro system based on human-derived cells. Toward this end, we have investigated the use of primary cultures of human (and rat) hepatocytes to screen NCEs for their enzyme-inducing potential. In an effort to accelerate and automate this screening procedure (*i.e.*, in an effort to develop a high-throughput screening [HTS] technique), we have examined whether cryopreserved hepatocytes can be used instead of freshly isolated hepatocytes to screen enzyme inducers. Inasmuch as most CYP enzyme induction involves transcriptional activation of *CYP* genes, we have also evaluated whether increases in mRNA levels can be used to identify enzyme inducers, and whether changes in mRNA levels can be detected with a rapid and highly sensitive assay based on branched DNA (bDNA) technology. Before the results of this promising line of research are described, it is informative to examine alternative approaches that might similarly lead to the development of HTS assays for enzyme inducers.

Receptor binding

The receptors that mediate the induction CYP1A, CYP2B, CYP3A and CYP4A have been identified as the *Ah* receptor, CAR$_\beta$, PXR and PPAR$_\alpha$, respectively. In principle, inducers of these enzymes could be identified in a receptor binding assay, perhaps one in which NCE's are screened for their ability to displace a radiolabeled ligand from these receptors (especially the human receptors). Indeed, there are several published procedures for examining ligand binding to the *Ah* receptor, although these tend to be rather complicated procedures to circumvent problems associated with the high degree of non-specific binding of *Ah* receptor ligands (such as polycyclic aromatic hydrocarbons and TCDD) to other proteins and lipids. Although this approach might prove useful, it is already apparent how false negative and positive results will arise. For example, omeprazole would test as a false negative because although this drug binds to the Ah receptor, it appears to bind at a site other than the "normal" ligand-binding site. Other *Ah* receptor ligands, such as (α-naphthoflavone, are antagonists, which points to the central problem with this approach: How can receptor agonists be distinguished from receptor antagonists? Another complicating factor concerns our incomplete understanding of the nature of ligand binding to CAR$_\beta$ (a constitutively active transcription factor which mediates CYP2B induction). Finally, the issue of "*context-dependent induction*" is one that would confront studies of ligand binding to PPAR$_\alpha$,which results in CYP4A induction in rodents but not humans. Nevertheless, if these issues can be addressed, it should be possible to develop an HTS assay for enzyme inducers based on receptor binding studies.

Reporter gene constructs and immortalized cell lines

Reporter gene constructs have the potential to provide a functional assay on which to base an HTS assay for enzyme inducers. Such an assay has been developed for CYP1A inducers by placing a reporter gene (such as luciferase) under the control of the xenobiotic-response element or XRE, which is a small stretch of DNA that binds to, and is activated by, the ligand-bound *Ah* receptor. Immortalized cell lines transfected with this construct express the reporter gene (which can be detected by the highly sensitive technique of chemiluminescence) when the transfected cells are exposed to *Ah* receptor ligands for several hours or longer. The success of this technique requires that the cells selected for transfection purposes express the *Ah* receptor, as well as its co-activating protein, *Arnt*. Many immortalized cell lines, including some of human origin, express the *Ah* receptor

and *Arnt*, and can therefore be used to screen CYP1A inducers. However, the same does not hold for the other receptors. Therefore, without suitable levels of CAR$_\beta$, PXR and/or PPAR$_\alpha$ (or their co-activating protein, RAX), it will not be feasible to use immortalized cell lines transfected with reporter gene constructs to develop an HTS assay for enzyme inducers.

One way to circumvent this problem is to transfect reporter gene constructs into primary hepatocytes, which do express the appropriate receptors and co-activators. This approach has been used successfully. We have explored this approach, but found the results (*i.e.*, expression of the reporter gene) varied from one experiment to the next. This approach suffers from other disadvantages. For example, the hepatocytes must be transfected before they are cultured, which can result in considerable cell stress. Only a fraction of the cells become transfected, which complicates any attempt to compare the expression of the reporter gene with the expression of the CYP enzymes themselves. The latter measurements, based on immunoblotting or assays of CYP enzyme activity, reflect CYP levels/activity in the bulk of the hepatocytes, whereas the former measurement reflects luciferase activity in the few hepatocytes that were transfected with the reporter construct. For this reason, in our efforts to develop a robust and rapid HTS assay of enzyme inducers, we have focused our attention on primary cultures of hepatocytes and the use of branched DNA (bDNA) technology to measure the levels of mRNA encoding inducible CYP enzymes.

Primary cultures of rat and human hepatocytes

We have established procedures for culturing rat and human hepatocytes under conditions that are suitable to screen NCE's for their ability to induce CYP enzymes. In this system, a typical induction experiment involves: (1) isolating the hepatocytes by collagenase digestion of the liver followed by Percoll™ centrifugation, (2) culturing the isolated hepatocytes in a sandwich configuration (with a collagen substratum and a Matrigel™ overlay), (3) allowing the cells to restore normal hepatocellular morphology and function (1 to 2 days adaptation), (4) treating the cells with NCEs and prototypical inducers (positive controls) for three days, and (5) isolating microsomes to determine CYP enzymes levels (by immunoblotting) and/or CYP activity (by various fluorimetric, radiometric, spectrophotometric and HPLC assays). By preparing and analyzing microsomes from cultured hepatocytes, it is possible to compare the results of the *in vitro* (cell culture) procedure with those from a traditional *ex vivo* procedure. Based on such comparisons, we have established that our cell culture procedures permit CYP enzyme induction to be studied in a manner that is qualitatively and quantitatively similar to that observed in *ex vivo* studies. There are several obstacles to adapting this *in vitro* procedure to an HTS assay. These include: (1) the time taken to isolate hepatocytes; (2) the erratic supply of human liver; (3) the adaptation period (typically 24 to 48 hours after seeding); (4) the duration of treatment with inducers (typically three days); (5) the time to prepare microsomes (several hours), and (6) the time to analyze the microsomes by immunoblotting or enzyme assays (which may take several days depending on the particular procedure). We have begun to address some of these limitations.

Cryopreserved hepatocytes

We have established procedures for freezing, storing and thawing rat hepatocytes under conditions that enable enzyme inducers to be screened in primary cultures prepared from cryopreserved rat hepatocytes. The ability to culture cryopreserved hepatocytes bodes well for the development of an HTS assay for enzyme inducers. Similar experiments with human hepatocytes have met with mixed success. In most cases, the cryopreserved human hepatocytes do not attach well to a collagen substratum, which precludes their use for enzyme induction studies. Nevertheless, the success achieved with rat hepatocytes suggests that it might be possible to establish procedures that enable human hepatocytes to be cryopreserved and subsequently used for enzyme induction studies.

Branched DNA (bDNA) assay of mRNA levels.

Having determined that CYP levels in microsomes from cultured hepatocytes treated with prototypical inducers are comparable to *in vivo* levels, we evaluated whether changes in CYP levels in cultured rat and human hepatocytes were mirrored by similar changes in corresponding mRNA levels. Primary cultures of rat and human hepatocytes were treated with prototypical CYP inducers, which were then analyzed for microsomal CYP1A, CYP2B and CYP3A activity, or were analyzed for mRNA levels by the Quantigene bDNA Signal Amplification Assay from the Diagnostic Division of Bayer Corporation. The bDNA assay measures mRNA levels in cell lysates based on a highly sensitive chemiluminescence assay that is adapted to a 96-well microtiter

plate (and more compact formats). Sample preparation is nominal compared with preparing microsomes. Likewise, the assay set-up and chemiluminescence detection procedure are substantially quicker than those required for immunoblotting or enzyme assays.

We established that the measurement of mRNA levels by the bDNA assay provides a reliable alternative to immunoblotting/enzyme analysis to detect the induction of CYP1A, CYP2B and CYP3A enzymes in both rat and human hepatocytes. Consequently, bDNA technology bodes well for the development of an HTS assay for screening enzyme inducers. For an automated HTS assay, hepatocytes would ideally be cultured in the same format as the bDNA assay. For example, both would be carried out in 96-well microtiter plates. In this case, it would be possible to screen for one or two mRNA species. However, it is noteworthy that the bDNA assay is so sensitive that the mRNA from a single, large culture dish (60 mm) can be used to analyze 20 or more mRNA species, which has enabled us to investigate the inducibility of several DMEs (including Phase II enzymes) in addition to CYP enzymes.

Ongoing and Future studies

There are several other opportunities for accelerating the procedure for screening enzyme inducers. Given that an increase in mRNA levels is an early event in the induction process, it might be possible to reduce the typical three-day treatment period to one day (or possibly less). However, it seems doubtful that the adaptation period (the time it takes the seeded hepatocytes to restore normal hepatocellular morphology and function) could be reduced to less that one day. Development of an HTS assay, particularly one that could be automated, would also be facilitated by the demonstration that hepatocytes cultured in a 96-well format (or smaller well size) respond to inducers no differently than do hepatocytes seeded in much larger culture dishes. This is not a trivial point because cell density, cell-cell contact and oxygen levels all affect the response of hepatocytes to inducers, and these important variables will likely change under different physical conditions. As already mentioned, another important advance in this area would be the development of techniques for cryopreserving human hepatocytes that, when thawed, would attach to a collagen substratum in culture. In summary, although a robust HTS assay for enzyme inducers has yet to be developed, considerable progress has been made toward this goal.

Panel Meeting

Alternative Test Kit for Skin Irritation and It's Problem

Chairperson: Hajime Kojima, *Nippon Menard Cosmetic, Co. Ltd.,*

PM. Utilization and Some Problems with a Skin Irritation Alternative Test Kit

Hajime Kojima

Research Laboratories, Nippon Menard Cosmetic, Co. Ltd.

In 1998, ECVAM(European Center for Validation of Alternative Methods) published details on *in vitro* skin corrosivity tests, which were the 3-dimensional cultured human skin model(culture model); EPISKIN™ and the rat skin transcutaneous electrical resistance test(TER). EpiDerm™, an other culture model also evaluated in the ECVAM validation study, was shown to be applicable for corrosivity testing for a wide range of chemicals. Furthermore notice of a pre-validation skin irritation study was published in 1999. The study includes four methods, EPISKIN™, Prediskin™, EpiDerm™ and non-perfused pig ear model. On the other hand, Corrositex™ was also evaluated by the Peer Review Panel in ICCVAM(Interagency Coordinating Committee on the Validation of Alternative Methods) .

In Japan, some skin irritation aleternative test kits are now on sale. Some kits such as the culture models, VitroLife™, Testskin™ etc., have not been validated but EpiDerm™ and Corrositex™. The future of this field in Japan is unclear.

In this panel meeting, I would like to discuss the present condition and future view concerning skin irritation

alternative test kits with researchers from kit suppliers, Kurabo Industries Ltd., Gunze Co. Ltd., and Toyobo Co. Ltd., and with participants in this annual meeting. The following three sub-titles have been prepared.

Sub-title
1) Characteristic of construction and percutaneous absorption of a kit
2) Prediction between *in vivo* and *in vitro*
   ① corrosion
   ② irritation
3) Reform and down-costing

PM-1. Potential of 3-Dimensional Human Skin Model for Percutaneous Absorption

Takuya Ishibashi and Hidekazu Takahashi
Tsuruga Institute of Biotechnology, TOYOBO CO., Ltd.

A 3-dimensional (3-D) human skin model with closely similar morphology to native epidermis has been developed and examined for its possible use as an alternative to *in vivo* skin.

The LSE is an organotypic skin culture model that has been developed by Bell. The LSE is a bilayered model consisting of dermal fibroblasts in a type I collagen lattice overlaid with a stratified, cornified epidermal layer. This model exhibits a number of morphologic and biochemical features of human skin. But the barrier function of the model is not complete as compared with intact skin.

