Proceedings of the 9th Annual Meeting of Japanese Society for Alternatives to Animal Experiments

Kyoto, November 29-30,1995

Special Lecture 1 The Way That Validation Studies Should Be

Hiroshi ONO

Hatano Research Institute, Food and Drug Safety Center, Hatano, Kanagawa 257, Japan

Validation is a scientific procedure for establishing an alternative to toxicity tests. Validation should be done through multi-laboratory collaboration in order to confirm the transferability of technology and inter-laboratory reproducibility. It requires a high level of quality of test performance.

Thus, a validation project should be attentively prepared and well conducted of results. Experiences in validation studies have taught us the need of quality assurance of the test performance through GLP-like mechanism. Criteria for evaluation of validation study should be provided, since a self-evaluation by the study group itself lacks objectiveness and seems insufficient in obtaining the acceptance. An independent body of scientists for evaluation of a study should be established. the clear presentation of the underlying mechanism in biological events of the test is important for the approval of the alternative test. The resources for validation studies also should be considered.

Special Lecture 2 To Which Extent And How Can Animal Experiments Be Replaced, Supplemented Or Modified In Pharmacological And Toxicological Research?

Diether NEUBERT and Stephan KLUG

Institute of Toxicology, University Medical Center, Benjamin Franklin, Free University Berlin, Germany

1.Introduction

In various countries objections to animal experimentation differ considerably:

- there are some countries where such a problem does not (yet?) exist, like in many roman countries,
- and others, like Great Britain, Germany and Switzerland, with fanatic animal rights movements, often not to the benefit of animals, where animal experimentation is largely restricted by law, with tendencies to even abandon such studies.

Furthermore, within a given country, people from rural areas or fishermen often have a different attitude toward animals than persons living in big cities. Those who own pets have different opinions than those who do not.

Our personal feeling is that, in the long run, experimentation with animals will be declining and be restricted. This holds especially true for studies associated with pain and discomfort for the animals, which should be avoided and restricted as far as possible. However, we will have to face the fact that this will inevitably lead to more studies performed in humans, which will raise concern in another group of people, and it may cause many new problems.

If one puts aside the emotional aspect, the crucial question remaining focuses on the possibilities for the near future to do with less or even without animal experimentation, but simultaneously keeping our present state of safety, as is

strongly demanded by society. It shall be the topic of this lecture to deal with this problem from a scientific point of view. Considerable reduction of animal testing has already been achieved within the last decades. For educational purposes, animal experiments can be largely avoided.

1.1.Definition of an "animal experiment"

As defined in Germany, animal experiments are interferences with (e.g. surgery on) or treatments of animals for experimental purposes, if they may be associated with pain, harm or injury. Presently, the killing of animals for dissecting organs is not considered an animal experiment, This has a tremendous impact on the possibilities to perform *in vitro*-studies (organ cultures or primary cell cultures).

1.2.History of "alternative" methods

"Alternative" methods have never been invented as such, although many institution claim that this has been the case. Most *in vitro* techniques were developed and initially used for purely scientific purposes, and because they were better suited than other methods for solving specific problems and not primarily for reducing animal experimentation. Nevertheless, reducing and modifying animal studies is a goal worthy to be pursued.

1.3.Methods suitable for answering defined questions

In scientific research, those methods must be used that are best suited to provide an answer to a defined question. This may be a whole animal experiment or an *in vitro* technique. The term "alternative", often used in this connection, is quite unscientific. It is purely political. From a scientific point of view there are no "routine" *vs* "alternative" methods, but only methods suited well or poorly to serve a defined purpose.

1.4.Problem of complexity

In vitro methods are, per definition, less complex than study designs using the whole organism. This may be advantageous for certain purposes, but creates serious limitations for other applications. Thus the problem of the biological complexity of a method plays a crucial role in the selection of a suitable model for a defined purpose. An opposite situation must exist in routine toxicology and basic research.

1.5.Different problems in pharmacology and toxicology

The principle questions to be asked in pharmacology and in toxicology are fundamentally different:

- In pharmacology the main question is: *does a given substance exhibit a defined effect?* Possible effects on several systems are studied sequentially using separate experimental designs.
- In toxicology the main question is: *does a given substance exhibit any adverse effect possible?*

2. Methods for pharmacological research

Methods for pharmacological research must include those for recognizing substance-induced effects of possible therapeutic significance, for elucidating mechanisms of action, and for performing pharmacokinetic studies. Information on kinetics must be available for experimental species and humans.

2.1.General principles

Because of the task to be performed, *in vitro* methods have been used as the favorite methods in pharmacological research (predominantly isolated organs). Without any doubt, they have been developed and extensively used for many decades, completely independent of any animal protection movements, because they were in many and well-defined respects superior to experimental whole animal designs.

There are problems which cannot be tackled with *in vitro* methods: e. g. possible effect on blood pressure, and most effects on the CNS can only be assessed *in vivo*. However, in the field of CNS-pharmacology amazing possibilities exist with respect to using *in vitro* techniques: e. g. the evaluation of antiepileptic effects using brain slices.

3. Methods for toxicological evaluations

Methods for toxicological research must include those for recognizing all types of relevant adverse effects, for elucidating mechanisms of action, and again, those for performing kinetic studies. For risk assessment, once more, kinetic data for the corresponding animal species and for humans must be available.

3.1.General principles

Because of the task to be performed, almost all routine assessments ("primary-stage testing") of possible substance-induced adverse effects are still performed *in vivo*. However, for additional and complementary studies, such as revealing possible modes of action or obtaining more detailed information ("secondary-stage testing") *in vitro* methods are used rather extensively and with considerable success in different areas of toxicological research. Also studies to reveal species differences in the metabolic conversion of a given substance can largely be performed *in vitro*.

3.2.Benefits and limitations of in vitro studies

Of course, *in vitro* methods must have clear-cut advantages and also pronounced limitations in their applicability. This holds especially true, when the predictive power, i. e. the possibility to predict possible effects in humans, is decisive for the interpretation of the data, as is the case in medical studies.

The areas of toxicological routine studies may be grouped as follows:

- fields in which *in vitro* methods are already used extensively,
- fields in which *in vitro* methods may be applicable in the future,
- fields in which it is very unlikely that *in vitro* methods can successfully be used to a great extent.

3.3.In which areas are in vitro methods routinely used?

There are few areas in which *in vitro* methods are presently used for routine risk assessments. The only toxicological risk assessment largely relying on *in vitro* methods is *mutagenicity* testing. This can be explained by two special aspects of this type of testing: (a) one is largely satisfied with a qualitative evaluation, and (b) it is assumed that mutagenicity is a general phenomenon. Since the latter assumption is only partly true, additional *in vivo* studies are generally requested by the regulatory agencies.

3.4.In which areas can in vitro methods not be used routinely?

There are a number of areas in toxicological research in which the future use of *in vitro*-methods appears quite feasible.

In the following, the different areas of toxicological research shall be discussed individually, with respect to the feasibility as well as to the pros and cons of using *in vitro* methods. It should be mentioned to begin with that the routine use for "primary-stage" testing appears very much restricted, in most of the toxicological field, if our present safety standards are to be kept.

3.4.1.Studies on acute toxicity

With the present strategy, replacement by *in vitro* studies is not possible. However, this strategy may be changed, since information for LD50 evaluations (largely required for administrative purposes), although not identical with acute toxicity, can be obtained with simpler methods (e. g. from cytotoxicity, although not at all identical with acute toxicity). Furthermore, for this type of testing, a considerable reduction in animals was possible (approximate LD50 and information on acute toxicity from a few animals).

3.4.2.Studies on organ toxicity

Several aspects of, more or less acute, organ toxicities may be assessed with *in vitro* techniques (hepatotoxicity, cardiac toxicity, nephrotoxicity, etc.). Manifestations of chronic toxicity may not be detectable (liver cirrhosis, etc.), but they often are the result of perpetuated lesion. Presently not assessable with in vitro methods are oto- and ocular toxicity, as well as disturbance of peripheral nerve function and certain CNS abnormalities. Chronic toxicity studies can be supplemented, not replaced by *in vitro* techniques.

3.4.3.Studies on carcinogenicity

If a qualitative information is considered sufficient, *in vitro* methods combined with mutagenicity tests may be able to replace the current (not very satisfactory) *in vivo* long-term tests in the future. However, although well-known for decades, little progress has been made in standardizing methods for detecting substance-induced malignant transformation *in vitro*. All systems use connective tissue cells. Not solved is the transformation of epithelial cells (carcinoma are more frequent than sarcoma), of metabolic activation *in vitro*, and it cannot be explained why human cells are problematic or not transformable.

3.4.4.Studies on reproductive toxicity

This is one of the most difficult field of toxicology, because abnormal development may be induced over extremely long periods (at least from the zygote to puberty), and numerous endpoints must be considered. *In vitro* techniques are only available for evaluating substance-induced effects during short periods of prenatal development, and no assessment of postnatal developmental changes can be achieved with *in vitro* methods. Furthermore, *in vitro* techniques for assessing possible effects on fertility are still inadequate and incomplete, However, many and very interesting *in vitro* methods (from whole-embryo culture, over organ cultures, organized cultures, to specific cell cultures) are available to be used for secondary-stage testing or for the screening of series of compounds when the teratogenicity of the original substance is known and an adequate *in vitro* system is available.

3.4.5 .Studies on immunotoxicity

This topic will be dealt with in another presentation at this symposium.

4. Summary and outlook

In vitro methods already have their establishes place in pharmacological or toxicological studies. They can altogether be considered as extremely valuable in supplementing *in vivo* testing, but cannot, even in the long run, replace it completely, unless a dramatic reduction in safety considerations is accepted. Restrictive laws in some countries will inevitable shift the necessary *in vivo* testing to other countries.

Educational Lecture The Role of Biostatistics in Toxicological Studies

Isao YOSHIMURA

Department of Management Science, Faculty of Engineering, Science University of Tokyo, Ichigaya, Shinjyuku-ku, Tokyo 162, Japan

This talk discusses the reason why there are inconsistent evaluations on the role and necessity of statistics for the analysis of toxicological date. Negative evaluations on the role of statistics primarily comes from the improper understanding that the statistical analysis implies applications of statistical test to toxicological date.

The recent development of statistics, however, has provided valuable tools for analysis beyond statistical test. Through proper use of such tools, toxicologists can get more reliable and useful knowledge with less errors. Some examples are shown in this talk.

SI-1 *In Vitro* Method For Immunotoxicity

Yoshiro KOBAYASHI[1] and Jun-ichi SAWADA[2]

[1]*Laboratory of Molecular Immunology, Toho University, and* [2]*National Institute of Health*

Immune response is regulated by a complex series of cell-to-cell communication, and therefore it is almost impossible to reconstruct the entire response *in vitro*. However, as *in vitro* methods have been valuable to elucidate the mechanism of immune suppressant, some effects of chemical compounds on immune system could be evaluated by *in vitro* assay system. From this point of view, we selected several *in vitro* methods of immune response, on which each speaker will present his/her own data for discussion over the possibility of constructing *in vitro* method for immunotoxicity.

SI-2 Effects Of Chemicals On Degranulation Of Rat Basophilic Leukemia Cells

Reiko TESHIMA

Division of Biochemistry and Immunochemistry, National Institute of Health Sciences, 1-18-1, Setagaya-ku, Tokyo, 158, Japan

Introduction

Mast cells play important roles in the immediate hypersensitivity reaction. Activation of these cells results in the release of compounds like histamine, serotonin and other mediators (Razin, et al., 1995) which causes inflammation, anaphylaxis and asthma. These cells provide a system in which intracellular signaling mechanisms can be studied in the *IgE* receptor-mediated signal transduction (Beaven and Metzger, 1993) Some agents which stimulate exocytosis from these cells are also known (Siraganian, 1988). I present the data on the effect of two types of agents, which increase cytosolic Ca^{2+} concentration ($[Ca^{2+}]i$), on the degranulation from RBL-2H3 cells, and propose a possible *in vitro* system to know the allergy-potentiating activity of chemicals.