Therefore we tried to improve the barrier function of the LSE and evaluate the improved model (LSE-high) for percutaneous absorption. The cumulative amount of each drug which permeated through LSE-high over 30 min was comparable to that through rat skin over 8 hrs.

PM-2-1. The Use of Human Skin Model for Skin Corrosivity Testing

Hisashi Torishima
Biomedical Department , Kurabo Industries Ltd.

By way of introduction ECVAM has supported a formal validation study on *in vitro* methods for predicting the skin corrosivity properties of chemicals. These serve as examples of alternative methods for *in vivo* toxicity testing.

In 1996-97, two of the *in vitro* tests in the study employed human skin models which were commercially available at that time, the Skin^2 ZK1350 and EPISKIN models. Both skin models had shown basic usefulness for corrosivity testing, and in particular, the EPISKIN corrosivity test had proved to be a scientifically valid test. However, the production of Skin^2 ceased in October 1996, and the EPISKIN model is currently not commercially available, despite plans to reintroduce it to the market.

In the ECVAM skin corrosivity validation study, BASF, Huntingdon Life Sciences (HLS) and ZEBET laboratories decided to conduct a study which had the objective to determine whether another commercially available human skin model, EpiDerm, could also be successfully used for predicting skin corrosivity. The study was performed according to the ECVAM prevalidation scheme, to allow for refinement of the test protocol and the prediction model, as well as for independent assessment of the performance of the refined methodology in a final blind trial in the three laboratories.

In conclusion, the present study proved that the EpiDerm skin corrosivity test provides an excellent prediction with a wide spectrum of chemicals, and holds promise as an alternative to the validated EPISKIN corrosivity test, which is currently not available. The concept of prevalidation applied to the present study allowed refinement of the EpiDerm skin corrosivity test, to overcome the weakness the Skin^2 corrosivity test had shown in the ECVAM skin corrosivity validation study, and to "catch up" with the EPISKIN corrosivity test, which was successfully validated in the same study.

PM-2-2. Can Skin Models Estimate *In Vivo* Skin Irritancy?

Katsuyasu Morota
Research and Development Division, GUNZE Ltd.

Our primary concern is "Can skin models estimate in vivo skin irritancy?" or "How can we trust the result?" Over the past decade, many reports have claimed that skin models can predict skin irritancy caused by chemicals. On the other hand, it is also known that some chemicals show up as false-positive or false-negative. This may be due to:

① wrong treatment of skin model
② structure deference of skin model
③ biochemical mode of action of irritant

In this session we would like to discuss the usefulness of skin models.

PM-3. 3) Reform and Down-costing

Hajime Kojima
Research Laboratories, Nippon Menard Cosmetic, Co. Ltd.

In this session, I would like to appraise a skin irritation alternative test kit. I will especially highlight some problems with the kit and the fact for 3dimensional cultured skin models do have deficiencies. There is a need for reform and down-costing in this area, as well as for improvement in test methods and consideration of new approaches such as the epicutaneous absorption assay

Mini Workshop
New Approaches to the Assessment of Acute Systemic Toxicity

Chairpersons:
Hiroshi Ono, *Food and Drug Safety Center*
Sumie Yamanaka, *Tokyo Dental College*

W-1. Novel Approaches to Acute Toxicity Tests : Prologue

Hiroshi Ono
Hatano Research Institute, Food and Drug Safety Center,

Alternatives to the traditional acute toxicity test, or LD50-test, have been substantiated, in OECD Guidelines for Testing of Chemicals by recent adoption of three new guidelines for acute oral toxicity testing. The three new methods, the Fixed Dose Procedure (No.420), the Acute Toxic Class method (No.423) and the Up-and-Down Procedure (No.425), though still undergoing some revisions, are going to replace the traditional acute toxicity test (No.401). The results of these tests have confirmed their applicability to classification schemes of chemicals currently in use. Though we can expect considerable reduction of the number of animals to be used, these are still animal experiments by nature, and though alternative tests may adequately fulfil chemical classification requirements, they will not replace all the uses of LD50 or of classical acute toxicity test. LD50 has been used for (1)classification and labeling of chemicals as for their hazard, (2)profiling the acute toxic effects of chemicals and obtaining knowledge relevant to accidental acute poisonings, (3)estimation of initial doses for ensuing longer-term toxicological studies, and for the early clinical study, (4)estimation of biological activities of certain pharmaceuticals, and for (5)comparison of toxic potencies among related chemicals.

Already considerable efforts have been dedicated to the development of in vitro alternatives to acute toxicity test, for instance with the MEIC project. The *in vitro* tests have always been evaluated by whether the results correspond well or not to LD50.

We may find a novel approach to hazard identification of chemicals by moving away from the criteria based on the mortality of animals. We should find a way to characterize the biological activities of chemicals at the initial phase of their intesation with humans, with appropriate sets of *in vitro* testing methods representing one of various cellular and subcellular activities.

W-2. Screening Assessment of Acute of Chemicals by Using Cultured Cell Lines : with Special Reference to Cytotoxicity and Induction of Apoptosis

Sumie Yamanaka

Department of Hygiene, Tokyo Dental College

We determined the basic cytotoxicity and the degree of induction of apoptosis for assessment of the toxic potential of chemicals *in vitro*. Cytotoxicity was evaluated by using five cell lines and three methods in order to estimate the toxicity of 28 chemicals. The IC_{50} values (the concentration of 50% growth inhibition) by cytotoxicity assay showed high correlation (r=0.87) with the oral LD_{50} values *in vivo*, which were obtained by sacrifice of many rodents. However, this correlation did not hold for skin irritants such as formaldehyde and hydrogen peroxide. The skin irritants had low IC_{50} values compared with high LD50 values because of direct membrane injury.

Furthermore, the apoptotic cells were distinguished from intact or necrotic cells by flow cytometry, and the relationship between the IC_{50} values of chemicals and the degree of apoptosis induction was determined. Mercury and titanium compounds induced few apoptotic cells only at concentrations near IC_{50} values. But in the case of Actinomycin-D, which was known as an apoptotic inducer, a high proportion of apoptotic cells was detected at concentrations much lower than the IC_{50}. The IC_{50} values of chemicals and the degree of apoptosis induction were evaluated by cytotoxicity test using flow cytmetry. Therefore, it was suggested that the degree of apoptosis induction could be an index of cytotoxicity as well as the IC_{50} value. Furthermore we should investigate the assay conditions, because the apoptotic cells measured by flow cytometry showed different features according to the cell lines used, the chemicals and reaction times, etc.

W-3. Prediction of Human Acute Systemic Toxicity from the Results of Cytotoxicity Obtained in the MEIC Programme

Noriho Tanaka

Laboratory of Cell Toxicology, Hatano Research Institute, Food and Drug Safety Center

There are some difficulties in predicting human acute systemic toxicity from the results of cytotoxicity *in vitro*. The reason is that each chemical displays a complexity of dynamic actions *in vivo* which can not be fully studied *in vitro* such as absorption in the gut, metabolism in the liver, blood protein binding, receptor toxicity, passage across blood-brain barrier, distribution in the organs, organ-specific toxicity and effects of low dose and long-term exposure, *etc.*

In recent research, the MEIC (Multicenter Evaluation of *In Vitro* Cytotoxicity) results provided valuable data to predict human acute systemic toxicity. In this study, 29 laboratories participated and tested 50 reference chemicals with known human acute toxicity using 61 cytotoxicity assays. *In vitro* cytotoxicity results (IC50) were compared with human acute lethal blood concentrations (LCs) by linear regression analysis. Rat and mouse LD50 values were also compared with human acute lethal doses. Rodent LD50 values predicted human lethal doses of the 50 chemicals relatively well (R^2 = 0.61-0.65). An average IC50 value for the ten tests treated for 24h using human cell lines predicted human LCs better than the other tests (R^2 = 0.74). When IC50 values for the 32 chemicals which can rapidly enter the brain were compensated for brain vulnerability, and IC50 values obtained for 48h treatment with the ten slow acting chemicals with a restricted passage into brain were used for

comparisons with 48h human LCs, the foregoing correlation improved substantially ($R^2 = 0.88$).Our results with the ATP content assay using HL-60 showed good prediction with human LCs of all chemicals ($R^2 = 0.72$) (Wakuri *et al.*, Toxic in Vitro 7, 517-521, 1993; Ekwall *et al.*, ATLA 28, 201-234, 2000).

W-4. Prediction of the Starting Dose for Acute Oral Toxicity (LD_{50}) Testing in the Up-and-Down Procedure (UDP) from Cytotoxicity Data

Manfred Liebsch, Elke Genschow, Willi Halle and Horst Spielmann

ZEBET, Center for Documentation and Evaluation of Alternative Methods to Animal Experiments at the BgVV, Berlin (D)

Three alternative methods to the "classical LD_{50} test" for determination of acute oral toxicity (OECD TG 401) have been accepted at the OECD level between 1992 and 1998, namely the Fixed Dose Procedure (FDP, OECD TG 420), the Acute Toxic Class Method (ATCM, OECD TG 423), and the Up-and-Down-Procedure (UDP, OECD TG 425). The latter two methods are sequential procedures based on consecutive dosing where each dosing is based on the outcome of the preceding dose, thus saving a considerable number of animals. Consequently, the number of animals saved with these two sequential procedures depends on how close the starting dose is to the 'true LD50' (UDP) or 'true toxicity class' (ATC).

To reduce the number of animals in acute oral toxicity testing, cytotoxicity data (IC_{50}) can be used to predict the starting dose for testing from the standard regression between IC_{50} and acute oral LD_{50} of the Register of Cytotoxicity (RC). In the RC the correlation between cytotoxicity, represented by the mean IC_{50}, and the acute oral LD_{50} of rats and/or mice was determined for 347 chemicals and drugs by applying the linear regression model for the log-transformed pairs IC_{50} versus oral LD_{50}. We have previously demonstrated that the standard regression line of the two toxicity parameters is characterised by an intercept $a = 0.625$ and regression coefficient $b = 0.435$ and that 252 of 347 chemicals (72.6 %) are located within a dose range differing not more than ± 0.699 (factor FG $\leq$ log 5) from the standard regression line.

We have previously suggested to reduce animal numbers in acute oral toxicity testing in the acute toxic class (ATC) method by using cytotoxicity data and the RC regression model for predicting the starting dose ATC method, which is a sequential testing procedure. In the present study we have used the RC and its IC_{50}/LD_{50} regression model to predict the LD_{50} from cytotoxicity data of 9 chemicals from an evaluation study of the up-and-down (UDP) procedure for acute oral toxicity testing in rats. For 7 of the chemicals, LD_{50} values (mg/kg) predicted from the RC were in the same dose range as LD_{50} values determined *in vivo*, while the dose range was differing by one order of magnitude for the two remaining chemicals. Thus the prediction of LD_{50} values from cytotoxicity data was promising in this limited data set.

To reduce animal numbers in the UDP method for acute oral toxicity testing we are, therefore, proposing a tiered *in vitro/in vivo* testing approach. As the first step cytotoxicity of a new chemical is determined. By applying the RC regression and adopting it to the sensitivity of a specific cell line, the LD_{50} (mg/kg) can be predicted from the IC_{50}. The predicted LD_{50} dose is then used as the starting dose in the UDP. The new tiered testing approach will contribute to a reduction of animal numbers in the UDP. Moreover, in the RC model the precision of the prediction is increasing with decreasing toxic potential and the majority of industrial chemicals (around 90%) are not toxic according to EU classification criteria. In view of the current discussion at the OECD level about adding a second part to the UDP which allows for calculation of the slope of the dose-lethality curve, the proposed tier testing approach might contribute even more to reduction of animals used.

W-5. Evaluation of Cytotoxicity by Quantitative Analysis of Apoptosis

Fumiyo Sato, Syozo Ajimi and Masanori Otsuka
Chemicals Evaluation and Research Institute

It has been proposed that cytotoxicity data can be used to predict the *in vivo* acute toxicity of chemicals. Recently, it has been demonstrated that some chemicals induce apoptotic cell death at relatively low concentrations or soon after exposure. In the present study, we quantitatively examined the survival rate and the incidence of apoptosis after exposure of cells to chemicals to investigate whether apoptosis could be applied as an early indicator for evaluation of toxicity.

Chinese hamster lung cell lines, CHL cells and V79 cells, were grown in Eagle's minimum essential medium supplemented with 10% newborn calf serum. After the CHL cells and V79 cells were exposed to 1-60 nM of actinomycine D (ActD) and 1-30 mM of thioacetamide (TAA) for 24 h and 48 h, respectively, the surviving cells were measured by the MTT assay. The apoptotic cells were also counted by staining with Hoechst 33258 and confirmed by the agarose gel electrophoresis. The IC_{50} value of ActD was estimated to be approximately 30 nM in both cell lines. On the other hand, the IC_{50} values of TAA were estimated to be 17 mM in the CHL cells and 35 mM in the V79 cells. There was no difference in the incidence of the apoptotic cells induced by ActD between the cell lines. On the contrary, the apoptosis was more frequently induced in the CHL cells by TAA than the V79 cells. This suggests that the induction of apoptosis might be related to the cytotoxicity of TAA.

The apoptosis and necrosis were then quantitatively assessed to elucidate a profile of cell death. The CHL cells were treated with 0-500 nM of ActD for 24 h, and the cells were double stained with acridine orange and ethidium bromide. The incidence of apoptotic cells and necrotic cells were calculated from 500 cells counted. . Apoptosis was seen from 25 nM, i.e. close to the IC_{50} value, and dose-dependently increased up to 100 nM. On the other hand, the incidence of necrotic cells increased up to 500 nM, i.e. the upper limit. The time-dependent effects of ActD on the death of CHL cells were examined. When the cells were treated at 25 nM, apoptosis was induced at 24 h and the incidence of apoptosis reached 39% at 48 h. While, in the cells treated at 50 nM, apoptosis was induced at 12 h and the incidence reached 60% at 24 h. Based on the above results, the time-course study of the induction of apoptosis may permit evaluation of toxicity at relatively low dose levels.

W-6. A Proposal from the Study of a Cancer Cell Line Panel-Feasibility of Bioinformatics Based on a Drug Sensitivity Data Base

Takao Yamori
Division of Molecular Pharmacology, Cancer Chemotherapy Center, Japanese Foundation for Cancer Research

Studies at the National Cancer Institute of US and in our laboratory have recently shown that a database including drug sensitivities of a panel of human cancer cell lines can provide unique and useful information concerning the molecular pharmacology of anticancer drugs. We established a panel of 39 human cancer cell lines including lung-, stomach-, colon cancers and so on, and then started to register their drug sensitivity in a database. We exposed the cell lines to various compounds including drugs for two days *in vitro* to determine the GI50 of each compound for each cell line, where GI50 means the concentration reducing the cell growth by 50 % of non-treated control. Each compound gave us a set of 39 GI50s with deviations over the cell line panel, which registered one by one. As the data base expanded, specific features appeared such that each set of GI50s showed a unique profile. Therefore, the profile is called a "finger print" of the corresponding compound. We have compared the fingerprints of about 200 standard compounds with known modes of action including anticancer drugs, and interestingly observed that a group of compounds sharing a certain mode of action indicated very similar fingerprints within the group. Accordingly, one could presume the mode of action of a given compound by investigating its finger print even when no information was available on its biological activity. Taking advantage of such finger prints, we have demonstrated that our database is useful for drug discovery in some ways. I will show some examples of interesting compounds. Furthermore, we expect that our database could be useful for correlating the drug sensitivities of cancer cells with certain gene expression profiles in them if we developed genomic analysis together. We have so far used the cancer cell line panel to characterize compounds

by pharmacological profiles as described above. In this presentation, however, I will change the focus and discuss whether we may use the cell line panel approach to characterize compounds by toxicological profiles.

Forum for Citizen
Animal Experimentations and Alternatives in Primary and Secondary Schools

Chairpersons:
Tsutomu Kurosawa, *Osaka University*
Hiroyoshi Ninomiya, *Azabu University*

F-1. Better Science Teaching with Humane Alternatives

Jonathan Balcombe
The Humane Society of the United States, 2100 L StreetNW, Washington, DC 20037 U.S.A.

The aim of this paper is to make the case that better education results when traditional animal-consumptive uses of animals are replaced with humane alternatives. Animal vivisection is very rare in North American and European primary and junior high schools. On these two continents, dissection is commonplace only in North American junior high schools. Therefore, this paper reports primarily on the advantages of alternatives at higher levels of education. Nevertheless, the advantages of alternatives apply at practically any level of education, and certainly at the primary and junior high school levels that form the focus of this conference.

Alternatives include computer-based materials, three-dimensional models, films and videotapes, self-experimentation and human studies, illustrated charts and diagrams, in-vitro and plant experiments, and of course, the study of living animals in ways that don't interfere with their natural way of life. Today, there are literally thousands of commercially available alternatives to animal-consumptive learning methods. The most popular alternatives involve Computer-Assisted-Learning (CAL).

There are many reasons why alternatives to animal-consumptive learning methods are desirable. Harming animals carries an ethical cost in animal suffering and death. There are also potential social costs of using methods that may teach children that harming animals is acceptable. Monetary costs may also be reduced by using alternatives, which, unlike animals, can be used repeatedly. All of these are good reasons to use alternatives, but obviously, they must also be educationally sound.

CAL alternatives to dissection provide several benefits not provided by dissecting a dead animal. Animations or film clips provide detailed information on the functioning of organs and other body structures. Built-in quizzes allow students to evaluate their learning progress. And students may repeat the dissection as many times as they want.

For the replacement of classroom vivisection exercises, CAL alternatives have further advantages. Students can study many variables at one time and can vary them on a large or small scale. Students can make 'fatal' fmistakes without losing the experiment. The computer can give feedback, provide hints and offer help. Lengthy preparations can be avoided, allowing students to focus on the intended lesson. Slow processes can be accelerated and fast ones slowed down. And once again, the exercise can be repeated any number of times. CAL also gives students experience with technology that will probably be important in their lives beyond the classroom.

Published studies comparing alternatives with traditional animal methods for both dissection and vivisection at school levels ranging from junior high to medical school suggest that the above advantages are real. Nine of 29 such studies have reported higher student learning performance using alternatives. Only one study reported favored dissection over an alternative, and the remaining studies showed no statistical difference. Collectively,

these studies favor use of alternative methods in life science education, especially when one considers the added benefits of using fewer or no animals, and providing a learning context that supports respect and concern for life.

One criticism of alternatives to animal-based learning methods is that they do not provide the student contact with real animals. Of course, contact with animals should be part of any student's learning experience. The Humane Society of the United States advocates the observational study of living animals in their natural surroundings. Only here can a student appreciate the whole, real animal in its full evolutionary context.

Student self-experimentation deserves emphasis along with CAL as a humane and effective alternative. Many excellent study modules have been designed in which the students play the roles of both scientist and study subject, investigating such phenomena as sensory perception, genetic variation, muscle-physiology, and reaction time. No student is harmed, these exercises are fun, and many can be adapted for primary and junior high school classrooms.

With the advantages that alternatives offer, the main forces keeping animal-consumptive methods in education widespread throughout the world are traditional and inertia. Science teachers tend to favor the methods by which they themselves learned. However, ethical concerns with harming animals, and the expense involved, are both growing. The replacement of traditional methods that harm animals with humane alternatives is expected to accelerate at all levels of education. As a shining example, a new North American veterinary school opening in 2001 will have an entirely non-consumptive curriculum. All levels of training will be supplemented by alternatives, and animals will be used only in the clinical setting, where they are in need of medical attention. If such an approach can be devised at this highest level of life science training, then surely it can be devised anywhere in the life sciences curriculum.

F-2. Education about Animals at Elementary School

Hiroki Kishimoto

Akashi Primary School attached to Faculty of Human Development, Kobe University

Introduction

It is that children do learning, or animal's existence is already a necessary thing in the elementary school.

As an agreement; rabbit breeding, insect observation for lower grades, taking familiar animals' pulse for middle grade, the dissection of the fish by the upper grades, and so on are typical.

They don't come only to learn them about the body of the creatures such as an animal, and then it is thought the thing that there is an aim in each child's deepening thought and understanding about "the life" what to be more important as for learning about the body of us, human.

However, there is not that effect, and suicide and a juvenile crime show a tendency of increasing. Of course, those problems must show a new aim though there is no problem only in the method with the education about the animal.

Therefore, I want to try to introduce the place of such thought, the case of one learning in the following.

Case Study

This case will be able to be said as the synthetic learning, which is a transition measure, by the thing, which is not in the subject now.

A unit name is a thing "human and animals". Deep relations with animals are searched for through the experience, and it aims that thoughts are deepened for themselves about those relations in this learning.

The scene when children had greatest interests of this learning is when we went out to the pet shop, the aquarium, the marine laboratory, the veterinary hospital, the stock farm, and so on and experience learning was done for 1 day. Children said, "Though it was tough, the milk which we got was very delicious." "It was fun because I could make friends with shop-owner and animals.", and "Although care was difficult, now I think horses are cuter than before. It seems that communication with the person of the area where communication can actually live with the animal affected a children's heart with strongly.

After that, we had a symposium about the relations between the animal and us human being. Children had

many opinions and we invited person who is working at Hyogo prefectural animal protection center, they gave us advices. We listened to the story about the disposal of the pet, and opinions were exchanged there. We had the place which "animal's death" was thought.

Consideration

When we learn about "the life", I think that animals had a big part at the elementary education. But, I think that it is the most important thing to have enough information about animals and lives together. Moreover, it is also important to talk to the person who is usually concerned with animals, and have time for children to consider with "the death" as well.

F-3. Alternatives to Live Animals Use in Japanese Primary Education

Asako Ogawa
AR Ishikawa

Some of us here may have had the experience of dissecting a live frog somewhere in our elementary years. Dissection was a very common practice in our days, but is it now? I've conducted an investigation on dissection and the use of alternatives in elementary schools by interviewing 6th grade homeroom teachers and science teachers, as well as talking to veterinarians, other experts associated in the field of education, and students.

Dissection is usually conducted in the 6th grade, when studying the human and animal body in science class. The result of my research is that especially these past few years, most teachers choose not to, although some still strongly emphasize the importance of actual experience through dissection.

Some of the reasons dissection hasn't been performed are as follow:
...frogs aren't as easily caught as in the old days
...they feel dissection is a sick practice (not just the teachers, but also many students)
...takes too much time
...the classroom smells awful after a dissection experiment

But the biggest factor may be the revision of the teachers' manual written by the Ministry of Education. It states that "dissection or specimen of a fish may be used for the observation of the interior of the body", not totally ruling out dissection, but also states that "the goal for this study is to educate the students' ability to pursue from different angles the interior and the mechanism of the body of human and other animals' body, and to be able to respect all lives."(EXPOSITION FOR ELEMENTARY TEACHER'S MANUAL FOR SCIENCE : Ministry of Education). Teachers feel dissection is inappropriate for this reason, and the students also claim that it isn't right to kill a frog when teachers tell them to take good care of the school rabbit, which is kept in many schools so that the students can learn to respect other lives.

Another factor may be the alternatives available to replace dissection such as video, pictures, and models. A teacher may use a dead fish bought from the supermarket and dissect it instead of using a live animal. This seems to work well as an alternative. Another example is to first have the students eat fried chicken, and then trying to build a chicken using the leftover bones. In "free schools", where the object of education is to develop and nurture each child's individuality, learning to cook fish and chicken are considered good alternatives, for not only can the students learn about the body of other animals, but also can understand where they stand in the food chain, and learn how they too are a part of nature.