Materials and Methods

We have examined the effect of pervanadate and 6 antioxidants on Ca^{2+} signals, protein tyrosine phosphorylation, inositol 1,4,5-trisphosphate (IP3) formation and degranulation of rat basophilic leukemia (RBL-2H3) cells. Ca^{2+} signals were monitored by using Ca^{2+}-indicating fluorescence probe fura-2 AM (Grynkiewicz, et al., 1985). Tyrosine phosphorylation was detected with anti-phosphotyrosine antibody (PY20, ICN) after electrophoresis and western blotting. IP_3 concentration was measured with IP3 assay kit (Amersham). Degranulation was monitored by measuring histamine or b-hexosaminidase released. Pervanadate was generated from a combination of H_2O_2 and vanadate (Vi) according to the previous paper(Teshima, et al., 1994). As antioxidants, 3 hydroquinone-type [DTBHQ (2,5-di-t-butyl-hydroquinone) (Kitajima, et al., 1995), DTAHQ(2,5-d-t-amylhydro-quinone), MTBHQ(2-t-butyl-hydroquinone)] and 3 phenolic [BHT(3,5-dibutyl-4-hydroxy-toluene),

DTBHA (3,5-dibutyl-4-hydroxyanisole), MTBHA (3-butyl-4-hydroxyanisole)] antioxidants were used.

Results and Discussion

Pervanadate induced concomitantly protein tyrosine phosphorylation, formation of IP_3, an increase in $[Ca^{2+}]i$, and histamine secretion in RBL-2H3 cells. These effects were clearly dependent on the ratio of H_2O_2/Vi. The secretion of histamine, IP3 formation, and sustained increase in $[Ca^{2+}]i$ were effectively induced by treatment of the cells with the pervanadate produced from 1 mM H_2O_2 and 1 mM Vi. These effects mimic the stimulative effects of an antigen (dinitrophenylated BSA) on Ca^{2+} signals and histamine secretion. Protein tyrosine phosphorylation and transient increase in $[Ca^{2+}]i$ were markedly induced by the pervanadate produced from 3 mM H_2O_2 and 1 mM Vi. The results indicate that protein tyrosine phosphorylation and a sustained $[Ca^{2+}]i$ increase is an important step for histamine secretion in RBL-2H3 cells.

Among 6 antioxidants, DTBHQ and DTAHQ (0.1-10 mM) induced rapid and sustained increase in $[Ca^{2+}]i$. The increase in $[Ca^{2+}]i$ was induced by emptying of intracellular Ca^{2+} stores, which seemed to be caused by inhibition of microsomal Ca^{2+}-ATPase. BHT and DTBHA (10-50 mM) induced slight increase of $[Ca^{2+}]i$. In contrast, MTBHQ and MTBHA (10-50 mM) had no activity to increase in $[Ca^{2+}]i$. DTBHQ and DTAHQ (10 mM) themselves elicited a low level of degranulation. However, the DTBHQ- and DTAHQ-induced degranulation was markedly enhanced by the addition of the protein kinase C activator, phorbol 12-myristate 13-acetate (TPA) (10ng/ml), in a concentration-dependent manner. Moreover, DTBHQ and DTAHQ enhanced the antigen-induced degranulation. BHT and DTBHA had a moderate enhancing effect of degranulation with TPA and antigen. MTBHQ and MTBHA had no enhancing effect of degranulation. The results suggest that DTBHQ and DTAHQ increases $[Ca^{2+}]i$ and enhances antigen-induced degranulation while DTBHQ and DTAHQ alone do not cause as much degranulation as antigen, which suggest that calcium signals are necessary but are not sufficient for maximum histamine secretion in RBL-2H3 cells.

From above experiments, It is suggested that knowing the effects of a chemical on signal transduction of RBL-2H3 cells involving Ca^{2+} signals and protein phosphorylation would be helpful for predicting allergy-inducing or allergy-enhancing effects of the chemical.

Acknowledgement

I would like to acknowledge Drs. Sawada, J., Akasaka, R., Kitajima, S., and Momma, J. forreviewing the manuscript.

References

Beaven, M.A. and Metzger, H., *Immunology Today* , **14**: 222-226, 1993.

Grynkiewicz, G., Poenie, M. and Tsien, R.Y., *J.Biol.Chem.* , **260**, 3440-3450, 1985.

Kitajima, S., Momma, J., Tsuda, M., Kurokawa, Y.,Teshima, R. and Sawada, J., *Inflamm. Res.* , **44**: 335-339, 1995.

Razin, E., Pecht, I. and Rivera, J., Immunology Today , **16**: 370-373, 1995.

Siraganian, R.P., *In: Inflammation: Basic principles and clinical correlates*, edited by Gallin, J.I., Goldstein,I.M. and Snyderman, R. 513-542. Raven Press, New York , 1988.

Teshima, R., Ikebuchi, H., Nakanishi, M. and Sawada, J., *Biochem.J.*, **302**: 867-874, 1994.

SI-3 Effects Of Steroids On Thymus Microenvironment In A Culture Model

Kou SAKABE and Kanji SEIKI

Department of Morphology, Tokai University School of Medicine, Bohseidai, Isehara, Kanagawa 259-11, Japan

Summary

Using rat TEC lines, the present study attempted to elucidate the mechanism of action of SH on the TEC growth and function. The findings were as follows: (a) the proliferation of TEC in response to SH was mediated through PKC activity introduced as a result of interaction between SH and plasma-borne inhibitors; (b) the strong inhibitory effect of SH on TEC proliferation might be mediated through the SH receptor pathway because the proliferative response was triggered by P and DHT, whereas the inhibitory response was triggered by P, DHT and E ; (c) moreover, the number and size of cytoplasmic fatty droplets apparently increased with E-treatment. These results clearly suggest that the control of TEC proliferation is a "shutoff" mechanism triggered by high plasma levels of SH. This further refers to the speculation that the development of the normal thymus may be due to a lack of this "shutoff" mechanism so that development occurs at the adequate plasma SH levels that are often observed before puberty. However, this development is inhibited at the high plasma SH levels after puberty and/or during pregnancy.

Introduction

Thymic epithelial cell (TEC) lines present a useful tool to explore the mechanism of steroid hormone (SH) action on thymus microenvironment in an "in culture-in animal" model. Because in the thymus, the proliferation, differentiation and function of TEC are intimately linked to their SH sensitivity (Sakabe et al., 1994) regardless of the stage and duration of postnatal development, or postpubertal involution due to exposure of the tissue to SH (Grossman and Roselle, 1983). Moreover, specific SH-induced biochemical changes are observed in this tissue during puberty and pregnancy as well as adulthood (Sakabe et al., 1987). Although many findings have been presented about the role of SH in physiological regulation, the mechanism(s) through which SH affect target cells are not fully understood. Using rat TEC line, the present study attempted to elucidate the mechanism of action of SH on the TEC growth and function.

Materials and Methods

Rat thymic epithelial cell line (TEC; IT-45R1) was originally established by Itoh et al. (1982). This cell line is known to produce thymic hormones and to stimulate prothymocyte proliferation. TEC culture was performed as follows: TEC were kept undisturbed for 5 days in medium (with 10% FCS) alone, then various steroids were added. At the end of the period necessary for exponential cell growth, the cultured cells were used for cell counting, BrdUrd uptake and protein kinase C (PKC) analysis.

Results and comments

The addition of progesterone (P) or dihydrotestosterone (DHT) to 10% FCS-supplemented medium generated a biphasic TEC proliferative response; the peak reached at $3x10^{-10}$ mol/l, above $3x10^{-9}$ mol/l resulted in a progressive loss

of cell yields (i.e., shutoff effect). On the contrary, estradiol (E) induced no cell proliferation at any hormone concentration over the control value; instead , it brought about an inhibitory effect.

The number of BrdUrd-positive cell was much more in P- and DHT-treated media than in control or E-treated medium.

PKC activity in P- or DHT-treated TEC was higher than that in controls, whereas the activity of this enzyme in E-treated cells was almost the same as the control value. PKC-immunoreactivity in the control or E-treated slides was only seen in mitotic cells, whereas in P- or DHT-treated slides, it was not only observed in mitotic but in non-mitotic cells as well.

The number and size of cytoplasmic fatty droplets apparently increased with E-treatment, however, P or DHT induced no fatty droplets at any hormone concentration.

The TEC line has been proven a reliable tool to explore various competitive hypotheses on the mechanism of SH regulation of cell growth and function. The results presented here strongly suggest complex interactions among the protagonists of this regulation. The present data have clarified the participation of distinct pathways favouring the acceleration and inhibition of TEC proliferation, which occur successively.

P and DHT administration generated a biphasic proliferative pattern whereby higher concentrations than those required for maximal cell yield resulted in a dose-dependent inhibitory effect. On the other hand, the time-dependent shutoff effect of SH on the growth of thymus after puberty or during pregnancy.

Acknowledgements

This work was supported in part by Grant-in-Aid for Science Research (C) from the Ministry of Education, Science and Culture to Kou Sakabe (#07670037). The authors thank Dr.Tetsuro Tamaki, Department of Physiology, Division of Human Structure and Function, Tokai University School of Medicine for his helpful advice.

References

Grossman, C. J., and Roselle, B. A., *J.Steroid Biochem.*, **19**, 461-467, 1983.
Itoh, T., Kasahara, S., and Mori,T., *Thymus*, **4**, 69-75 (1982).
Sakabe,K., SeikiK., and Fujii-Hanamoto, H., *Thymus*, **8**, 97-107, 1986.
Sakabe,K., SeikiK., and Fujii-Hanamoto, H., *Med.Sci.Res.*, **15**, 1185-1186, 1987.

SI-4 A Simplified Or Alternative Test For Contact Hyper-Sensitivity Assay

Naonobu YOSHIZUKA[1], Ryuichi KASHIMA[1], Atsushi OHUCHI[1], Yuichi ITOH[1], Yuzo IKEDA[1], Yoshiro KOBAYASHI[2]

[1]Biological Science Laboratories, Kao Corporation, 2606, Akabane, Ichikai-machi, Haga-gun, Tochigi, 321-34, Japan , and [2]Department of Biomolecular Science, Faculty of Science, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274, Japan

Introduction

Contact hypersensitivity assay (CHA) is one of the most important safety evaluation tests of product and its ingredients. From the animal welfare point of view, in vitro test method should be developed for CHA. In the induction phase of sensitization, it is considered that haptens through the skin bind to some proteins in Langerhans cells (LCs) of the epidermis. Haptenated proteins are processed and presented to T cells in local lymph node together MHC

class II molecules. First we studied a lymph node cell (LNC) proliferation in delayed type hypersensitivity (DTH) reaction. Recently, the murine LNC proliferation assay has been used to predict strongly sensitizing chemicals. However, the sensitizing concentrations in the murine LNC proliferation assay are sometimes much higher than those in the conventional guinea pig (GP) test. We therefore have investigated a LNC proliferation assay in GPs and its potential as a screening assay under sensitizing conditions in routine animal test has been studied. Second we report the characterization of carrier protein for hapten which has been hypothesized by Landsteiner and John (1936) to mediate DTH reaction following application of simple chemicals, such as 2,4-dinitro-chloro-benzene (DNCB), in the APCs.

Methods

Sensitization procedure

24-hr occlusive patch sensitization. A 24-hr occlusive patch (24 cp) was applied to an area (2 x 4 cm) of clipped and shaved scapular region with the occlusive patch unit containing 0.2 ml of a test solution. Control group animals were treated with the vehicle only. Intradermal injection sensitization. The test solution (0.1 ml/site) was injected id into two sites of area 2 x 4 cm in the scapular region. Control group animals received the vehicle only. Combination sensitization (id injection and 24 cp). A combination sensitization procedure involved simultaneous id injection and 24 cp.