The fact that fewer and fewer teachers choose dissection is good news for the animals and also for people like me working towards better treatment for animals, and the end of vivisection. But this investigation revealed some disturbing points such as irresponsible and incorrect explanation from teachers conducting dissection, telling students that "the frog will be fine after the experiment because I will sew him up" or "it's okay to dissect because they don't feel pain", and the reason for not dissecting being not the welfare of the animal but because the students don't like cleaning up after an experiment. The students' right to say no to an experiment he or she doesn't want to participate in is becoming common knowledge in the U.S. and many European countries; in Japan, just speaking up may be the reason for "ijime", or ill treatment from other classmates. This is another disturbing matter.

Respecting other lives means learning to feel the pain of others and to be able to empathize with others. We can also say that alternatives to laboratory animals have begun to be taken seriously because we feel strongly

and more than ever the need to respect other lives. The teachers' decision to use alternatives instead of dissection is a good sign that this is beginning to be taken seriously in elementary classrooms, but for it to sink into the students we must try harder.

F-4. Observation of Pig Kidney as an Example of Alternative to Animal Experiments in High School Biology Education

Kazushige Iijima
Seisen Junior and Senior High School

Teaching the structure of kidney and the mechanism of urine production is one of the essential subjects for the biology class in high school education. Nevertheless, kidneys have not been observed in most Japanese high schools so far. Thus, the author proposes an observation method of pig's kidney in helping students understand the structure of kidney and the mechanism of urine production in general.

Pig's kidneys are eaten as food, but they are not so popular, presumably because of their urinous smell. Pig's kidneys are a by-product of producing pork, so there is no need to kill a whole pig just for the purpose of experimentation.

From the standpoint of alternatives to animal experiments, the present proposal is regarded as an example of "replacement."

1. Observe the entire appearance of the kidney. Slice the kidney to show a longitudinal section for observation.
2. Inject carbon ink into the renal artery using a syringe. Slice a specimen off the renal cortex, and observe the preparation with an optical microscope. The glomeruli and capillary vessels, filled with carbon ink, can clearly be observed.
3. Cut the renal cortex into small pieces, and filter them using a series of screens with meshes changing from larger to smaller ones. Finally, the glomeruli will remain on the finest screen. By means of this method, the number of glomeruli contained in a single kidney, i.e., the nephron number, can be calculated. As a result, the author obtained a nephron number of 670,000.

Poster Presentation
Ist Group

Chairperson: Kuwagata Makiko, *Food and Drug Safety Center*

P-1. *In Vitro* Embryotoxicity Screening Test of Dental Monomers by Differentiation of ES Cells

Koichi Imai[1], Horst Spielmann[2], Tohru Hayakawa[3], Hitoyo Kikutake[3] and Masaaki Nakamura[1]
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The effects of 9 monomers for dental use on the differentiation of embryonic stem (ES) cells of mouse (cell line D3) into contracting myocard and on cytotoxicity of the ES cells and mouse 3T3 fibroblasts were examined. Of the monomers tested, MTYA was found to be weakly embryotoxic. On the other hand, BPE-1300, BSNa, GMR, NPG, PTSNa, 4-AETA, 4-META and QTX were not embryotoxic. Further testing with different kinds of materials is needed before evaluating the usefulness of the testing method using ES cells.

P-2. Application of Whole Embryo Culture as an Alternative Method for Embryotoxicity Test : Research on Determining the Developmental Stage of Embryos Used for Cultivation

Mari Kato[1], Masaharu Akita[1], Atsushi Yokoyama[2] and Yukiaki Kuroda[3]

[1]Kamakura Women's College, [2]Life Science Laboratory of Kanagawa, [3]National Institute of Genetics

The rat whole embryo culture method is used for testing the embryotoxicity and teratogenicity of chemicals. One of the problems, however, is the difficulty to determine the accurate stage of rat embryos covered by yolk sac until gestation day 11.

In order to minimize the stage differences of embryos used to start cultivation, the wet weight and size of embryo crown-rump length, number of total somites and total protein content were determined. Also the relationship between the growth of embryos and each parameter measured was examined. As a result, the highest correlation coefficient, 0.72, was obtained between the wet weight and the crown-rump length. Then embryos with wet weight of 130-139mg were cultured for 48 hrs and compared to embryos randomly collected at 11.5 days' gestation. In this experiment, the minimum variance was obtained in the heart rate and the diameter of yolk sac of embryos after cultivation for 24 hrs, and the heart rate, the crown-rump length and the total number of somites after cultivation for 48 hrs of embryos with a restricted wet weight, 130-139mg, compared with those in randomly collected embryos. These results suggest that the selectively uniform embryos could be used by collecting embryos with a restricted wet weight whereby consistent development and growth of embryos with a minimum variance can be obtained.

P-3. Determination of Turning Period in Cultured Rat Embryos

Atsushi Yokoyama[1], Masaharu Akita[2] and Yukiaki Kuroda[3]

[1]Lifescience Laboratory of Kanagawa, [2]Kamakura Women's College, [3]National Institute of Genetics

Rat embryos at 9.5 days of gestation were cultured for 48 hours using a rotary incubator. At the initiation of culture, during inversion and at the end of culture, the embryos were keptin a constant direction in a special agar-containing dish set up for taking photographs. Using thephotographs, the rate of occupancy (O) and the rate of voids (V) of each embryo in yolk sac were analyzed by a computer graphic analyzer. The embryos at the initiation of culture (total number of somites 7) showed value (O) of 22.4% and value (V) of 76%. The inversion of cultured embryos started at an average of 22 hours in culture and was almost completed 28 hours in culture. The embryos at that time (total number of somites 15 to 21) showed values (O) of 29% to 33% and values (V) of 71% to 67%. Moreover, the embryos at the end of culture (total number of somites 33) showed value (O) of 37% and value (V) of 63%. This parameter is a suitable method for the confirmation of the development of 9.5-day gestation rat embryos in rotation culture.

P-4. Whole Embryo Culture of Twin Rat Embryos

Atsushi Yokoyama[1], Masaharu Akita[2], Yukiaki Kuroda[3] and Robert, B, Tucker[4]

[1]Lifescience Laboratory of Kanagawa, [2]Kamakura Women's College, [3]National Institute of Genetics, [4]Jefferson Med. College

Embryos with fusion placenta (twins) were cultured in whole embryo culture. Rat embryos with fusion placenta were cultured in a serum bottle containing 6ml rat serum in a rotating culture system (20 r.p.m.) at 38℃ for 48 hrs. (A) Eight cases showed fusion placenta (1:1). (B) Fusion placenta with a ratio of 4:1 were observed in two cases in 4256 rat embryos. Rat embryos in the case (A) were observed to proceed with normal development. On the other hand, embryos in case (B) were small and displayed a reduction in the embryonic blood circulation, had edema, and died after 24 hours in whole embryo culture. This case of an embryo with a fusion placenta ratio of4:1 will be reported and the causation by the reduction of placental ability on cultured rat embryos

will be discussed.

P-5. Whole Embryo Culture Method as an Alternative for Embryotoxicity Test

Atsushi Yokoyama[1], Masaharu Akita[2] and Yukiaki Kuroda[3]
[1]Lifescience Laboratory of Kanagawa, [2]Kamakura Women's College, [3]National Institute of Genetics

In an experiment to examine the effect of methylazoxymethanol (MAM) on cultured rat embryos, its effect was assessed at a concentration of 250 μg/ml. Culture conditions of whole embryos were based on the procedure described by New (1978). Each rat embryo on day 11 gestation(plug day = 0) was cultured in a 25 ml culture bottle containing 5 ml of heat-inactivated rat serum for 48 hr at 37℃. As the growth markers of rat embryos, the crown-rump length was measured and the number of somites counted. Protein content of embryos was also determined by Lowry's method. Total protein content of embryos treated with MAM was 30-40% lower than that in control embryos. The crown-rump length of embryos treated with MAM at high concentration was 25% shorter than that in control embryos. Furthermore, the body width examined preliminarily was also found to be reduced. The anomaly observed in external morphology of cultured rat embryos was the microcephaly, with an incidence of about 90% in embryos treated with MAM. In particular, the 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 on embryos rather than via the dam, and that its effect was related to the growth of prosencephalon and morphogenetic development of the head.

P-6. *In Vitro* Toxicity of Mitomycin C on Cultured Rat Embryos : Dependence on Age of Embryo

Masahiro Koida.[1], Takaaki Mizumoto[1], Masaharu Akita[2] and AtsushiYokoyama[3]
[1]Taiho Pharmaceutical Co.,Ltd., [2]Kamakura Women's College, [3]Lifescience Laboratory of Kanagawa

Rat embryos, at 9.5, 10.5, 11.5 and 12.5 days of gestation, were exposed to 0.1, 0.5 or 1.0 μg/mL Mitomycin C(MMC) for 24 hours, respectively.

The heart beat rate, angiogenesis, number of somites and crown-rump length and external appearance of the embryo were recorded immediately after MMC exposure for 9.5 and 11.5- day embryos and after MMC exposure followed by 24-hour culture in rat IC serum for 10.5 and 12.5-day embryos.

No significant findings were observed in all stages of the embryos treated with 0.1 and 0.5 μg/mL MMC.

Embryo death, delayed development , partial hematoma and decreased heart beat rate were only observed in 11.5-day embryos treated with 1.0 μg/mL MMC.

P-7. Effects of Thalidomide and Its Metabolite on Growth and Differentiation of Rat Embryos in Whole Embryo Culture

Masaharu Akita[1], Atsushi Yokoyama[2] and Yukiaki Kuroda[3]
[1]Kamakura Women's College, [2]Lifescience Laboratory of Kanagawa, [3]National Institute of Genetics

For alternatives to embryotoxicity testing, three methods, viz., whole embryo culture test, micro mass culture test and embryonic stem cell test have been developed. It was reported by researchers in various research institutes that there are correlations among these three methods and *in vivo* tests of embryotoxicity. The purpose of alternatives for animal testing is not only a reduction and a replacement of *in vivo* embryological testing, but also to provide detailed analytical research on embryotoxicity. Whole embryo culture tests might be able to offer more detailed analytical research than *in vivo* tests. In the present study, we examined the effects of thalidomide on rat embryos in whole embryo culture in the presence and absence of rabbit S9-mix, human cytochrome P-450

and UGT1.

There were no differences in the blood circulation, the crown-rump length, and the total number of somites and the morphogenesis (especially the morphogenesis of fore limb buds) between embryos in the experimental group with UGT1 and those in the control (not treatment) group. However, embryos cultured with S9-mix showed reduced morphogenesis in fore limb buds compared with embryos in the control group. Also, embryos cultured with P-450 showed a major reduction in morphogenesis in fore limb buds compared with those cultured with S9-mix.

From the above and preceding results, it was considered that anomalies in cultured rat embryos were induced by thalidomide and its metabolites, and that the effects of metabolites of thalidomide were more severe than those of thalidomide itself. Our results suggested that whole embryo culture tests may be able to analysis of more detailed changes than *in vivo* tests, and this *in vitro* system may be useful as an alternative for embryotoxicity tests.

P-8. Histological Study of Effects of Bisphenol A on Rat Embryos in Whole Embryo Culture

Masaharu Akita[1], Atsushi Yokoyama[2], Shigekazu Shimizu[3], Yoshihiro Nozaki[3] and Yukiaki Kuroda[4]

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Although endocrine disrupting chemicals were examined from various viewpoints, many problems were still remained to be resolved. Over the past year, we have examined the embryological toxicity of 2,2'-bis(4-hydroxyphenyl) propane (bisphenol A), an endocrine disrupting chemical. We reported to the 13th annual meeting of the Japanese Society for Alternatives to Animal Experiments, that cultured rat embryos treated with 100 ppm bisphenol A in the medium showed malformations. In the present study, we examined the histology of internal organs of cultured rat embryos treated with bisphenol A. Rat embryos on day 11.5 of gestation (plug day = 0) were cultured for 48 hours in medium containing 1 or 100 ppm of bisphenol A. The embryos were fixed in Bouin's fluid and stained with H.E. for histological examination of internal organs.

General edema and swelling were found in all embryos treated with bisphenol A at a concentration of 100 ppm. Morphology of the eyeballs and the nasal cavity was unclear. Apoptosis-like changes in epithelial cells of the neural tube were found with a decrease in the number of dividing cells, swelling of the interstitial cells with lysis of apoptosis-like cells as well as a decrease in cell density, and detachment of interstitial cells due to general edema and swelling. As to animals treated with bisphenol A at a concentration of 1 ppm, no histological changes were detected. In conclusion, it is considered that bisphenol A at a concentration of 100 ppm produced general histological changes in internal organs of rat embryos, but did not at a concentration of 1 ppm.

P-9. Effects of Prostaglandin E_2 on Blood Vessels in Rat Embryos in Whole Embryo Culture

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As reported in several papers (Matsuoka, 1971, Fujita, 1973, 1989), prostaglandinshavetheeffects of interrupting pregnancy and inducing toxicity action in various animal species, but the mechanism of their action is still unclear. The present study was conducted to clarify the effects of prostaglandin E_2 (PGE_2) on cultured rat embryos. Conceptuses were removed at gestational day 11.5 (plug day=0) from dams and transferred into Tyrode's solution. Embryos were cultured for 72 hours at 38℃ with 95%O_2 and 5%CO_2 using a rotation culture system. PGE_2 was obtained from SIGMA Chemical Company. The presence of malformations and the vein of yolk sacs or embryos were determined by using a dissecting microscope with micrometer. PGE_2-treated

embryos exhibited 40% contraction in the vein of yolk sac phase after 24 hours. In the vein of embryos, the PGE_2 treatment group showed a significant decrease in the diameter of the vein. On the other hand, marked teratogenic action of PGE_2 was observed at 1 or 10 μg/ml. The typical pattern of PGE_2-induced malformation was facial anomalies, for example cleft lip and defect of the mandibular process. Therefore, our report suggested that the contractile response of the facial vein or yolk sac vein was a causative factor for PGE_2-induced malformation.

2nd Group

Chairperson: Noriho Tanaka, *Food and Drug Safety Center*

P-10. Interlaboratory Validation Study of *In Vitro* Alternatives (HET-CAM) to the Draize Eye Irritation Test

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The object of this study is the development and validation of new alternatives to the eye irritation(Draize test). Among the alternatives to animal tests, alternative tests of eye irritation have been the most actively studied. The hen's egg chorioallantoic membrane (HET-CAM) test is an alternative to the Draize rabbit eye irritation method. It is accepted widely by several countries as an alternative eye irritation method. The HET-CAM test employs the vascular membrane of a fertile hen's egg to assess eye irritation potential. We established a collaboration with 6 cosmetic research centers and studied validation (inter-laboratory, intra-laboratory validation) of the HET-CAM method. To support this method, we combined it with a cytotoxicity test (NRU-assay) as a battery system. For the validation study, we selected 25 materials for pre-validation, 20 materials in a 1st-validation study, and 12 materials in a 2nd validation study; materials selected were cosmetic ingredients. The correlation study to compare HET-CAM assay, Draize test and cytotoxicity was processed with linear correlation coefficient and rank correlation coefficient values. We did analysis correlation of total results (1 test, 2 test) of HET-CAM from the 6 cosmetic research centers. In conclusion, there are good correlations between the HET-CAM assay, the cytotoxicity assay and MMAS, suggesting that these are good methods to replace the animal tests. However, considering the variation in interlaboratory validation results, these methods require additional validation studies.

P-11. Establishment of Short Time Exposure (STE) Method for Evaluating the Eye Irritation Potential of Cosmetic Ingredients

Yuichi Ito, Yukie Komatsuzaki, Osamu Morita, Minehiro Okuda and Hiroyuki Suzuki

Safety and Environmental Research Center, Kao Corporation

For evaluating the eye irritation potential of cosmetic ingredients, cells *in vitro* are usually treated with test materials for a long time (24-48 hrs). However, an accidental ocular exposure to final products lasts a short period.

In this study, we conducted cytotoxicity testing by exposing SIRC cells to test materials over short time periods to mimic the actual conditions of human exposure. We examined 31 test materials (surfactants, polyols, acids, alkalines and alcohols etc.) selected from cosmetic ingredients evaluated in the NIHS/JCIA validation study[1]. SIRC cells were treated with test material in EMEM/FBS medium or physiological saline for 5 minutes.

Cell viability was measured by MTT assay and compared with the Draize eye score[1].

For 22 test materials, a good relationship between results of STE method and Draize eye scores was obtained; acids, alkalis and alcohols did not exhibit a good relationship. These results suggest that STE method is a useful tool for predicting the eye irritation potential for certain types of cosmetic ingredients.

1) Ohno Y. et.al., *Toxicology In Vitro* **13**,73-98,1999

P-12. Improvement of Cytotoxicity Method for Prediction of Chemicals' Eye Irritation Activity

Kyoko Goto, Yasushi Shimada and Yoshihisa Watanabe

Central Research Laboratories, Hokko Chemical Industry Co., Ltd.

The cytotoxicity test by Crystal violet staining assay using CHL cells (CHL-CV) was performed as an alternative method for the prediction of Draize eye irritation on various chemicals. We determined the 50% inhibition concentration (IC_{50}) of test chemicals against CHL cells. The results *in vitro* were compared with those of eye irritation tests.

We investigated time and dose relationship for 22 chemicals. The cells were treated with test chemicals for 3, 6, 24 or 48 hours. The correlation coefficient between log (IC_{50}) scores of short time-treatment methods and the maximum average scores (MAS) was higher than the correlation coefficient between log (IC_{50}) scores of long time-treatment methods and MAS.

We further investigated a 3 hours-treatment method with 91 chemicals. The correlation coefficient value (r) was -0.44. In the case of chemicals with large log P value, almost all were evaluated as "non or slightly irritant" *in vivo*. In the case of 63 chemicals with log P <4, the correlation coefficient value was -0.71.

These results suggested that a 3 hours-treatment method using CHL-CV cells is useful as a prediction test of eye irritation for chemicals with log P <4.

P-13. Alternative to Primary Draize Skin Irritation Test Using Cultured Human Skin Model : Comparison of Chemical Application Procedure

[1]Katsuyasu Morota, [1]Shin-Ichiro Morita, [2]Hajime Kojima, [2]Satoru Nakata, [2]Hiroaki Konishi and [1]Isao Masamichi

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Chemical application procedure is very important when estimating primary skin irritation using cultured human skin model. In this study, MTT50 procedure and TIME50 procedure were compared. With the MTT50 procedure, too short an application period can give false-negatives and too long an application period can give false-positives. Appropriate conditions give good correlation with *in vivo* data.

P-14. Skin Corrosivity Tests Using Cultured Skin Model

[1]Katsuyasu Morota, [1]Shin-Ichiro Morita, [2]Hajime Kojima, [2]Satoru Nakata, [2]Hiroaki Konishi and [1]Isao Masamichi

[1]Reseach and Development Division, GUNZE Ltd., [2]Biochemical Research Institute, Nippon Menard Cosmetic Co.,Ltd.

In this study, 22 ECVAM-selected chemicals (9 are corrosive, 13 are non-corrosive) were tested for their corrosivity using a cultured skin model. Twelve chemicals, including all the corrosive chemicals, revealed less than

10% cell survival. This corrosive chemicals can be detected with this method, but it is difficult to distinguish severe-irritant and corrosive chemicals.

P-15. Evaluation of Phototoxicity of Fragrances Using *In Vitro* 3T3 NRU PT Assay (1) Comparison between the *In Vitro* Test and IFRA Restriction

Makoto Mizuno, Yuuko Okamoto and Kenji Ohkoshi
Fundamental Research Laboratories, KOSÉ Corporation

The *in vitro* 3T3 mouse fibroblast neutral red uptake phototoxicity (*In vitro* 3T3 NRU PT) assay has been validated in an EU/COLIPA validation study, and the draft OECD guideline for this assay has been submitted as an *in vitro* phototoxicity test.

In this study, we used this *in vitro* test to assess the phototoxicity of fragrances that are restricted for use in cosmetics by the International Fragrance Association (IFRA).

Twenty-one fragrances were tested and phototoxic potential assessed by Photo-Irritation-Factor (PIF). The results of twelve fragrances corresponded with IFRA restriction or *in vivo* data. Of the others which did not correspond to these data, most were natural citrus oils. It is thought that bergapten is the main phototoxic substance in these citrus oils, thus we measured this compound in natural perfumes. The level of bergapten in these samples was much lower than expected, and it is supposed that most of them are non-phototoxic *in vivo*. Furthermore, our results showed a good correlation between PIF and the level of bergapten.

In conclusion, it is considered that *in vitro* 3T3 NRU PT assay is a reliable method to evaluate the phototoxicity of fragrances.

P-16. Evaluation of Phototoxicity of Fragrances Using *In Vitro* 3T3 NRU PT Assay (2) Effects of Reactive Oxygen Species

Yuuko Okamoto, Makoto Mizuno and Kenji Ohkoshi
Fundamental Research Laboratories, KOSÉ Corporation

Phototoxic reactions begin when a molecule absorbs a photon. There are three main types of photochemical reactions that are induced by photosensitisation. Type 1 reactions involve an electron transfer between the excited triplet state of the substance and substrate, including molecular oxygen. This is followed by the reaction of super oxide anion. Type 2 reactions involve an energy transfer between the excited state of the substance and molecular oxygen, producing singlet oxygen. Singlet oxygen is able to react with various biological substrates. Type 3 reactions are direct reactions of the excited photosensitiser with certain biological substrates.

In this study, we evaluated the role of reactive oxygen species in the phototoxicity of fragrances using the 3T3 NRU PT assay. Fragrances which had previously been shown to be phototoxic *in vitro* were assessed by using scavengers for reactive oxygen species and the results were compared with studies conducted without scavengers.

The positive reactions of two fragrances (marigold absolute and methyl N-methyl anthraninate) were inhibited by only histidine. These fragrances may be thought to be type 2 substances. The phototoxicity of cumin oil and bergapten was not inhibited by any of the scavengers used. These fragrances contain a sort of psoralens, and might be classified as type 3 substances. On the other hand, the effect of musk ambrette was weakly affected by mannitol and SOD. These results suggest that the 3T3 NRU PT assay could be used to evaluate the photodynamic mechanisms of fragrances.

3rd Group

Chairperson: Yasuo Ohono, *National Institute of Health Science*

P-17. Primary Irritation Test of Cosmetic Ingredients Using the Nitrocellulose Replica Method

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The Nitrocellulose-replica method (NCR method) as a semi-opened system on human skin is able to detect detailed morphological changes of skin surface by microscopic scoring after exposure to irritants without erythema induction. In this study, we compared an animal (guinea pig: GP) test and the human NCR method using volatile ingredients and liquid oils of cosmetics.

For volatile ingredients, there were severe irritations (strong erythema and edema) using a 24h closed patch test (CPT) on GP and human. On the other hand, primary irritation by volatile ingredients could be evaluated by the NCR method without severe skin reactions. The results of 7 days' accumulative open application test on GP correlated well with data for the NCR method. For liquid oils, irriation score of the NCR method correlated with molecular weight of the oils.