Lymph node cell proliferation assay in GPs

On the day of the experiment, subscapular lymph nodes were individually excised and pooled in PBS^-, supplemented with penicillin G and streptomycin for 10 min. at 4°C. After through washing with PBS^-, LNC suspensions were prepared by grinding lymph nodes, filtering, washing with PBS^- and resuspending the cells in serum-free RPMI1640 medium. Viability was more than 95%. LNCs were transferred to each well in 96-well microplates and cultured for 20 hr at 37°C, pulsed with [^{3}H] methyl thymidine([^{3}H]TdR). [^{3}H]TdR incorporation was determined. Cell proliferation was recorded as the ratio between the mean [^{3}H]TdR incorporation LNC in sensitized animals and the mean [^{3}H]TdR incorporation into LNC in control animals (stimulation index, SI).

Priming cells with haptens

APCs adhered on the dish, were cultured with 100 mg hapten/ml in serum free RPMI1640 at 37°C for 1 hr. After incubation, the primed cells were recovered with rubber policemen and washed three times with ice-chilled Hanks' balanced solution.

Affinity chromatography

Affinity chromatography was performed according to the method previously described (Mescher, et al., 1983) using anti-DNP antibody conjugated Sepharose 4B column.

Results and Discussions

The efficacy of the LNC proliferation assay in the GP as a first stage screening method of predicting sensitizing potentials of chemicals was studied using several haptens. Animals were sensitized with each hapten, and then the LNC suspension was prepared, followed by incubation with [^{3}H]TdR. LNCs from animals sensitized with DNCB, for example, proliferated maximally and

significantly at day 5. Significant LNC proliferation and high SI values were obtained successfully by a combination of 24 cp and id injection method. On the contrary, a primary irritant, such as sodium dodecyl sulfate, failed to induce the proliferation (Kashima et al., 1994).

We purified dinitrophenylated protein from DNCB-primed APCs with monoclonal anti-dinitrophenyl (DNP) antibody-conjugated Sepharose 4B column. The molecular weight of dinitrophenylated protein was 66kDa in mouse Langerhans cells, mouse peritoneal exudate cells (PECs), GP PECs or human peripheral blood monocyte. The 66kDa protein thus obtained could induce a DTH like reaction in GPs at lower concentration than for DNP-GP serum albumin. When GP PECs were primed with each of nine chemicals similar to DNP with different sensitizing potentials, the amount of each 66kDa protein on the Western blot membrane highly correlated with in vivo skin sensitizing score. Taken together, our results suggest that the 66kDa-protein may function as the so-called "carrier protein".

References

Landsteiner, K., and John, J., *J. Exp. Med.*, **64**, 625-639 , 1936.
Mescher M. F. , S. Kathryn C., Sullivan, C. P., Turkewitz, A. P., Herrmann, S. H. , *In: Methods in Enzymology* , eds. Langone J. J., V. H. Van, **92**, 86-109, 1983.
Kashima R., Okada, J., and Ikeda, Y., *Fd. Chem. Toxic.*, **32**,831-836, 1994.

SI-5 Possibility Of Developing *In Vitro* Methods For Predicting Human Immunotoxicity

Diether NEUBERT and Reinhard NEUBERT

Institute of Toxicology, University Medical Center, Benjamin Franklin, Free University Berlin, Germany

Introduction

Alterations of components and functions of the immune system have become of great scientific interest and of considerable public concern. Methods for revealing such alterations have been suggested on several occasions, but have not been accepted as standard procedures e.g. by regulatory agencies. Furthermore, both of which are of equal importance in a risk assessment.

Difficulties arising in attempts to reveal possible alterations of components and functions of the immune system are modified, and they include:

•Reactions of the immune system are complex, and many interlinks within the system, and with components outside of the system(endocrine, CNS, etc.) exist.
•It is the physiological function of the system to adapt to intrinsic as well as pathological situations, and therefore, a great flexibility is characteristic for the system with considerable inter- and intra- individual variability of the "normal" ranges of various components and functions.
•The term "immunotoxic" is ill-defined, since many substance-induced variations cannot be considered as "toxic". A change of e.g. 20% of a typical lymphocyte subpopulation may be a clear-cut biological effect, but is certainly not to be considered as toxic. Therefore, we prefer the neutral term: "alteration of components and functions of the immune system". The possible toxicological significance, especially for the situation in humans, will often remain obscure.

•It is largely unknown, which "battery" of variables of the immune system should be evaluated in routine studies. It is certainly impossible to assess all the facets of this system. Therefore, important aspects may be missed, in any of the test batteries suggested.
•It is unknown whether a testing of adverse effects on the immune system can be integrated into the routine long-term toxicity studies. Although this has been suggested, the feasibility was not tested.
•It is unknown which animal species allows the best prediction of adverse effects with relevance for the situation possibly existing in humans. There are strong indications that rodents may not be the most suitable species, and that studies in primates may be required. Furthermore, for a medical risk assessment it is necessary to confirm or reflect hypothesis from animal studies by comparable studies in humans, which often will not be possible(especially not on the basis of rat studies).
•The developing immune system may be especially susceptible to possible adverse effects. The information presently available on this topic is score, since too few substances have been evaluated and no standardized procedures have been agreed upon.

Possibilities for developing in vitro systems for predicting immunotoxicity in humans

While most methods for assessing immunological functions are aimed at taking advantage of the complexity of the system, it is tempting to develop *in vitro* systems for predicting, at least some, substance-induced alterations in the human immune system. However, this approach should not be overestimated.

Preferentially, such test systems should use human cells as a target, for obvious reasons.

The following test systems appear feasible for assessing possible substance-induced effect:

•Lymphocyte proliferation after stimulation with various stimulating agents (mitogens, recall antigens, anti-CD3, lipopolysaccharides [LPS], etc.). Performed with ^{3}H-thymidine this is a standard test today. However,, there are many more possibilities:
•Proliferation may be measured to T cells as well as of B cells.
•The expression of cell surface markers can be measured in the course of the proliferation and differentiation (using monoclonal antibodies[mAbs] and flow cytometry). Such surface markers may be activation markers(e.g. CD25, HLA-DR), or a variety of adhesion receptors (e.g. integrins), or other receptors. Such studies are not confined to lymphocytes, but may be extended to neutrophiles etc. Corresponding studies have been performed.
•A special system is the use of immune T cells (umbilical cord blood), and to measure the maturation processes (e.g. occurrence of $CD^{4+}CD45RA^{-}$ $CD45RO^{+}CD29^{+}$) during culture *in vitro* after stimulation. Again, this approach has been used in our laboratory.
•Production of specific cytokines (e.g. interleukins, THFa, etc.), and release into the culture medium. This may be performed with lymphocytes, monocytes, and other cells (including established human permanent cell lines). Cytokine production may be stimulated by various agents, and the components are measure by ELISA or similar methods (again using specific mAbs).
•Besides while blood cells other cells of the immune system may be used for *in vitro*-studies. Such cells include thymus cells (e.g. from humans), or even

better organ clutters of thymus. Thymus organ cultures of rodent fetuses are well-established and have been used for assessing substance-induced effects (e.g. of "dioxins", antiviral substances, etc.). Similar cultures are also possible with lymph nodes.
•Studies on the cytotoxicity of cells *in vitro*. Several standardized assays are available, especially for testing cytotoxicity against tumor cells.

However, one has to be aware that the results of such studies cannot directly be extrapolated to the situation possibly existing *in vivo*. This is due to the fact that many of the proliferation stimulators are artificial and the concentrations used in vitro may not be reached *in vivo*. Furthermore, care should be taken to use concentrations of the chemicals to be tested that are relevant to the situation *in vivo*.

Aside from this, numerous methods are available for mechanis-tic studies (e.g. on signal transaction, etc.).

Possible compromises and future developments

Especially rewarding may be methods in which *in vivo* and *in vitro* studies are combined ("*ex-vivo*"). The whole repertoire mentioned under (2) may be performed with cells after treatment of experimental animals of humans with minimal stress to the animals or volunteers (use of generally non-toxic doses and a few blood drawings).

On the other hand, it should be stressed that certain very important function on the immune system may not be evaluated with *in vitro* techniques. These include:

•Host resistance against microorganisms and tumors,
•induction of autoimmune diseases,
•induction of allergic reactions.

Therefore, as in other fields of medicine, a meaningful combination of *in vitro* and *in vivo* methods will provide the optimal information required for a risk assessment with relevance for tumors.

SII-1 *In Vitro* Evaluation Of The Efficacy Of Hair Growth Products

Seiji ARASE

Department of Dermatology, School of Medicine, University of Tokushima, 3-18-15, Kuramoto-cho, Tokushima 770, Japan

We have succeed in culturing various types of cells which compose human hair follicle such as outer root sheath cells, matrix cells, and dermal papilla cells., We have also established the organ culture system of whole follicles and trues, we examined the effects of hair growth (care) products tested in our *in vitro* systems might be useful for the evaluation of their clinical effects.

References

Arase, S.et al., *J. Dermatol.*, **17**: 667-676, 1990.
Arase, S. et al., *Skin Pharmacil.*, **7**, 12-15, 1994.
Itami, S. et al., *Ann. New York Acad. Sci.*, **642**, 385-395, 1991.

SII-2 Evaluation Of Anticancer Agents By Collagen Gel Droplet Embedded Culture Of Human Cancers

Makoto INABA[1] and Hisayuki KOBAYASHI[2]

[1]*Cancer Chemotherapy Center, Japanese Foundation for Cancer Research, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170, Japan and* [2]*Research Laboratory, Nitta Gelatin Inc., 22-2 Futanata, Yao, Osaka 581, Japan*

Introduction

A collagen gel droplet embedded culture drug sensitivity test (CD-DST) was developed as an *in vitro* drug sensitivity test to select effective drug(s) for individual cancer patients. Herein, its methodology and characteristics are reviewed and its feasibility as a screening method of anticancer agents are discussed with regard to alternatives to animal testing.

Materials and Methods

Procedure of CD-DST

Three major procedures of CD-DST are briefly described, (1) preculture, (2) collagen gel (CG) embedded culture and (3) image analysis as an assay method (Kobayashi et al. , 1995; Koezuka et al. , 1993). The cell suspension prepared from tumor tissues by mechanical and enzymatic dispersion is precultured for 24 or 48 hr in CG flask, and viable tumor cells are selected. CG droplets including about 3 x 10^3 cells each prepared by gelling a 30 ml admixture of cancer cell suspension and CG solution are cultured in serum-free DF (10) medium. After 24 hr, the drug solution is added to the medium, and cells are exposed to the drug for the required time. Following the culture for 7-10 days, colonies are stained with neutral red and fixed. The total volume of cancer cell colonies is estimated by an image analysis system in which fibroblastic cells are eliminated by means of size selection. The *in vitro* cytotoxic effect is expressed as a ratio of the total colony volume of the treated cells to that of the untreated cells.

Characteristics of CD-DST

CG droplet embedded culture of isolated tumor cells, use of serum-free medium and colony volume assay by an image analysis are 3 major unique methods applied to CD-DST. By the CG embedded culture method cloning efficiency was significantly higher than that with soft agar culture of lung, breast, colon and stomach cancers were 0.899, 0.464, 1.35 and 0.690, while those in soft agar were 0.061, 0.015, 0.098 and 0.015, showing an increase of 14.7, 30.9, 13.8 and 46.0-fold, respectively(Koezuka et al. ,1993). Not only cloning efficiency but also growth rate was much higher in CG culture than soft agar culture since colonies in CG culture were markedly larger. It should be also noted that CG droplet culture produced *in vivo* - like three-dimensional outgrowth. On the other hand, use of serum-free medium and final assay by image analysis enabled CD-DST to assess total colony volume of only cancer cells effectively eliminating fibroblastic cells. These features provided CD-DST with several practical merits : (1) CD-DST is possible with a small number of sells, (2) it has high success rates, 89% for lung cancer and 80% for breast cancer, (3) response of cancer cells to drugs is much more sensitive than organ culture on collagen gel matrix, and (4) accurate quantitation of cancer cell growth (Kobayashi, 1995).