These results suggested that the NCR method is a useful and sensitive evaluation system without the disadvantage of severe irritations on animals.

P-18. Improvement of the Neutral Red Uptake Method on Cytotoxixity Assays

Masatoshi Hoya, Takao Ashikaga, Mariko Sugiyama, Masaaki Mori, Hiroshi Itagaki and Yoshio Katsumura.
Shiseido Life Science Research Center.

We have reported that the three dimensional tissue equivalent constructed from collagen and human dermal fibroblasts is useful to predict irritation potential of chemicals. We adopted the MTT assay, which is widely used in some commercial kits, as an endpoint of this collagen gel assay.

For some reducing agents, the cell survival rate on collagen gel could not correspond with the accumulated *in vivo* results. MTT assay is based on the reduction of the soluble yellow MTT tetrazolium salt to a blue insoluble formazan product by mitochondrial succinic dehydrogenase. Therefore, it is suggested that the MTT assay on collgen gel is unsuited to evaluate the cytotoxic abilities of any reducing agents.

In this study, we evaluate the neutral red uptake assay (NR assay) for the cytotoxic endpoint on collagen gel. Classification of irritancy predicted from the NR assay correlated with the accumulated *in vivo* results. Since topical protocol NR assay needs complicated procedures, we tried to develop a simplified protocol using isopropanol as a fixing and extracting agent.

This new protocol of NR assay may be applicable to cytotoxicity tests using three dimensional tissue equivalents or monolayered cells.

P-19. Cytotoxicity Testing of Orthodontic Light-Cured Resins Using Human Skin Model

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We examined cytotoxicity levels of orthodontic bonding resins and glassionomer cement irradiated with plasma arc light and conventional halogen light using TESTSKIN (living skin equivalent). Six seconds of

plasma arc light and 20 seconds of conventional halogen light irradiation showed similar levels of cytotoxicity. There is no significant difference in the levels of cytotoxicity when irradiated with plasma arc light for 6 seconds or 9 seconds. However, there is a high level of cytotoxicity when irradiated for only 3 seconds with plasma arc light. Therefore when evaluating cytotoxicity levels, necessary irradiation time using plasma arc light should be 9 seconds or more.

P-20. Use of the MelanoDerm Human Skin Model to Assess the Cytotoxicity Potential and Efficacy of Skin Lightening Products

Thomas J. Stephens, Stephen Richards and Monya Sigler
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A commercially available human skin model (MelanoDerm) containing a 10 to 1 ratio of human keratinocytes to melanocytes was used to assess the efficacy of skin lightening agents and skin lightening products as well as their cytotoxic potential. The tissues are grown from cell cultures to which _FDF has been added to trigger the melanogenic machinery within the melanocytes to produce melanin in the absence of light. The tissues have an air to tissue interface allowing the direct dosing of the test materials undiluted onto the top surface of the skin. Samples of skin lightening agents and products were applied to tissues 3 times a day at 2-3 day intervals for a total of 14-21 days. Cytotoxicity of test agents and products was assessed using the MTT method, while skin lightening benefits were documented with photography of the whole tissue, histology and by measuring the melanin content of treated and control tissues. Results showed that 1% kojic acid was non-cytotoxic and very effective in lightening tissues while other agents and products produced varying responses.

P-21. Evaluation of Absorption, Metabolism and Toxicity Expression of Benzo[a]pyrene in Simple Double-Layered Culture System Using Small Intestinal Epithelia and Liver Cells

Motoyuki Suzuki, Osamu Fukuda, Yasuyuki Sakai and Akiyoshi Sakoda
Institute of Industrial Science, University of Tokyo

To mimic the actual metabolic processes of toxicants ingested by humans, we developed a simple double-layered cell culture system using Caco-2 (human colon carcinoma cells) as representative of small intestine and Hep G2 liver cells as representative of the target organ. Benzo[a]pyrene was selected as a model toxicant because its metabolites formed by cytochrome P450 have a carcinogenic and an endocrine-disrupting activity. Benzo[a]pyrene (0-100 μM) was added to the apical side of the Caco-2 cell layer. After 7 days of culture, we measured viabilities and CYP (1A and 3A) activities of both Caco-2 and Hep G2 cells in the presence and absence of inducers specific for both CYP subfamilies. Although both 1A and 3A were similarly induced in Caco-2 and Hep G2 cells, only the induction of 1A enhanced the benzo[a]pyrene effect on Hep G2 viability. The ED_{50}s in the single-layered culture system (w/o Caco-2) were about 9 and 16 μM under the induced and non-induced conditions, respectively. In the double-layered systems, however, toxicity was only observed under the induced condition and the ED_{50} was about 35 μM. The ratio of produced resolfin in the apical side to that in the basolateral side of the Caco-2 layer was about 2 : 1 in all the experiments performed. This suggests that toxic metabolites of benzo[a]pyrene formed in the Caco-2 layer are preferentially transported to the apical side and that the benzo[a]pyrene toxicity is markedly lowered in the double-layered system.

P-22. Transdermal Studies for Several Preservatives in Cosmetics Regarding OECD Guideline (a New Proposal) for Percutaneous Absorption: *In Vitro* Method

Hiroshi Tokunaga, Yonson Chang, Tadashi Uchino and Masanori Ando
National Institute of Health Sciences

OECD published the Second Proposal of the Guideline for the Percutaneous Absorption: *in vitro* Method on July 1996. Dr. Ono, the Director of Pharmacology in the National Institute of Health Sciences, has already canvassed opinion from the Japanese related groups and reported to OECD on October 1996. There is as yet no response from OECD but we organaized an investigating group for the Second Proposal of OECD Guideline. We report on the transdermal studies of cosmetic preservative, sodium benzoate(BA), salicylic acid(SA), methylparaben(MP) and ethylparaben(EP) with the Percutaneous Absorption: *in vitro* Method of the Second Proposal of OECD Guideline.

After mounting the guinea-pig abdominal skin on the Franz vertical and static diffusion cell, 1.0ml of 0.05w/v% test solution is added to the donor side and 19.0ml of saline or 20% propyleneglycol solution to the receptor side. During 2 to 8 hours at 32℃, 0.2 ml of receptor's fluid is removed from the receptor. The amount of test sample in the receptor's fluid is determined by HPLC. The permeated amount of test samples depended on the permeation time and increased linearly. Flux of BA, SA, MP and EP were 4.70, 44.94, 4.74 and 5.98 μg /cm2/hr, respectively. When spiking those preservatives into the commercial cosmetic lotion, flux(%) of BA, SA, MP and EP against the control was 217, 46, 66 and 72 %, respectively. These data suggested that the flux of test samples in cosmetics might depend on the solubility of test samples in the vehicle.

P-23. Effect of Penetration Enhancers on the Skin Permeation of Drugs : Comparison of the Effects Using LSE-High and Excised Hairless Rat Skin

Tetsuya Watanabe[1], Tetsuya Hasegawa[1], Hidekazu Takahashi[2], Takuya Ishibashi[2] and Kenji Sugibayashi[1]
[1]Josai University and [2]Toyobo Bio Laboratory

The purpose of this study is to evaluate a three-dimensional cultured human skin, LSE-high, as a model membrane for studying the effect of permeation enhancers. Two-chamber diffusion cells (effective diffusion area: 0.95 cm^2) were used and LSE-high was mounted between the half-cells. Excised hairless rat skin was also used for comparison. Aqueous suspension of isosorbide-5-mononitrate (ISMN) or isosorbide dinitrate (ISDN), a model hydrophilic or hydrophobic compound, respectively, in 0.9% NaCl (2.5mL) was applied to the stratum corneum side of the skin, and their skin permeation was followed from several enhancer systems. Diisopropyl adipiate, isopropyl myristate, lauryl alcohol, *d*-limomen, 1-methyl-2-pyrrolidone and oleic acid were tested at a concentration of 3%, and *l*-menthol was tested on different concentrations. Skin permeation of each drug was followed by periodical sampling from the receiver solution and kinetically analyzed.

Every enhancer showed a marked penetration-promoting effect on the LSE-high permeation as well as the rat skin. Interestingly, the rank order was almost the same to that for the rat skin. Among the enhancer systems, 3% and 10% *l*-menthol exhibited the highest effect for the skin permeation of ISMN and ISDN, respectively. LSE-high may be a useful tool when testing the promoting effect of absorption enhancers on the skin permeation of drugs.

P-24. Environmental Fate and Effects of Pharmaceuticals and Physiologically Active Agents on Organisms in the Environment in Japan

Sekizawa, J., Imai K., Matsuki Y. and Yoshioka T.
National Institute of Health Sciences, Hatano Research Institute on Drug and Food Safety, Oita University

Discharge and disposal of pharmaceuticals and physiologically active agents to the environment attracts concerns on the potential effects of these agents on organisms in the environment and on human health. We searched the literature concerning environmental fate of pharmaceuticals which pose a risk when administered during pregnancy, and concerning effects of pharmaceuticals and hormonally-active agents on organisms in the environment. Combined with the knowledge of the amount of these chemicals used in Japan, we estimated the potential risk of these agents to organisms in the environment in Japan.

4th Group

Chairperson: Makoto Hayashi, *National Institute of Health Science*

P-25. Development of a Novel *In Vitro* Pyrogen Test Using a Human Monocytic Cell Line

Toshimi Murai, Yukari Nakagawa and Hideko Maeda
Osaka Branch, National Institute of Health Sciences

Parenteral pharmaceuticals are tested for pyrogenic contamination which usually takes the form of bacterial endotoxin. The bacterial endotoxin test (*Limulus* amoebocyte lysate test) has been adopted as an official test which can replace the rabbit pyrogen test. The *in vitro* test is accepted generally and used widely as a simple and highly-sensitive method for detecting endotoxin. However, considering that the bacterial endotoxin test does not detect pyrogens other than endotoxin, and the phylogenetic distance between *Limulus* and higher vertebrates, it is desirable that a new *in vitro* test system using human cells be established.

Macrophages are primary target cells for endotoxin *in vivo* and the endotoxin-activated macrophages produce pyrogenic cytokines such as IL-6, IL-1 and TNF as endogenous pyrogens. We intended therefore to develop an *in vitro* test system for detecting pyrogenic substances based on measurement of cytokine production by human cell lines derived from the monocyte/macrophage lineage.

Mono-Mac-6 human monocytic cell line was subcloned by limiting dilution and a clone MM6-CA8 was selected for its superior response to endotoxin and peptidoglycan by increasing production of pyrogenic cytokines. The MM6-CA8 cells were evaluated for their suitability for use in the *in vitro* test system by comparing their response to various pyrogens with that of human whole blood (HWB) from normal healthy donors.

MM6-CA8 cells respond to various pyrogens including endotoxin, peptidoglycan, muramyl dipeptide (MDP), poly(I)·(C), etc. by increasing production of IL-6, IL-1 and TNF. The sensitivity of MM6-CA8 cells to those pyrogens was almost the same as that of HWB, and their response characteristics to each pyrogen were basically identical. These results indicate that MM6-CA8 cells will make a sensitive and reliable *in vitro* test system for detection and quantification of various substances that are pyrogenic for humans.

P-26. Low-Molecular Peptide Model of Protein Denaturation by UVA Irradiation and the Effect of Antioxidants

Takao Ashikaga[1,2], Akira Motoyama[3], Hideyuki Ichikawa[2], Hiroshi Itagaki[1], Yoshio Katsumura[1] and Yoshihisa Sato[2]

[1]Shiseido Life Science Research Center, [2]Institute For Advanced Skin Research Inc., [3]Shiseido Pharmaceutical Research Center, Japan

As a simple model of ultraviolet (UV)-induced protein denaturation, Amyloid P hexapeptide (APH, Phe-Thr-Leu-Cys-Phe-Arg) was exposed to UVA irradiation. A new product was isolated by HPLC and identified by fast atom bombardment mass spectrometry (FAB-MS) as the disulfide dimer. Thus, UVA induced disulfide bond formation. We also examined the effect of replacing the Cys residue of APH with other amino acid residues (methionine, tryptophan, tyrosine and histidine) which might be susceptible to oxidative damage. In the case of [Trp^4]-APH, the amount of the analog was decreased at 40 J/cm^2 or greater. When APH and [Trp^4]-APH were mixed and UVA-irradiated, the combination afforded a greater number of products than the sum of those obtained when they were separately irradiated. This finding suggests that denaturation of proteins by UVA may involve very complex reactions of multiple amino acid residues. Since the UV-induced APH dimer formation could be easily evaluated by HPLC, we used this system to examine the effect of antioxidants on the UVA-induced reaction. The dimer formation was inhibited by dithiothreitol, N-acetylcysteine, cysteine and glutathione but was promoted by azide and catechin. This system is expected to be useful for analysis of the mechanisms of protein denaturation by UVA exposure, and may allow the identification of new protective agents against phototoxicity or photoallergy.

P-27. Evaluation of Contact Sensitivity by Using Expression of Cytokine mRNA

Keiko Suzuki, Shunsuke Yamazaki, Takashi Shinkawa and Hiroshi Kakishima

Kanebo LTD., Cosmetics Laboratory

The Guinea Pig Maximization Test is one of the most important safety evaluation tests of allergic materials, but there are many problems with this test. The development of an alternative method is hoped for from the viewpoint of animal protection.

In this study, we evaluated a screening method utilising expression of cytokine mRNA in order to predict contact sensitivity from chemicals. We tested 9 materials or cosmetic products.

As a result, we detected skin sensitization of not only a severe or moderate level but also of a weak level by evaluating expression of cytokine mRNA. We concluded that this screening system is a useful predictive method for contact sensitivity from chemicals.

P-28. Development of Detection System for Estrogenic and Androgenic Chemicals and Application of the Method to High Throughput PreScreening for EDCs

Hidekazu Takahashi , Yoshihiro Soya , Shigeaki Nishii , Kazuhiro Matsui , Takuya Ishibashi and Yoshihisa Kawamura

Tsuruga Institute of Biotechnology , Toyobo Co.,Ltd.

Many chemicals released into the environment potentially disrupt the endocrine system in wildlife and human beings. Uterotropic assay and the Hershberger assay have been used as methods of screening for Endocrine disrupting chemicals (EDCs). However, for screening EDCs, *in vitro* methods have received attention because their sensitivity, specificity, rapidity, simplicity and cost are more suitable than *in vivo* methods. In this study we have developed a nonisotopic high-throughput receptor-based assay to estimate the binding affinity (likely dissociation constants of receptor-chemical complexes) of chemical substances for receptors. The

assay method is very simple (only dispense and incubate), and doesn't need special equipment. The level of binding affinity between chemical substances and those receptors was able to be estimated easily by using absorption data, concentration of chemicals and that of receptors.

The binding affinity between several chemicals and ERα was determined for DES(2.3nM), genistein(61nM), zearalenone (142nM), and Bisphenol A(6300nM), and binding affinity for the androgen receptor was determined for mibolerone (3.5nM),p,p'-DDD(5390nM), p,p'-DDE (3534nM), p,p'-DDT(3714nM).

The features of our method are the following: 1) It can distinguish the denatured reaction of these receptors by chemicals from the binding reaction between the receptor and chemicals. 2) Using binding affinity, it is possible to calculate the IC_{50} of chemicals for receptors also. 3) It is able to measure many samples simultaneously, so it accomplishes the high-throughput screening of chemicals. 4) It will be adaptable to include metabolic activation using rat liver S9.

This assay method should serve as an useful tool as a prescreening test which is based on the *in vitro-in vivo* strategy to assess the effects of chemicals on endocrine function.

P-29. Application of Xenopus Oocyte Expression System to Toxicological Evaluation

Yasuo Ohno and Kenichi Nakazawa
Division of Pharmacology, National Institute of Health Sciences

The effects of chemical compounds on cloned mammalian membrane receptors were examined in a Xenopus oocyte expression system to determine whether or not this system is suitable for toxicological evaluation as an alternative to animal experiments. When rat clones were used, nicotinic acetylcholine receptor, extracellular ATP receptor and 5-hydroxytryptamine receptors were expressed in Xenopus oocytes. The ion channel function of the ATP receptor was blocked by trivalent cations including lanthanum or divalent cations. From experiments using artificially mutated ATP receptors, it was suggested that this block is mediated through the 333rd residue asparagine. Like the rat clone, human nicotinic acetylcholine receptor clone was also expressed in Xenopus oocytes. Chemical compounds including apomorphine blocked the expressed human nicotinic acetylcholine receptor, and this block depended on the constituent subunits of the receptor. These results indicate that the Xenopus oocyte expression system is suitable as an alternative method for toxicological evaluation. We are now examining whether or not this system can be applied for nuclear receptors such as estrogen receptor, in addition to membrane receptors described above.

P-30. Quantitative Evaluation of *In Vitro* Chromosomal Aberration Test Results

Makoto Hayashi
Division of Genetics and Mutagenesis, NIHS, Tokyo, Japan

For the tests of chemical safety, usually regulatory guidelines require the data of genotoxicity. The main endpoints of genotoxicity are gene mutation and chromosomal aberration, and tests to evaluate these endpoints are included in the standard battery. No threshold rule is usually adopted in the genotoxicity tests. However, a practical threshold can be estimated. Generally, until now, a qualitative evaluation of chemical genotoxicity has been applied for chemical safety. The Ministry of Health and Welfare (MHW) and the Ministry of International Trade and Industry (MITI) adopted the Chemical Substances Control Law, promulgated in 1973, and amended in 1986, to protect humans from exposure to hazardous substances through the environment that have the properties of persistence and bioaccumulation. If newly produced or imported chemicals (>1 ton/year) are neither degradable nor bioaccumulated, then screening toxicity tests are required. The screening tests consist of the 28-day repeat-dose toxicity test (corresponding to OECD guideline 407, the recent version), the reverse mutation assay in bacteria (OECD guideline 471), and the chromosomal aberration (structural and numerical) test in cultured mammalian cells (OECD guideline 473). We have applied the semi-quantitative evaluation method for designation. To evaluate in vitro chromosomal aberration test results semi-quantitatively, we have used "the estimated dose at which chromosomal aberrations are observable in 20% of metaphases" (D_{20}, mg/ml). Since the

guideline was revised in 1997, the test protocol of *in vitro* chromosomal aberration has been changed. In the present study, I evaluate the deviation of D_{20} values related to the test protocols defined in these old and new guidelines. With the new guideline, the continuous treatment is required only when the short treatment protocol with and without exogenous metabolic activation system give negative results, though the old guideline required always short and long treatment. The D_{20} values were compared on approximately 50 chemicals that gave positive results by both short and long treatment protocols without exogenous metabolic activation system. According to the present analysis, we could conclude that the differences in test results between short and long treatment protocols were not biologically significant.

5th Group

Chairperson: Akihiro Kurishita, *P&G,Co.Ltd.*,

P-31. Permanent Female and Male EGC-Lines of Balb/cJ Mice : an Alternative Concept for Reproductive Toxicity Testing ?

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ZEBET, Center for Documentation and Evaluation of Alternative Methods to Animal Experiments at the BgVV, Berlin (D)

While several *in vitro* tests are available for replacing animal experiments in base-level screening of new substances, few have been defined adequately for quantitative studies in reproductive toxicology. In order to offer a sensitive and predictive *in vitro* method to assess the genotoxic potential of chemical agents on male and female reproduction, we established primordial germ (PG) cell-derived permanent female and male embryonic germ (EG) and embryonic stem (ES) cell lines of the mouse (strain Balb/cJ). All EG and ES cell lines were characterised (by PCR and karyotype) and periodically checked for quality criteria like alkaline phosphatase activity and mean generation time (MGT) to ensure clone stability.

The differences in developmental sensitivity of EG cells, ES cells and differentiated fibroblast cells of the mouse cell line 3T3 regarding genotoxicants were comparatively tested under identical test conditions. Cytotoxicity assay was based upon determination of growth inhibition (MTT-test) and genotoxic effects were determined by sister chromatid exchanges (SCE) induced by standard reference mutagens like Ethylnitrosourea (ENU), Methylnitrosourea (MNU), Methylmethansulfonate (MMS), Hydroxyurea (HU) and Mitomycin C (MMC). After calibrating the *in vitro* _EG cell assay by testing positive and negative control substances, we are now starting to clarify if our *in vitro* test system can reliably and reproducibly discriminate between known genotoxic and non toxic agents. Furthermore, a distinction between different degrees of genotoxic effects is mandatory for our assay. To classify the genotoxic potential of all tested chemicals we will develop a biostatistical prediction model on the basis of concentration-response curves and exclusion of a major impact of cytotoxicity. Finally we would like to establish an additional *in vitro* test focusing on EG cell progression through meiotic prophase as a predictive endpoint for sex-specific genotoxicity testing.

P-32. Results of the ECVAM Validation Study of Three *In Vitro* Embryotoxicity Tests

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There is a strong demand to validate existing *in vitro* alternatives to living mammals for testing the potential reproductive toxicity of chemicals. Thus in 1996 ECVAM awarded a contract to ZEBET to co-ordinate the experimental validation of three well established *in vitro* embryotoxicity tests, the postimplantation rat whole embryo culture (WEC) test, the micromass (MM) test and the embryonic stem cell test (EST). Since 1997, ZEBET has co-ordinated the study as the main contractor in co-operation with the lead laboratories for the WEC and MM test, A. H. Piersma (RIVM, Bilthoven, NL) and N. A. Brown (SGHMS, London, UK), respectively. In addition, ZEBET served as the lead laboratory for the EST.

Following a call for applications in 1997, four laboratories were selected for each test early in 1998 according to experience and sufficient representation of industry and EU-member countries. Since three laboratories have performed two of the *in vitro* tests in parallel, the study was conducted in 9 European laboratories by testing 20 coded chemicals in the three *in vitro* embryotoxicity tests. The preliminary phase of the formal validation study was finished in January 1999 and the definitive phase of the study by the end of the year 1999.

The 20 test chemicals comprising different embryotoxic potential (*non, weakly* and *strongly embryotoxic*) were backed by high quality *in vivo* data in the most important laboratory animal species as well as in human. The results obtained in the prevalidation phase of the study were used to define biostatistically based prediction models (PMs) in order to identify the embryotoxic potential of test chemicals for the WEC test, the MM test and the EST. Since the PM of the WEC test took into account only parameters of growth and development, but not cytotoxicity data, a second PM (PM2) was developed for the WEC test by incorporating cytotoxicity data of the differentiated mouse fibroblast cell line 3T3, which were derived from the EST. This approach, which has previously never been used, resulted in an increase to 84% correct classifications in the WEC test.

The evaluation of the PMs applying the results of the definitive phase of the blind validation trial lead to the following conclusions:

- The three *in vitro* embryotoxicity tests WEC test, MM test and EST provide an overall accuracy to predict three classes of embryotoxicity (*strong, weak* and *non embryotoxic*) of about 80% using the PMs defined in this study.
- A predictivity of 100% was obtained with strong embryotoxic chemicals in each of the three *in vitro* tests.
- Differences in the performance of the three *in vitro* tests are due to the specificity and sensitivity of the classifications predicted, in particular for weak and non embryotoxic chemicals.