Application to screening of anticancer agents

CD-DST can be applied to not only a clinical drug sensitivity test but also *in vitro* drug screening test for the development of new anticancer agent. The latter is expected to contribute as an alternative to animal experiments. However, for anticancer agents which are generally known to have only limited selective toxicity against cancer, screening by only an *in vitro* test is impossible. Of importance is how much we can decrease the experimental use of animals. This aspect is discussed about two stages of screening of anticancer agents:

(a) Primary screening

In vitro cytotoxicity assay using a number of cultured lines of various human cancers is widely carried out as a primary screening for anticancer agents, but it has some difficulties predicting *in vivo* anticancer efficacy based on *in vitro* cytotoxicity date. In this regard, CD-DST as a primary screening tool is also within limitations, although primary culture of human tumor xenografts might show different profiles of drug sensitivity reflecting heterogeneous cell population from established culture lines. Human tumors which show marked *in vitro* sensitivity to a test compound are worthy of *in vivo* testing.

(b) Secondary screening

Therapeutic efficacy of the candidate compound against various tumors must be investigated in this stage. It is very important to evaluate what types of human cancers are responsive *in vivo* to it. A large number of nude mice would be needed for random tests on various human tumor xenografts. However, if we test *in vitro* sensitivities of all these human tumors by CD-DST and conduct *in vivo* experiments in the order of sensitive tumors, it is quite possible to diminish the number of nude mice used. To establish this *in vitro-in vivo* sequential testing, we must recognize a significant correlation between *in vitro* sensitivity and *in vivo* response. Then we treated various human tumor xenografts *in vivo* with 3 typical anticancer agents (mitomycin C, cisplatin and Adriamycin) at their MTDs. On the other hand, we conducted CD-DST for

these tumor lines under drug exposure condition which is pharmacokinetically equivalent with the respective MTD. As a result we found an 83% correlation rate between *in vitro* and *in vivo* growth inhibition(Inaba et al., 1996). In conclusion, it is possible to reduce the number of mice used for screening of anticancer agents, particularly in a secondary evaluation, by effective use of prominent *in vitro* testing such as CD-DST.

References

Inaba, M., Tashiro, T., Sato, S. et al., *In vitro - in vivo* correlation in anticancer drug sensitivity test using AUC-based concentrations and collagen gel droplet embedded culture, *Oncology*, in press, 1996.

Kobayashi, H., Tanisaka, K., Kondo, N. et al. , Development of new in vitro chemosensitivity test using collagen gel droplet embedded culture and its clinical usefulness, *Jpn. J. Cancer Chemother.*, **22**: 1933-1939, 1995. (in Japanese with English abstract)

Koezuka, M., Kondo, N., Kobayashi, H. et al., Drug sensitivity test for primary culture of human cancer cells using collagen gel embedded culture and image analysis, *Int. J. Oncol.*, **2**: 939-959, 1993.

SII-3 Pharmacological Significance Of *In Vitro* Drug Metabolism Studies

Tsuyoshi NAKAMURA[1], Ken-ichi NUNOYA[1], Yasuyuki KOsaka[1], Michihiro CHIDA[1], Katsunori NAKAMURA[1], Kohki TAKAHASHI[2], Kazuaki INOUE[3], Noriaki SHIMASA[4], Kanzo KIMURA[5], Kan CIBA[6], Takashi ISHIZAKI[6], and Tetsuya KAMATAKI[1]

[1]Division of Drug Metabolism, Faculty of Pharmaceutical Sciences, Hokkaido University, [2]Tanabe Seiyaku Co., Ltd., [3]Department of Pathology, Hokkaido University Hospital, [4] Daiichi Pure Chemical Co., Ltd., [5] Sumitomo Pharmaceutical Co., Ltd., [6] Research Institute of Medical Center of Japan

Research efforts made in past decades accumulated on the development of *in vitro* alternative screening system, which might extrapolate to *in vivo* metabolism in humans. Recently, human livers are used frequently to determine the metabolic profiles, rate of metabolism and the specific enzymes involved, early in the development of new drug. Several different *in vitro* models, including human liver microsomes, hepatocyte cultures, and precision-cut liver slices can be used to conduct comparative drug metabolism studies (Sakuma et al., 1994, 1995) .

Microsomes expressing human CYP enzymes in B-lymphoblastoid cells, inset cells, *E. coli* and yeast (Fukuta et al., 1992)were also adopted. Each preparation has advantages for particular studies. Strategies to determine the CYP enzymes which involved in the metabolism of promethazine and other drugs and its pharmacological significance are presented using *in vitro* alternative screening systems.

References

Fukuta, H. et al., *Mutation Res.*, **269**: 97-105, 1992.

Sakuma , T. et al., *Mol. Pharmacol.*, **45**: 228-236, 1994.

Sakuma , T. et al., *Biochem. Mol. Biol. Intern.*, **35**: 447-455, 1995.

SII4 Evaluation Of Antiviral Activity Of Medicinal Herbs

Kimiyasu SHIRAKI and Masahiko KUROKAWA

Department of Virology, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-01, Japan

Introduction

Medicinal herbs have been historically used for the treatment of various kinds of diseases. Aspirin (salcin), reserpine, curare, colchicine, caffeine, atropine, etc. have been developed from them. Plants have their own nucleic acid, protein, carbohydrate, and lipid metabolisms as well as a hormone system. Anti-herpes simplex virus (HSV) activity of about 200 medicinal herbs has been evaluated in an *in vitro* culture system and an HSV-infected therapeutic activity in infected mice (Kurokawa et al., 1993; Kurokawaet al. ,1995; Kurokawa et al., in press). During this process we have first screened anti-HSV activity in the plaque reduction assay in vitro and then the adsorbability of their anti-HSV activity in the herbal extract (Kurokawa et al., 1994; Kurokawa and Shiraki, 1994). The serum pharmacology facilitated the selection of active anti-HSV herbs in this study. In contrast, Kakkon-to which is composed of 7 medicinal herbs, showed strong therapeutic activity but no direct anti-HSV activity *in vitro* (Nagasaka et al., 1995). Its anti-HSV activity was mediated by the enhancement of the delayed type hypersensitivity (DTH) specific to HSV antigen.

Materials and methods

we have used typical and easily available traditional medicines which have been orally administered for the treatment of various diseases. Hot-water (HW)-extracts of dried traditional medicines were lyophilized and used for the experiments(Kurokawa et al., 1993; Kurokawa et al. ,1995; Kurokawa et al., in press).

Duplicate cultures of Vero cells in 60 mm plastic dishes were infected with 100 plaque forming units (PFU) /0.2 ml of HSV. Then the cells were overlaid with nutrient methylcellulose medium containing HW-extracts. The infected cultures were incubated for 2-days at 37°C The cells were fixed stained, and the number of plaques was counted.

Cytotoxicity of HW-extracts was evaluated by the extent of omission of uninfected cells from the surface of stained dishes in plaque reduction assay, the thymidine uptake assay, and a growth inhibition assay.

To examine the adsorbability of their anti-HSV activity in the herbal extract, female Hartley guinea pigs weighing 300-350 g were starved for 24 h before administration of HW- extracts (Kurokawa et al., 1994; Kurokawa and Shiraki, 1994). As it is difficult to predict the part of alimentary tract at which the antiviral components of HW-extracts are absorbed, the abdomen of anesthetized guinea pigs was opened and each 10 ml (20 mg/ml) of HW-extracts, acyclovir (antiherpetic agent), or water was carefully injected into the stomach, and small and large intestines (total dose, 600 mg/guinea pig). The opened abdomen was closed by clips. Their sera were collected at 2 h after injection and inactivated at 56°C for 30 min.

HSV-infected cells were incubated in the medium containing 25% guinea pig serum containing absorbed fraction of HW-extracts. After 24 h incubation, virus yields were determined and the adsorbability of anti-HSV activity in the herbs was evaluated.

HSV suspension (1 x 10^6 PFU) was applied to the naked right midflank of female BALB/c mice (6-week-old). HW-extracts were orally administered at 8 h before and three times daily for 10 successive days. The development of skin lesions and mortality were monitored.

Results

Thirty-six HW-extracts reduced the plaque formation of HSV and we selected 35 of them as candidates for the next absorbability assay, because one HW-extract had been used up. Thirty-five-HW-extracts were examined for their anti-HSV activities in the fraction absorbed from alimentary tracts. All guinea pigs administered with *Mylabris sidae Fabr.* were dead by 2 h after injection. Twenty-one HW-extracts reduced HSV virus yields to less than 70% of the control and were selected as candidates for the next HSV infection model. Ten HW-extracts which showed cytotoxicity in plaque reduction assay were included in the selected candidates, suggesting possible exclusion of cytotoxic substances during absorption.

Therapeutic efficacious of 21 HW-extracts were examined in a cutaneous HSV infection model in mice. In groups treated with 5 HW-extracts (30 mg / mouse / day), most of mice were dead before skin lesions could be scored, because of their possible toxicity. Ten HW-extracts significantly prolonged mean survival times and / or delayed the development and progression of skin lesions. *Phellodendron amurense* reduced the mortality significantly. Thus, The 10 HW-extracts exhibited significant therapeutic efficacy in an animal HSV infection model. The other 14 which were not selected by the absorvavility assay, were evaluated in cutaneous infection model. Only 2 of them showed therapeutic activity.

Kakkon-to which had no anti-HSV activity in the HW-extract showed significant therapeutic activity in a cutaneous infection model(Nagasaka et al., 1995). NK cell activity and the levels of interferon and cytokines were not affected by Kakkon-to. The DTH reaction specific to HSV which is an important host defensive activity against cutaneous a HSV infection was significantly enhanced by kakkon-to. Therefore it mainly contributed to cure the lethal HSV infection.

Discussion

We have screened medicinal herbs for anti-HSV activities and selected 12 harbs with therapeutic activity in a mouse HSV infection model. In this process we have learned the advantage and little limitation of the serum pharmacology in screening the absorbability of active compounds in the herbs. There was unexpected toxicity of herbs which did not exhibit strong cytotoxicity *in vitro*. However three step screening, activity *in vitro*, serum pharmacology, and then final assay *in vivo* was more efficient in screening the activity in HW-extract and reducing the number of experimental animals than two step assay, activity *in vitro* and then directly *in vivo*.

However the activity of Kakkon-to could not be predicted from the assay *in vitro*. And its action was limited in the infection site and no systemic reaction was observed.

We have to develop a new system in vitro to screen the activity *in vivo* which could not be predicted from the assays *in vitro* in this study.

References

Kurokawa, K., Hase, K., Xu, H., Yamamura, J., Koyasu, M., Sato, H., Kadota, S., Hozumi, T., Namba, T., and Shiraki, K. , *J. Med. Pharm. Soc.* , *Wakenyaku*, **10**: 71-85, 1994.

Kurokawa, K., Ochiai, H., Nagasaka, K., Neki, M., Xu, H., Kadota, S., Sutardio, S., Matsumoto, T., Namba, T., and Shiraki, K., *Antiviral Res.*, **22**: 175-188 , 1993.
Kurokawa, K., and Shiraki, K. , *J. Traditional Med.*, **11**: 71-85, 1994.
Kurokawa, M., Nagasaka, K., Hirabayashi, T., Uyama, S., Sato, H., Kageyama, T., Kadota, S., Ohyama, H., Hozumi, T., Namba, T., and Shiraki, K., *Antiviral Res.*, **27**: 19-37, 1995.
Kurokawa, M., Sato, H., Ohyama, H., Hozimi, T., Namba, T., Kawana, T., and Shiraki, K. : J. Traditional Med., in press.
Nagasaka, N., Kurokawa, M., Imakita, M., and Shiraki, K., *J. Med. Virol.*, **46**: 28-34, 1995.