The most important result of the present validation study, is that for the first time, three *in vitro* embryotoxicity tests have been established that are backed by validated test protocols, and which will be available through ECVAM as INVITTOX protocols: **1.** the WEC test using cultures of whole rat embryos, **2.** the MM test employing primary cultures of dissociated limb bud cells of rat embryos, and **3.** the EST, which is using two established mouse embryonic cell lines and which does not require sacrifice of pregnant animals.

The three validated *in vitro* embryotoxicity tests are meeting three essential criteria of validated alternative toxicity tests. Firstly, SOPs were established, which are now available to the public. Secondly, sound biostatistical PMs have been established and validated. The PMs for all three tests provide an accuracy of close to 80% and, more importantly, 100% predictivity for strong embryotoxic chemicals. Thus, they can routinely be used to identify strongly embryotoxic chemicals, e.g. when screening new substances. Thirdly, the three *in vitro* tests were experimentally validated in a blind ring trial according to the validation scheme recommended by the EU, the OECD and the US NIEHS (Balls *et al.* 1990 & 1995; OECD, 1996; NIEHS, 1997).

Thus, this study clearly demonstrated that the ECVAM strategy for prevalidation and validation of *in vitro* toxicity tests is sound. This view is supported by the participants of the ECVAM validation study and also by the members of the ECVAM Scientific Advisory Committee ESAC.

P-33. ECVAM "Catch-up" Validation Study on the Use of EpiDerm for Skin Corrosivity Testing

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In 1996-97, ECVAM supported a formal validation study on *in vitro* methods for predicting skin corrosivity. Two of the *in vitro* tests included employed human skin models, the Skin[2] ZK1350 and EPISKIN models. In the ECVAM validation study, BASF, Huntingdon Life Sciences (HLS) and ZEBET had tested the Skin[2] human skin model, the production of which ceased in October 1996 whilst the validation study was still in progress. Since both of the skin models had shown basic usefulness for corrosivity testing, and in particular, the EPISKIN corrosivity test had proved to be a scientifically valid test (which is meanwhile officially accepted in the EU), the three laboratories decided to conduct a study which had the objective to determine whether another commercially available human skin model, EpiDerm, could also be successfully used for predicting skin corrosivity. This new type of "catch-up" validation study was performed according to the ECVAM prevalidation scheme, to allow for refinement of the test protocol and the prediction model, as well as for independent assessment of the performance of the refined methodology in a final blind trial in the three laboratories.

In phase I of the study, ZEBET (Laboratory 1) drafted a standard operating procedure (SOP), including a prediction model. It was a major task to simplify an existing EpiDerm test protocol, which had used the time-course of cytotoxicity as its endpoint. To evaluate the predictivity of the simplified method, which used only a 3-minute exposure to test chemicals, 50 chemicals representing a wide spectrum of chemical entities were tested, revealing that the test sensitivity was too low (65%), whereas the specificity was very high (88%). In addition, acceptance criteria for the negative and positive controls were established.

The main goal of phase II was to produce sufficient data for assessing the reproducibility of the EpiDerm skin corrosivity test after transfer to Laboratory 2 (HLS). Repeated testing of several chemicals in both laboratories revealed excellent intra-laboratory and inter-laboratory reproducibility. In addition, chemicals classified as "non-corrosive" (NC), with a 3-minute exposure in phase I, were re-tested by ZEBET with extended exposure periods of 1 hour and 4 hours. The results revealed that test sensitivity could be significantly increased if chemicals classified NC with a 3-minute exposure were tested with a 1-hour exposure. Thus, a refined SOP was drafted, according to which all chemicals had to be tested with exposures of 3 minutes and 1 hour, and data of these two exposure times were used in the refined hierarchical prediction model.

In phase III, the blind trial, BASF (Laboratory 3) joined the study. ECVAM selected 24 chemicals from the test chemical set used in the ECVAM skin corrosivity validation study, and BIBRA International (UK) purchased, coded, and distributed the chemicals. Each chemical was tested twice, independently, according to the principles of GLP, and coded data were submitted to the Humboldt University (Berlin, D) for biostatistical analysis. The analysis revealed that the final test protocol and the refined prediction model PM2 provided a highly balanced prediction of 88 % sensitivity and 86 % specificity, which is regarded the best predictivity an *in vitro* skin corrosivity test can be expected to achieve.

In conclusion, the present study proved that the EpiDerm skin corrosivity test provides an excellent prediction with a wide spectrum of chemicals, and could be used within the context of the new Annex V (EU Dangerous Substances Directive) test method (human skin model assay) on skin corrosion. The results obtained were reproducible, both within and between laboratories, and the EpiDerm test was applicable to testing a diverse group of chemicals (both liquids and solids), including organic acids and bases, neutral organics, inorganic acids and bases, electrophiles and phenols. The concordances between the skin corrosivity classifications derived from the *in vitro* data were very good, and the test was able to distinguish between corrosive and non-corrosive chemicals for all of the chemical types studied.

Due to the excellent outcome of this "catch-up" validation study the ECVAM Scientific Advisory Committee

(ESAC) in March 2000 endorsed the EpiDerm skin corrosivity test as a validated assay, which led to a statement signed by ECVAM and the DG ENV that the test can be used in context of the recently published Annex V Method B40 (skin corrosion) of *Directive 67/548/EEC.*

P-34. Alternatives to Animal Experimentation and Mass Media : Reactions of Media and Public

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Research into alternatives to animal experimentation is not widely broadcast. The results of these research activities therefore should be actively publicised.

Recently our research efforts to develop laboratory animal simulators have received attention from the media, and the results of our research have appeared in several news media. With this experience, we have tried to analyze the attitude of the media and the subsequent reaction of the public. In the present study we delineate the reactions of the media and the public towards alternatives to animal experimentation.

Materials and methods

Four pressmen inquired with us about alternatives to animal experimentation, in particular the development of laboratory animal simulators. These pressmen were from the Yomiuri, Mainichi , Asahi and Osaka Daigaku newspapers. News items appeared in these four Japanese newspapers, in one English newspaper and on one English web site. When meeting these pressmen, we have emphasized 6 main interests in our research activity and have analyzed the appearance of associated keywords in each news item. We have received several responses to the news items from various sources.

Results

The largest item contains 3 color photos occupying a full page of newspaper, and all of the news items contain various sized color or black and white photos. The news items were different in terms of keywords emphasized by us. Three enquiries from overseas were related to the purchasing simulators but none from domestic. Two TV media asked about laboratory animal simulator related to alternatives to animal experimentation and about the possibility of interviews to produce a TV program. One news agency interviewed us by telephone concerning laboratory animal simulators.

Discussion

The opinion of a citizen expressed to the media triggered the interests of pressmen. The news item which appeared in the media further attracted attention of pressmen. The results obtained in the present study may contribute to determining the best way to report our research activities to the media. We discuss the attitude of press men and the contents of new items.

P-35. The ZEBET Database on Alternatives: Now Available on the Internet

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In February of the year 2000 ZEBET (German Center for the Documentation and Validation of Alternative Methods) at the Federal Institute for Consumer Health Protection and Veterinary Medicine (BgVV) put the *ZEBET database* on alternative methods to animal experiments on the Internet in English via DIMDI, the

German Institute for Medical Documentation and Information (*http://gripsdb.dimdi.de/engl/guieng.html*).

The access is free, moreover DIMDI's complete service is available to visitors of the ZEBET database. The ZEBET database contains documents on alternatives to testing in animals, which have been carefully evaluated by ZEBET's staff according to the "3Rs"-concept established by Russel and Burch in 1959. Therefore, methods documented in the ZEBET database must meet at least one of the following criteria: "Refinement" of an experiment by minimizing pain and suffering of animals, "Reduction" of the number of animals used, "Replacement" of an animal experiment by a non-animal method. In addition, the ZEBET-database provides information on the current stage of development and validation of a method and on the acceptance for either scientific or regulatory purposes.

Each document is characterized by the following criteria: the title of a method, keywords, assessment, summary and bibliographic references. To search DIMDI's database and host-system the *grips* software has to be used. Examples are given for searching in the ZEBET database. Currently 125 alternative methods are meeting the criteria of the ZEBET database. Fifty of them are available online on the internet via DIMDI, the remaining 75 documents will be available by the end of the year 2001. International fellow organizations, e.g. FRAME (Fund for the Replacement of Animals in Medical Experiments) in the UK and CAAT (Johns Hopkins Center for Alternatives to Animal Testing) in the USA, have established links on their web sites to provide visitors free access to the ZEBET database.

P-36. Prediction of the Starting Dose for Acute Oral Toxicity (LD_{50}) Testing in the Up-and-Down Procedure (UDP) from Cytotoxicity Data

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Three alternative methods to the "classical LD_{50} test" for determination of acute oral toxicity (OECD TG 401) have been accepted at the OECD level between 1992 and 1998, namely the Fixed Dose Procedure (FDP, OECD TG 420), the Acute Toxic Class Method (ATCM, OECD TG 423), and the Up-and-Down-Procedure (UDP, OECD TG 425). The latter two methods are sequential procedures based on consecutive dosing where each dosing is based on the outcome of the preceding dose, thus saving a considerable number of animals. Consequently, the number of animals saved with these two sequential procedures depends on how close the starting dose is to the 'true LD50' (UDP) or 'true toxicity class' (ATC).

To reduce the number of animals in acute oral toxicity testing, cytotoxicity data (IC_{50}) can be used to predict the starting dose for testing from the standard regression between IC_{50} and acute oral LD_{50} of the Register of Cytotoxicity (RC). In the RC the correlation between cytotoxicity, represented by the mean IC_{50}, and the acute oral LD_{50} of rats and/or mice, was determined for 347 chemicals and drugs applying the linear regression model for the log-transformed pairs IC_{50} versus oral LD_{50}. We have previously demonstrated that the standard regression line of the two toxicity parameters is characterised by an intercept a = 0.625 and regression coefficient b = 0.435 and that 252 of 347 chemicals (72.6 %) are located within a dose range differing not more than ±0.699 (factor FG ≤ log 5) from the standard regression line.

We have previously suggested to reduce animal numbers in acute oral toxicity testing in the acute toxic class (ATC) method by using cytotoxicity data and the RC regression model for predicting the starting dose ATC method, which is a sequential testing procedure. In the present study we have used the RC and its IC_{50}/LD_{50} regression model to predict the LD_{50} from cytotoxicity data of 9 chemicals from an evaluation study of the up-and-down (UDP) procedure for acute oral toxicity testing in rats. For 7 of the chemicals LD_{50} values (mg/kg) predicted from the RC were in the same dose range as LD_{50} values determined *in vivo*, while the dose range differed by one order of magnitude for the two remaining chemicals. Thus the prediction of LD_{50} values from cytotoxicity data was promising in this limited data set.

To reduce animal numbers in the UDP method for acute oral toxicity testing we are, therefore, proposing a tiered *in vitro/in vivo* testing approach. As the first step cytotoxicity of a new chemical is determined. By apply-

ing the RC regression and adopting it to the sensitivity of a specific cell line, the LD_{50} (mg/kg) can be predicted from the IC_{50}. The predicted LD_{50} dose is then used as the starting dose in the UDP. The new tiered testing approach will contribute to a reduction of animal numbers in the UDP. Moreover, in the RC model the precision of the prediction is increasing with decreasing toxic potential and the majority of industrial chemicals (around 90%) are not toxic according to EU classification criteria. In view of the current discussion at the OECD level about adding a second part to the UDP which allows for calculation of the slope of the dose-lethality curve, the proposed tier testing approach might contribute even more to reduction of animal use.

P-37. Current Validation Studies on Alternatives to Animal Experiments in Europe

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See abstract of **PL** (page 104)

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AW=Award Lecture, F=Forum, P=Poster Discussion, PL=Plenary Lecture,
PM=Panel Meeting, S=Symposium, SS=Satellite Session, MW=Mini Workshop