SII-5 Roles Of Diazepam Binding Inhibitor (DBI In Alcohol Dependence: Analysis Using Primary Cultured Neurons

Seitaro OHKUMA, Masashi KATSURA and Kinya KIRIYAMA

Depertment of Pharmacology, Kyoto Prefectual University of Medicine, Kawaramachi-Hirokoji, Kamikyo-Ku, Kyoto 602, Japan

Introduction

Although a large number of neurochemical and neurophysio-logical investigations have been reported to clarify the molecular mechanisms for the establishment of alcohol dependence, the exact mechanisms are not clear at present(Kuriyama and Ohkuma, 1990). Several clinical reports have revealed that administration of benzodiazepines (BDZs) to alcoholics showing motor and psychiatric abnormalities such as convulsion, irritation and anxiety during alcohol withdrawal relieve these signs and symptoms. DBI is an endogeneous polypeptide with property as an anxiety product and is reported to be significantly elevated in cerebrospinal fluid from alcoholics. Based on these data, it is assumed that DBI may be involved in the expression of alcohol dependence and the expression of withdrawal signs. In this study, we attempted to examine the level of DBI mRNA in the brains of ethanol (EtOH)-treated and -withdrawn mice and primary cultured cerebral cortical neurons prepared from mice to clarify this possibility.

Materials and Methods

The preparation of primary cultured mouse cerebral cortical neurons was carried out according to the method previously reported(Ohkuma et al., 1995). The treatment of mice with EtOH was preformed by the method of Goldstein (1972). The mice continuously exposed to EtOH vapor for 7 days were used as EtOH-dependent mice. The mice showing withdrawal signs such as convulsion 8 hours after the termination if the continuous exposure to EtOH vapor were used as EtOH-withdrawn mice. Neurons were exposed to EtOH by direct addition of EtOH into measured by the previously reported method(Katsura et al., 1994).

Results and Discussion

In the cerebral cortex from EtOH-dependent mice DBI mRNA expression increased significantly in comparison with that from mice without EtOH exposure. DBI mRNA expression increased significantly in comparison with that from mice without EtOH exposure. DBI mRNA expression was further enhanced in the cerebral cortex of mice after withdrawal from EtOH exposure. These increase in DBI mRNA expression were abolished by simultaneous administration of flunitrazepam (an agonist of BDZ receptors) during the exposure to EtOH vapor (Fig. 1). On the other hand, the expression of b-actin

mRNA was not altered in the cerebral cortex of both EtOH-dependent and -withdrawn mice.

Exposure of neurons to EtOH for 3 days Elevated significantly the level of DBI mRNA and DBI mRNA expression in neurons 8 hours after the termination of EtOH exposure further increased. Simultaneous administration of flunitrazepam, Ro 15-4513 (an inverse agonist of BDZ receptors), and Ro 15-1788 (an antagonist of BDZ receptors) with EtOH also suppressed the increase in DBI mRNA (Fig. 2). b-actin mRNA level in neurons was not altered by EtOH treatment.

The results showing that increase in DBI mRNA during the establishment of alcohol dependence and the appearance of withdrawal signs suggest that DBI is involved in the events responsible for the establishment of alcohol dependence and/or expression of withdrawal signs. In addition, the increased expression of DBI mRNA during and after the treatment with alcohol may be induced by the interaction of EtOH with BDZ receptors. The results presented here also suggest that primary cultured neurons are considered to be an appropriate tool to investigate the mechanisms for the establishment of alcohol dependence.

References

Goldstein, D.B., *J. Pharmacol. Exp. Ther.*, **180**: 103-215, 1972.

Katsura,M., S.Ohkuma, S., Chen, D.-Z., Tsujimura, A., and Kuriyama, K., *Neurosci. Lett.*, **168** : 1-4, 1994.

Kuriyama,K., and Ohkuma,S., *Alcohol Alcoholism*, **25**: 239-249, 1990.

Ohkuma, ., Katsura, M., Guo, G.-L., Hasegawa, T., and Kuriyama, K., *Mol. Brain Res.*, **31**, 185-193, 1995.

W1 First Validation Study Of Cytotoxicity Tests Under JSAAE Project

[1]Tadao OHNO and 98 co-workers

[1]RIKEN Cell Bank, The Institute of Physical and Chemical Research(RIKEN) and The List of co-workers is available on request.

In October, 1992, JSAAE organized a first step inter-laboratory validation study on 5 cytotoxicity assays with two cell lines each: colony formation (CF) assay, crystal-violet staining (CV) assay, lactate dehydrogenase (LDH) release assay, neutral red uptake (NR) assay, and MTT assay. From the results, (1) CF, CV, MTT, and NR assays are recommendable from the view point of performance of the assays (Table I); (2) CV and MTT assays gave smaller intra-

Table 1. Data files of final difinitive tests submitted to The Working Group

Assay	Cells	Number of data files					Performance rate of		
		a*	b	c	d	e	runnings$ b/a	candidate files$$ d/a	finally accepted e/a
CF	HeLa.S3(sc)	168	130	0	130	118	77%	77%	70%
	BALB 3T3 A31.1.1	168	149	0	149	130	89	89	77
CV	HeLa.S3(sc)	112	98	7	91	82	88	81	73
	CHL	112	97	7	90	85	87	80	76
LDH-1#	HeLa.S3(sc)	126	70	13	57	42	56	45	33
	SQ-5	126	70	20	50	27	56	40	21
LDH-2A	HeLa.S3(sc)	126	68	14	54	34	54	43	27
	SQ-5	126	67	28	39	30	53	31	24
LDH-2B	HeLa.S3(sc)	126	62	21	41	17	49	33	13
	SQ-5	126	62	28	34	18	49	27	14
LDH-2C	HeLa.S3(sc)	126	68	14	54	29	54	43	23
	SQ-5	126	61	21	40	25	48	32	20
MTT	HeLa.S3(sc)	140	123	14	109	97	88	78	69
	SQ-5	140	117	14	103	92	84	74	66
NR	HeLa.S3(sc)	168	154	7	147	128	92	88	76
	NRCE	168	139	7	132	112	83	79	67
Total number of files		2184	1535	215	1320	1066			

* a, expected; b, submitted; c, unacceptable; d, acceptable before ED50 calculation; e, finally accepted by the logistic analysis program, LAP-JSAAE, and the intra-laboratory variation analysis (see Table 5 and 7).

$ runnings = b/a x 100

$$ candidate files = d/a x 100, accepted by The Working Group before ED50 calculation.

\# Different series in the LDH release assay, see Materials and Methods.

The figures in the table were updated and amended after our presentation in the meeting INVITOX'94 (Zürich, Switzerland, 1994) to which data were submitted and published in the proceedings (Ohno, T., *et al.*, Toxicology In Vitro, 9, 571-576, 1995). Difinition of performance rates were changed in order to reflect reality from that described in the proceedings. Other than these final test data files, cumulative 157 laboratories submitted 2275 data files of preliminaly tests.

laboratory varidation of ED50 values of the same but differently coded chemical, Tween 20; (3) CV assay with CHL cells revealed to have given the smallest mean fourth-spread of log(ED50), therefore this assay was considered to give the smallest inter-laboratory varidation; (4) CF assay with HeLa S3 (sc) cells resulted in the largest [difference of medians of log(ED50) values of the least toxic and the highest toxic chemicals/mean fourth spread], followed by MTT with SQ-5 and CV with two cell lines. (5) Considering time consuming. simplicity, precision, and effect of cell lines in each assay, we recommend that CV assay is the most practical as the cytotoxicity assay. Whole results will be published in AATEX soon.

W2 Characteristics And Problems Of Cytotoxicity Methods Involved In The 1st JSAAE Varidation Study

[1]Noriho TANAKA and 98 co-workers

[1]Hatano Research Institute, Food and Drug Safety Center (FDSC) and The list of co-workers is available on request.

The 1st JSAAE validation study was carried out to validate on cytotoxicity tests which will be proposed as one of core battery for alternatives to the Draize eye test. Forty-two laboratories participated in this study and 5 cytotoxicity tests were validated by using 2 cell types and 7 coded chemicals for each test.

In this presentation, we will review on the practical issues for conducting a validation study and the characteristics of each cytotoxicity assay.

W3 Inter-Laboratory Validation Of Alternative Methods To Eye Irritation Test For Safety Evaluation Of Cosmetic Ingredients

Hajime KOJIMA[1], Tohru INOUE[2], Toyozo KANEKO[2], Yoshihiro MORIKAWA[1], Takemi YOSHIDA[5], Makoto HAYASHI[3], Junko MOMMA[2], Tadao OHNO[6], Akio FUJII[1], Mitsuteru MASUDA[1], Junichi AKIYAMA[1], Norikazu IKEDA[1], Hiroshi ITAGAKI[1], Yutaka IMANISHI[1], Masahito USAMI[1], Kenji OHKOSHI[1], Hidenobu OKUMURA[1], Hiroshi KAKISHIMA[1], Yutaka KASAI[1], Akihiro KURISHITA[1], Kaoru SAIJO[6], Kazutami SAKAMOTO[1], Katsuhiro TAKANO[1], Hisashi TATSUMI[1], Naoko TANI[1], Katsuyoshi CHIBA[1], Tsuneaki NAKAMURA[1], Kiyoji MATSUKAWA[1], Rie WATANABE[1] and Yasuo OHNO[4]

[1]Japan Cosmetic Industry Association(JCIA), 4th floor, Hatsumei Bldg., 9-14 2-chome Toranomon, Minato-ku, Tokyo 105, Japan, [2]Div. Toxico., [3]Div. Genet. Mutagen. and [4]Div. Parmacol. Natl. Inst. of Health Sci., [5]Dep. Boiochem. Toxicol. Sch. Pharm. Sci. Showa Univ., [6] RIKEN Gene Bank, Inst. Phys. Chem. Res.

Introduction

The project entitled "The study on the test methods to evaluate safety of cosmetic containing a new ingredients" started in 1990. The objects of this project are to investigate the possibility of replacing *in vivo* eye irritation test and to introduce appropriate methods for safety evaluation of cosmetic new

ingredients in the guideline. After extensive literature review, we decided to conduct an inter-laboratory validation of several alternative methods to Draize eye irritation test.

Materials and Methods

The test methods were (1) CAM test (HET-CAM and trypan blue staining), (2) Hemolysis test, (3) Hemoglobin denaturation test, (4) MATREX^{TM} test, (5) Skin^{2TM}(ZK1100) test, (6) HeLa cells-MTT reduction tests, (7) SIRC cells-neutral red uptake test, (8) CHL cells-crystal violet staining test, (9) CornePack^{TM} test (Normal rabbit eye epithelium cells-neutral red uptake test), and EYTEX^{TM} test. Thirty-nine chemicals, including 14 chemicals for the third validation, have been selected mainly from the wider range of cosmetic ingredients and have been blindly evaluated for their eye irritation potentials.

Results

The first (Ohno et al., 1994) and the second phase validation have been finished, and the third one will be finished by the end of 1995. From the results obtained in the first and the second phase validation, it became clear that EC50 values obtained from cytotoxicity tests using a culture medium with serum[(6), (7) and (8)] and CAM try pan blue staining test showed a good correlation to *in vivo* Draize test. Thus, we temporarily consider that a battery of several *in vitro* methods may constitute a refined evaluation system of eye irritation, However, several limitation have became apparent. These are (1) cosmetic dye as acid red may interfere with measurement of endpoints in HET-CAM, hemolysis, hemoglobin water insoluble compounds may cause variation of the data relatively large in case of CHL cells-crystal violet staining tests, (3) special care must be needed in MATREX^{TM} test, Skin^{2TM} test, and HeLa cells-MTT tests for the substance as calcium thioglycolate which directly reduces MTT, (4) benzyl-alcohol had a tendency to become false negative in many test.

Discussion

After the third phase validation is finished, we will finally evaluate the predictive ability of these *in vitro* methods and consider how to incorporate those methods into the guideline of eye irritation test.

Acknowledgments

This study was partly supported by a Research Grant for Health Science from the Ministry of Health and Welfare.

References

Ohno, Y., Kaneko, T., Kobayashi, T., Inoue, T., Kuroiwa, Y. Yoshida, T., Momma, J., Hayashi, M., Akiyama, J., Atsumi, T., Chiba, K., Endo, T., Fujii, A., Kakishima, H., Kojima, H., Masamoto, K., Masuda, M., Matsukawa, S., Ohkoshi, K., Okada, J., Sakamoto, K., Takano, K. and Takanaka, A. , First Phase Validation of the *In Vitro* Eye Irritation Tests for Cosmetic Ingredients, *In Vitro Toxicology*, 7: 89-94, 1994.

W4 Studies On Scoring-Variances In Draize Eye-Irritation Tests

Toyozo KANEKO[1], Junko MOMMA[1], Junko KUMAKAWA[1], Tohru INOUE[1], Yasuo OHNO[2], Toshiro SUZUKI[3], Hiroshi KAKISHIMA[4,5], Tsuneaki NAKAMURA[4,6], Hisashi TATSUMI[4,7], Shigenobu HAGINO[4,8]

[1]*Div. of Toxicol. and* [2]*Div. of Pharmacol.,Natl. Inst. of Health Sci., Tokyo 158, Japan.* [3]*Seigiken Research Center Co.,Ltd.,*[4]*Japan Cosmetic Industry Assoc.(JCIA),*[5]*Kanebo Ltd.,*[6]*Lion Corp.,* [7]*Sunstar Inc.,*[8]*Shiseido Co.,Ltd.*

Introduction

Validation of various *in vitro*-alternative methods for Draize eye-irritation test is now an important subject, especially for the safety evaluation of the cosmetic ingredients. According to our earlier experiences in comparative studies between Draize scoring and various alternative methods, it was found that more attention concentrating on an analytical reevaluation was needed to see detailed gross findings as well as histological studies on the ophthalmic tissues of the tested rabbit, because unequivocal large variances commonly seen in Draize scoring make such validation process problematical.

Materials and Methods

Twenty five-cosmetic ingredients selected for the validations were analyzed in the present study; ten for the first phase validation (Ohno et al., 1994), of which tested compounds can be found elsewhere; and the following fifteen compounds for the second validation: Sucrose Fatty Acid Ester(S2-1), Glycerin (S2-2), Acid Red 92(S2-3), Polyoxyethylene Sorbitan Monooleate (20E.O.)(S2-4), Calcium Thioglycollate (S2-5), Disteraryldimethyl-ammonium Chloride (S2-6), 2-Ethyl-hexyl p-Dimethyl-amino Benzoate (S2-7), Cetyl-pyridinium Chloride (S2-8), Methyl p-Hydroxybenzoate (S2-9), Iso-propyl Myristate(S2-10), Poly-ethylene Glycol 400 (S2-11), Silicic Acid (S2-12), Benzyl Alcohol (S2-13), Sodium Salicilate (S2-14), m-Phenylene Diamine (S2-15).

These were dissolved or suspended in distilled water, and 0.1ml each was applied to the right eye three NZW rabbits. Along with these standard examinations, additional experiments were con-ducted with special interests focusing on, 1) scoring variance among the examiners, 2) variance among the tested rabbits in the same strain, JW, 3) strain difference in irritation sensitivity between NZW and JW, and 4) comparison between bilateral eyes to see possible technical variance.

Results and Discussion

The AOI scores of each test chemicals for concentration at 1, 10 and 100%, respectively, were as follows: S2-1, ND (=not determined), 11.0, 28.3; S-2, ND, 0.0, 4.7 ; S2-3, 0.7, 25.0, 71.0 ; S2-4, ND, 0.0, 4.7 ; S2-5, ND, 4.0, 79.7 ; S2-6, ND, ND, 96.3 ; S2-7, ND, 0.0, 0.0 ; S2-8, 2.7(=0.1%), 34.7, 94.7, ND ; S2-9, ND, ND, 8.7 ; S2-10, ND, 0.7, 0.0 ; S2-11, ND, ND, 4.0 ; S2-12, ND, ND, 2.7 ; S2-13, 0.0, 23.0, 31.0, ; S2-14, ND, 0.0, 83.7 ; and S2-15, ND, 4.3, 80.7. Among the results, the variances were seemed to diverse greatly in the cases revealing moderately irritant compounds, where the AOI scores appeared to be somewhere between 25 and 50.

The study to compare such variance of examiners and that of individual rabbits is concern, the results showed clearly that the variance from the latter was much larger than that from the former, especially in the scores from the recovery phase. Concerning the strain difference between NZW and JW, the JW was much sensitive, especially in group streated with moderately irritant substances. No significant difference was observed in the study comparing the scores of bilateral eyes each other. However, it is of interests that the irritation scores were slightly higher in the group treated bilaterally as compared with the case treated solely on the right side. Lastly, despite such

variances in the Draize scores, the scores of each compound were found to have possessed a good correlation to the precise histological criteria; thus the Draize scores can be assumed to be fundamental bases for evaluating and validating new alternative methods.

Acknowledgments

This study was supported in part by a Research Grant for Health Science from the Ministry of Health and Welfare, and a Research Grant of the Japanese Society for Alternatives to Animal Experiments.

Reference

Ohno,Y. et al., First-Phase inter-laboratory validation of the *in vitro* eye irritation test for cosmetic ingredients, *In Vitro Toxicol.* , 7: 89-94, 1994.

W5 Investigation Of Closeness Between Draize Eye Irritation Scores And Observed Ed50s In *In Vitro* Assays

Isao YOSHIMURA

Department of Management Science, Faculty of Engineering, Science University of Tokyo, Ichigaya, Shinjyuku-ku, Tokyo 162, Japan

The correlation between ED50s in *in vitro* assays and the Draize eye irritation scores in *in vivo* tests were evaluated based on the validation study reported by Ohno et al. (W-1) in this workshop. Although the result was not so informative due to the limited number of test chemicals used in the study, correlation coefficients were as high as 0.90 when HeLa was used as cells on crystal violet , MTT and colony formation methods.

P1 Tumor-Promoting Activities Of Benzotriazole UV-Absorbers

Hiroki DEGAWA, Toshie TSUCHIYA and Akitada NAKAMURA

Division of Medical Devices, National Institute of Health Sciences, 1-18-1, Kamiyoga, Setagaya-ku, Tokyo 158, Japan

Introduction

Recently, UV-absorbers in the form of sunscreen agents are being used increasingly in facial cosmetics, contact lenses, etc. to shield skin and various organs from the harmful effects of UV radiation as well as to protect the ingredients from degradation. Therefore, it is important to clarify the safety of these absorbers. In this report, we firstly demonstrated the tumor-promoting activities of various benzotriazoles UV-absorbers using the in vitro system.

Materials and Methods

Seven benzotriazoles were tested by the V79 metabolic cooperation assay. Further tumor-promoting activity was examined using the two-stage transformation assay in Balb/3T3 cells *in vitro*.

Results and Discussion

By the V79 metabolic cooperation assay, five compounds indicated positive activities. HBPBT showing a typical structure of benzotriazloe UV-absorber also inhibited the gap-junctional intercellular communication in V79 cells.

The numbers of transformed foci were not increased by the addition of HBPBT during the initiation stage, but increased by the addition of that during the promotion stage after the cells had been initiated with 3-methylcholanthrene (MCA). The present results indicate that HBPBT has a tumor-promoting activity in vitro, and that its tumor-promoting action must be caused by the inhibitory action on the intercellular gap junctional communication.

Reference

Tsuchiya, T. et al., *J. Biomed. Mater. Res.*, **29**: 121-126, 1995.

P2 Effects Of 2,3,7,8-Tcdd On Growth Of *Xenopus Laevis* Larvae

Shin MIMA, Michiko SAKAMOTO, and Takashi TANIMURA

Department of Anatomy, School of Medicine, Kinki University

Xenopus laevis embryo were exposed to 200ppb TCDD or 0.25% DMSO(control) from days 0 to 5 after fertilization, and then they were maintained in TCDD-free dechlorinated water from days 5 to 13 after fertilization. Larvae were sampled on respective days and recorded for death, edema, body weight and body length. Protein, RNA and DNA contents in the TCDD-treated and control larvae were measured from day 8 to 12 after fertilization. Body weight and body length of larvae were reduced significantly from days 5 and 9 after fertilization, respectively. Larval death and edemas were observed from day 10 after fertilization. On day 8-10, RNA/DNA and protein/DNA were reduced in the TCDD treated larvae. These results suggest that TCDD affects RNA and protein synthesis earlier than the appearance of larval death and edema.

P3 Susceptibility To Di-N-Butyltin Dichloride Of Day-8 Embryos Cultured *In Vitro*

Takayuki IWASE[1], Makoto EMA[2], Yumiko IWASE[1], and Yoshiyuki OGAWA[2]

[1]Toxicology Lab., Yokohama Research Center, Mitsubishi Chemical Corp., 1000 Kamoshida, Aoba-ku, Yokohama, 227, Japan and [2]National Institute of Health Science, Osaka Branch, 1-1-43, Houenzaka Chuo-ku, Osaka, 540, Japan

Introduction

Organotin compounds, including di-n-butyltin dichloride (DBTC), are used as heat and light stabilizers for poly (vinyl chloride) plastics, and as catalysts and vulcanizing agents for other industrial products (WHO, 1980). DBTC is a potent teratogen when administered to pregnant rats on days 7-15 of gestation (Ema et al., 1991). Also rat embryos are highly susceptible to teratogenic effects of DBTC with maternal exposure on days 7 and 8 of gestation (Ema et al., 1992). The present study was conducted to assess the embryotoxicity and dismorphogenic potential of DBTC using gestation day 8 rat embryos in a whole embryo culture system. Moreover, the usefulness of the embryo culture system for predicting developmental hazards was examined.

Materials and Methods

Wistar rat embryos were explanted on day 8.5 of gestation (plug day = day 0), of the primitive streak stage, and cultured for 68 hr according to the procedures of Freeman et al(1987) and Fuginaga et al.(1991). DBTC was added to the culture medium at the beginning of this period to give final concentrations of 3, 10 and 30 ng/ml. Embryos were exposed to DBTC for the first 24 hr or 68 hr in culture.

Results and Discussion

At the end of the culture period, a concentration-dependent increase in incidence of embryos showing dysmorphogenesis was noted, and this was significant for those exposed to DBTC at 10 and 30ng/ml. Dysmorphogenesis including turning defects, craniofacial anomalies (cleft prosencephalon, facial clefts, facial deformation, facial asymmetry), and open anterior neuropores. Moreover, at each concentration, embryos exposed to DBTC for the first 24-hr or 68-hr showed similar frequencies of dysmorpho-genesis. These results indicate that the first 24-hr of exposure is essentially sufficient for inducing anomalies in rat embryos cultured from day 8.5 of gestation. This corresponds well into *in vivo* results obtained previously using rats (Ema et al., 1992).

It is concluded that *in vitro* exposure of gestation day 8 rat embryos to DBTC interferes with their development. Moreover, the data provide evidence in support of the efficacy of the post-implantation whole embryo culture system for predicting developmental hazard potential.

References

Ema, M. et al., *Toxicol. Letter*, **58**: 347-356, 1991.

Ema, M. et al.,*Toxicology*, **73**: 81-92, 1992.

Freeman,S.J. et al. ,*In"Biochemical Toxicology. A Practical Approach"*, Eds. Snell, K., et al. pp. 83-107. IRL Press, Oxford , 1987.

Fujinaga, M. et al. ,*Teratology*, **43**: 95-100, 1991.

P4 Inhibitory Effects Of Cocaine On Gap Junctional Inter-Cellular Communication In Chinese Hamster V79 Cells

Yumiko IWASE and Takayuki IWASE

Toxicology Laboratory, Yokohama Research Center, Mitsubishi Chemical Corporation, 1000, Kamoshida-cho, Aoba-ku, Yokohama, 227, Japan.

Introduction

Cocaine has been reported to be a human teratogen (Fries et al., 1993) and may cause single ventricle in development in the fetal heart (Shepard et al.,1991). Some teratogens are known to inhibit the gap junctional intercellular communication (GJIC) in V79 cells (Torrason et al.,1992) and connexin43, one of the most prevalent connexin proteins of gap junction, plays an essential role in heart development (Severs and Cardiovasc, 1994). Its absence induces cardiac malformations in mice(Reaume et al., 1995). In the present study, the effects of cocaine on GJIC in V79 cells were therefore tested using metabolic cooperation and dye transfer assays.

Materials and methods

Chinese hamster V79/HPRT$^+$ and V79/HPRT$^-$ cells were obtained from the Japanese Cancer Resources Bank. They were maintained in Eagle's minimum essential medium, containing a 100% increase nonessential amino acids, 1 mM sodium pyruvate and 3% fetal bovine serum. The cultures were grown at 37^{O}C in a humidified incubator with 5% CO_2.

The metabolic cooperation assay was performed using the method of Yotti et al. (1979) with some minor modifications. In the dye transfer assay, $2x10^5$ V79/HPRT$^+$ cells were seeded on 35mm dishes and treated with cocaine under various conditions. The numbers of communicating cells were then counted after microinjection of 5% Lucifer yellow CH solution.

Results and Discussion

Cocaine inhibited metabolic cooperation between V79/HPRT$^+$ and V79/HPRT$^-$ cells at a noncytotoxic concentration. In the dye transfer assay, the GJIC inhibition caused by 250 mg/ml cocaine reached a maximum after a 3-hour-treatment, with the same level of influence continuing up to 24-hour-treatment.

With a 3-hour-treatment, the drug interfered with intercellular communication dose-dependently, 500 mg/ml of cocaine causing a 57% inhibition of GJIC. However, this did not persist and the effect had disappeared after a 6-hour-recovery period. From these results, it is concluded that cocaine exerts inhibitory effects on GJIC and these may play a role in its ability to induce morphological defects.

References

Fries, M.H. et al.,*Teratology*, **48**: 413-420, 1993.
Reaume, A.G. et al.,*Science*, **267**: 1831-1834, 1995.
Severs, N.J. and Cardiovasc, J., *Electrophysiol.*, 5: 462-475, 1994.
Shepard, T.H. et al., *Teratology*, **43**: 113-117, 1991.
Torrason, M. et al., *Toxic. in Vitro*, 6: 165-174, 1992.
Yotti, L.P. et al., *Science*, 206, 1089-1091, 1979.

P5 Whole Embryo Culture From The Egg Cylinder Stage To Detect Developmental Toxicity In Rats

Hiromasa TAKASHIMA, Makiko OGAWA, and Tetsuji NAGAO

Hatano Research Institute, Food and Drug Safety Canter, 729-5 Ochiai, Hadano Kanagawa 257, Japan

Introduction

Some of nitrosourea antineoplastic agents, such as nimustine, induce structural abnormalities in rat fetuses when administered i.p. to pregnent rats as early as on day 6 of gestation (Takashima et al., 1993). In the present study we attempted to culture rat embryos at the stage of egg cylinder (embryonic day 6.5) (Takashima et al., 1989) to detect the early developmental toxicity of nimustine.

Materials and Methods

Experiment 1

In the first experiment, pregnant rats were treated i.p. with nimustine 5 mg/kg on day 6 of gestation and the embryos were collected on embryonic day 7.5 for subsequent 84 hour culture. On embryonic days 10.0, 10.5 and 11.0, yolk sac diamerter and number of somites were observed. Then, protein contents were measured after the culture period.

Experiment 2

In the second experiment, embryos were cultured for 108 hours from embryonic day 6.5, and exposed to nimusutine at 2.0, 3.5, 5.0 or 7.0 μg/ml from embryonic days 6.5 to 7.5 or from embryonic days 7.5 to 8.5. Yolk sac diameter, number of somites and protein contents were measured in the same schedule as experiment 1.

Result

Experiment 1

The embryonic mortality was increased and all parameters related to the development of embryos was retarded by the treatment. These results suggest that the embryos already had premortal or preteratogenic damages by the time they were removed from the uterus.

Experiment 2

The results were summarized in Table 1. The difference in *in vitro* embrio-

Exposure (embryonic days)	6.5 - 7.5				7.5 - 8.5			
Concentration (μg/ml)	2.0	3.5	5.0	7.0	2.0	3.5	5.0	7.0
Mortality	-	-	-	↑	-	-	-	-
Yolk sac diameter	-	↓	↓	N.D.	-	-	-	↓
No. of somites	-	↓	↓	N.D.	-	-	-	↓
Protein contents	-	↓	↓	N.D.	-	↓	↓	↓

↑: Increase ↓: Decrease -: Not affected N.D.: Not detected

Table 1 Number and Protein Contents of Embryos Exposed to Nimustine

nic susceptibility to nimustine depending on mbryonic age agrees well with that shown in the *in vivo* study (Experiment 1). These results suggest the possibility to detect developmental toxicity by whole embryo culture from the egg cylinder stage.

Reference

Takshima, H., Kuwagawa, M., Nagao, T., and Mizutani, M., Culture of rat whole embryo at the stage of egg cylinder, *AATEX*, **2**: 82-83, 1993.

Takashima, H., Shimizu, Y., Wada, A. and Mizutani, M. , Characteristics of teratogenicity of nitrosourea derivatives, ACNU and MCNU, in rats, *Teratology*, **40**: 684-685, 1989.

P6 *In Vitro* Study On Optic Vesicle Hypoplasia Induced By 5-Fluorouracil In Rats

Makiko OGAWA, Hiromasa TAKASHIMA and Tetsuji NAGAO

Hatano Research Institute, Food and Drug Safety Center, 729-5 Ochiai, Kadano, Kanagawa 257, Japan

Introduction

Our previous study indicated that the hypoplasia of the optic vesicle was induced after exposure of 5-flurouracil (5-FU) to *in vivo* and *in vitro* rat embryos through the common mechanism. The present study was performed to reveal the mechanism of micro/anophthlmus induces by 5-FU using the whole embryo culture.

Materials and Methosds

Embryos (Crj:CD) on embryonic day 9.5 (ED 9.5; plug day = ED 0) were cultured for 53 hours in media containing 0 -0.30 mg/ml of 5-FU. At the end of culture, embryos were assessed for morphological and histological development, and apoptosis was observed by Nile Blue Sulphate (NBS) staining(Scotte et al., 1977). To examine the migration of cranial neural crest cells (NCC), rats on gestational day 9.5 were treated with 5-FU i.p. and the neuroepithelium of ED 10.0 embryos was marked with Dil. After marking, embryos were culture for 38 hours and the distribution of Dil-labelled cells were observed under a fluorescence microscope (Ninomiya et al., 1994).

Results and discussion

Treatment with 5-FU suppressed growth of embryos, and the incidence of optic vesicle hypoplaisa was increased in concentration dependent manner. 5-FU treated embryos showed no formation of the lens placode, and embryonic cells deaths were not increased. However, pattern of NBS incorporation into optic vesicle cells was different from controls. These results suggest that 5-FU-induced optic vesicle hypoplasia resulted from apoptosis around the optic vesicle and/or abnormal migration of cranial NCC. Pattern of migration of NCC in the posterior midbrain in 5-FU treated embryos was similar to controls.

References

Scotte, W.J., Ritter, E. J., and Wilson, J. G., *J. Embryol. exp. Morph.*, **42**: 93-104, 1977.

Ninomiya, Y., Osumi-yamashita, N., Imai, H., and Eto, K., *Exp. Med.*, **12**: 199-215, 1994.

P7 Effects Of Thiabendazole On Cultured Rat Embryos

Atsushi YOKOYAMA[1], Masaharu AKITA[1] and Yukiaki KURODA[2]

[1]Kamakura Women's College, 1420 iwase Kamakura-shi, Kanagawa 247, Japan and [2]Azabu University, 1-17-71 Fuchinobe Sagamihara-shi, Kanagawa 229, Japan

Introduction

Although thiabendazole (TBZ) is approved for the use as a fungicide of imported citrus fruits, its safety is open to examination. In the present study, we conducted embryo culture for elucidation of teratogenic activity of 5OH-TBZ.

Meterials and Methods

The rat embryos on day 11.5 of gestation (plug day = 0) were cultured for 48 hours. As a drug, 5OH-TBZ was added to the medium at a concentration of 40 mg/ml.

Results and Discussion

Although the embryos after culture for 48 hours showed the heart rate of 180±12 beats/min., the total number of somites of 51±1 and the crown-rump length of 7.9±0.1 mm in the control group, the 5OH-TBZ-treated group showed no change in the heart rate, but the total number somites was 7% smaller and the crown-rump length was 8% shorter, showing inhibition of systemic growth, and a decrease in blood circulation was also observed.

From the above results, 5OH-TBZ significantly inhibited systemic growth in the cultured embryos, having the different effect from the hypogenesis of extremities produced by its original compound.

P8 The Study Of Salicylate-Induced Cleft Lip On Cultured Rat Embryos

Atsushi YOKOYAMA[1], Masaharu AKITA[1], and Yukiaki KURODA[2]

[1]Kamakura Women's College, and [2]Azabu University

Introduction

Sodium salicylate, a metabolite of aspirin, was shown to have embryo toxicity in pregnant rats. As the typical type of malformation, cleft lip and cleft palate were accounted in about more than 70%. *in vivo* test system, and bilateral and unilateral cleft lip were indentified. This finding may be due to some influences of maternal body. Therefore, we confirmed the effects of sodium salicylate on rat cultured embryos in the *in vitro* test system.

Materials and method

The rat embryos of 11 day of gestatoin were cultured for 72 hours. Sodium salicylate was given at 300 mg/ml, which was the concentration for developing the malformation *in vivo*.

Results and Discussion

As result, bilaterial cleft lip was hardly observed, but unilaterial cleft lip was ovserved in 45% of cultured embryos. Among them, the disorder at the left side accounted for 15%, and that at the right side accounted for 85%. For the

other effects on the growth index of the cultured embryos, heart beat was significantly decreased compared with the control group. Malformation including short tail, kinky tail and edema was combined. From the above, sodium salicylate developed right lateral cleft lip as its direct effect. It is considered that this finding may be associated with the developmental process of the left and right, lateral and medical nose of rat embryos.

P9 Rat Embryo Cultures In Semi Fetal Calf Serum

Atsushi YOKOYAMA[1], Masaharu AKITA[1], and Yukiaki KURODA[2]

[1]Kamakura Women's College, 1420 wase Kamakura-shi, Kanagawa 247, Japan and [2]Azabu University, 1-17-71 Fuchinobe Sagamihara-shi, Kanagawa 229, Japan

Introduction

At present, we have attempted the use of various sera for cultures of rat embryos. In the present study, we report the results of culture usung semi fetal calf serum purchased from Mitsubishi Chemical Co., Ltd.

Materials and Methods

Semi fetal calf serum produced in the Nakashibetsu cattle (PFCS), manufactured and marketed by Mitsubishi Chemical Co., Ltd., was mixed with rat serum at the ratios of 50%, 80% and 100%, and 100% rat serum was used for the control group, the rat embryo on 11.5 days of gestation old (plug day =0) was cultured for 48 hours.

Results and Discussion

After 48-hour culture, the medium group mixed with 50% PFCS showed the heart rate of 180±11 beats /min., the total number of somites of 49±1 and the crown-rump length of 7.7±0.1 mm, indicating no significant differences compared with the control group. In the medium group mixed with 80% PFCS, however, although there were no differences in the heart rate and the crown-rump length, the total number of somites showed 10% lower value than that in the control group on the average, delayed development of the frontal region of head was identified in the external morphology. From the above results, it was suggested that PFCS of Mitsubishi Chemical Co., Ltd. can be used for the culture of rat embryos on 11.5 days of gestation to replace 100% rat serum for culture embryos 24 hours before openimg of yolk sac to culture for 48-hour in medium with 50% rat serum.

P10 Rat Late Embryos Cultured In The Serum Free Medium (AKY-020)

Masaharu AKITA[1], Atsushi YOKOYAMA[1], Keiko YOSHIDA[1], and Yukiaki KURODA[2]

[1]Kamakura Women's College, 1420 Iwase Kamakura-shi, Kanagawa 247, Japan and [2]Azabu University, 1-17-71, Fuchinobe Sagamihara-shi, Kanagawa 229, Japan

Introduction

We have attempted the use for serum free medium for whole embryos culture since reports in the 9rd Japanese Society of Alternatives to Animal

Experiments. Embryo used for culture were from early to medium phase of organogenesis. In the present study, we attempted of the cultured rat embryos obtained from the late phase of organogenesis.

Materials and Methods

The rat embryos on day 12.5 of gestation (plug day = 0) was cultured for 24 hours. As the medium, the following 3 media were used: I : 100% rat serum (control group), II : 100% AKY-020, III : 80% AKY-020+ 20% Cosmedium 001 (manufactured by CosmoBio Inc.)

Results and Discussion

It was suggested that, similarly to cultures in rat serum, serum free medium AKY-020 may be used for culture of rat embryos on day 12.5 of gestation. From the above, it was considered that cultures of embryo obtained from the late phase of organogenesis might be possible without rat serum.

This research was supported in the part by Research Fund from JSAAE.

P11 The Study Of The New Method Make The Most Of Exo Utero Method

Atsushi YOKOYAMA[1] , Masaharu AKITA[1] , and Yukiaki KURODA[2]

[1]Kamakura Women's College, 1420 Iwase Kamakura-shi, Kanagawa 247, Japan and [2]Azabu University, 1-17-71 Fuchinobe Sagamihara-shi, Kanagawa 229, Japan

Introduction

We developed the method to anesthetize the dam continuously to conduct the exo utero method so as to visualize and maintain the embryo. And we compared it the whole embryo culture.

Materials and Methods

(1) In order to anesthetize the dam, administer pentobarbital i.p.. (2) Keep the dam warm to monitor the body temperature and the number of respiration. (3) Secure the blood vessel in the femoral region. (4) Open the abdomen, remove and observe uterus and perform the exo utero operation. (5) Suspend the somatic skin and soak the inside with an artificial amniotic fluid. (6) Inject and drain the artificial amniotic fluid with a micropump.(7) In the blood vessel secured in (3), install a cannula for continuous anesthesia. (8) Confirm the growth of embryo after 6 hours.

Results and Discussion

Vascular circulation and heart rate of the embryos were normal. Although there was no difference in the crown-rump length, however, an increase in the total number of somites was observed. Considering the overall morphology, prominence of the head proceeded.

P12 Establishment Of An Organ Culture System For Fetal Rat Palates

Naoki OHYAMA[1,2], Chisato MORI[2], Hiroshi NAITOH[2], Hidekazu IRIE[2], Noriko NAKAMURA[2], and Kohei SHIOTA[2]

[1]*Toxicology Laboratory, Yokohama Research Center, Mitsubishi Chemical Corporation, 1000 Kamoshida-cho, Aoba-ku, Yokohama, Kanagawa 227, Japan and* [2]*Department of Anatomy and Developmental Biology, Faculty of Medicine, Kyoto University, Kyoto 606-01, Japan*

Introduction

Recently, a novel technique was established for cultivating fetal mouse palates in a chemically-defined serumless medium(Siota et al. 1990). In the present study we tried to develop an organ culture system for the fetal rat palate which was modified after the mouse technique, because the rat is preferred to the mouse in reproductive and developmental toxicological studies. In addition, we compared the occurrence of programmed cell death, one of the significant, events in palatogenesis, in the developing palate of mouse and rat fetuses both *in vivo* and *in vitro*.

Materials and methods

The maxillary regions of day-12 and day-13 ICR mouse fetuses were dissected and cultivated in a chemically-defined serumless medium by a suspension culture technique (Shiota et al., 1990). The maxillary regions of day-14 and day-15 Wistar rat fetuses were also cultivated according to the modified method. At the end of culture, the palatal explants were observed for the condition of palatal shelf closure and the length of the palatal shelf was recorded. Some palates were serially sectioned and stained with hematoxylin and eosin. Some cases were subjected to histo-chemical examination for DNA end labeling (TUNEL) (Mori et al., 1994).

Results

The opposing palatal shelves came in contact and palatal fusion was archived in about 90% of cultured fetal mouse palates. Most of the cultured palates of day-14 rat fetuses failed to fuse, but those of day-15 fetuses closed successfully *in vitro*. The palatal fusion rate of day-15 rat fetuses cultured in 95% O_2 was better than that cultured in 50% of O_2. Addition of rat serum to the culture medium showed an inhibitory effect on palatal shelf closure. The rate of palatal closure was not different among 48 hr, 72 hr and 96 hr cultures. Programmed cell death was found at some later stages of palatal fusion both *in vivo* and *in vitro*. TUNEL-positive cells appeared at earlier stages of palatogenesis in fetal rat palates than in the corresponding fetal mouse palates.

Discussion

We established an organ culture technique for fetal rat palates which was modified after a similar method for fetal mouse palates. The result of the present study suggest that this in vitro organ culture system for fetal rat palates may be useful for studying the mechanism of normal and abnormal palatogenesis and for assessing the developmental toxicity of chemicals.

References

Mori, C., Nakamura, N., Okamoto, Y., Osawa, M., and Shiota, K.,*Anat. Embryol.*, **190**: 21-28 , 1994.

Shiota, K., Kosazuma, T., Klug, S., and Neubert, D., *Acta Anta.*, **137**: 59-64, 1990.

P13 *In Vitro* Cytotoxicity Of MEIC Chemicals On Human Cytotoxic T Lymphocytes

Tadao OHNO, Il-Kuk CHANG, Berm Soo KIM, Shu Qin LIU, Kaoru SAIJO, Midori HAYASHIDA and Mayako KATO

RIKEN Cell Bank, The Institute of Physical and Chemical Reserch (RIKEN), 3-1-1 Koyadai Tsukuba Science City, 305, Japan

To detect immunotoxicity of chemicals, effects of eight MEIC chemicals were examined on cytotoxic activity of human cytotoxic T lymphocytes (CTL) which specifically kill human squamous carcinoma (SQ-5) cells in vitro. WEIC chemicals showed apparent inhibition on CTL-mediated cytotixic activity and the activity of CTL showed decrease with increase of the chemical concentration. Result imply that induced and cultured human CTL are one of useful candidate for in vitro evaluation of chemical toxicity in humans.

This work was supported in part by Special Co-ordination Funds for Promoting Science and Technology from the Science and Technology Agency of Japan.

P14 Evaluation Of Alternative To Skin Irritation Testing: Interleukin-1a And Interleukin-8 Release By Human Keratinocyte Cell Culture Treated With Surfactance

Michiko SHIBATA, Takanari TSUDA, Hiroshi ITAGAKI and Yoshihiro MORIKAWA

Shiseido Safety and Analytical Research Center, 1050 Nippa-cho, Kohoku-ku, Yokohama 223, Japan

The effect of cosmetic surfactants on interleukin-1a (IL-1a) and interleukin-8 (IL-8) release from human keratinocyte cell culture was studied to investigate the feasibility of the irritation potential of chemicals. After exposure of cells to surfactants, the amounts of IL-1a and IL-8 released into culture medium were measured by enzyme-linked immunosorbent assay (ELISA). Cytotoxicity of surfactance was evaluated by neutral red uptake assay. At near-cytotoxic concentration of test chemicals, IL-1a release was increased 7 to 15 times. IL-8 release was increased with alkyl- amidobetaine (AAB) and polyethylene glycol fatty acid ester (PGFAE) which would not be picked up by cytotoxicity testing. After SLS treatment of cells, IL-1a release is associated with membrane damage, whereas IL-8 release continued for 24 hr, suggesting that IL-8 was produced within the cells. The patterns of IL-8 mRNA expression by RT-PCR method correlated with the results of IL-8 release measured by ELISA. These results suggest that IL-8 release by AAB and PGFAE may account for the skin irritation potential of these chemicals, of which the cytotoxicity was weak. We conclude that measurement of IL-8 release may be useful to predict the irritation potential of chemicals which would not be picked up by cytotoxicity testing.

P15 Alternative To Primary Draize Skin Irritation Test Using Three-Dimension Cultured Human Skin Model

Katsuyasu MORITA[1], Shin-ichiro MORITA[1], Hajime KOJIMA[2], Hiroaki KONISHI[2] and Hiroaki TAKASAWA[1]

[1]Kyoto Research Laboratory, GUNZE Ltd., 1 Ishiburo Inokura-Shinmachi, Ayabe city, Kyoto 623 Japan and [2]Biochemical Research Institute, Nippon Menard Cosmetic Co., Ltd., Gifu 503 Japan

Introduction

During last decade some three-dimensional cultured human skin models were proposed for an alternative to animal skin irritation test. It is important that the fittest method is chosen for using such skin models. In this study, we investigated the application period of test materials. Triton X-100 and SLS were chosen as an indices. Triton X-100 show the same cytotoxicity and eye irritancy with SLS, but weak at skin irritancy.

Materials and Methods

We made a cultured skin model which is mainly constructed from human skin cells and two types of collagen sponges. This skin model has well-differentiated epidermis and dermis. Triton X-100 and SLS were diluted with PBS. 25ml of each concentration of them were placed on applicator pad (Ø6mm) and applied to surface of skin model. The application period was tested from 1 hour to 24 hours. After application, we evaluated their cytotoxicity by MTT method.

Result and Discussion

When the application was 1 hour, Triton X-100 showed weaker toxicity than SDS. This result agree with that of skin irritation test *in vivo*. However Triton X-100 showed stronger toxicity when the period was more than 2 hours. This result contrary to that of skin irritation test *in vivo*. It means false-positive.

It is said that Triton X-100 hardly pass through corneous layer and therefore show weak skin irritancy. In the model it showed weak toxicity within 1 hour, too. On the other hand, as there is not dilution system by blood stream like *in vivo*, Triton X-100 accumulated and showed fales-positive when application period was more than 2 hours. As a result, 1 hour application will be fine for alternative to primary Draize skin irritation test. But Triton X-100 may be toxic when apply it to damaged skin or apply it long time. 24 hours application may fit in such cases.

In this study, toxicity of 10 materials and 18 cosmetic products were also assessed in this model.

P16 *In Vitro* Alternative To Phototoxicity Test

Mariko SUGIYAMA, Hiroshi ITAGAKI, Yoshihiro MORIKAWA

Shiseido Safety and Analytical Research Center, 1050 Nippa-cho, Kohoku-ku, Yokohama, 223, Japan

We have been investigating alternative methods to predict phototoxicity. In our previous report, the battery system in which the RBC photohemolysis assay and yeast growth inhibition assay were combined, was shown to be

useful for predicting phototoxic potential of new chemicals as an alternative to animal testing. However, EU/COLIPA recommended in vitro cell culture method using BALB/c 3T3cells for a candidate as an alternative to phototoxicity study in the joint validation project. In this study, we tried to evaluate cell culture method using NB1RGB cells with the modified protocol which was used in validation study under the joint management of the EU/COLIPA. Twenty-three chemicals were tested and the results of those chemicals were compared with phototoxic reactions in guinea pigs. Although the results obtained from using NB1RGB cells were well collected with those from using BALB/c 3T3 cells. two chemicals were classified as false negative in 23 chemicals.

The cell culture method seems to be a reliable and useful candidate as an alternative to animal experiment, but, in this study, we consider the battery system in more useful for predicting phototoxic potential of new chemicals.

P17 Application Of Human Oral Mucosal Model To Toxicity Testing

Ichiro MASUDA, Kazuko SATO, Michiyo TAKAKUWA and Atsushige SATO

Department of Biomaterials Science, Faculty of Dentistry, Tokyo Medical and Dental University, 5-45, 1 Cho-me, Yushima, Bunkyo-ku, Tokyo, 113, Japan

Introduction

The Oral mucosal test have been used for determining the irritation potential of dental materials. We developed *in vitro* models of human oral mucosa as an alternatives to oral mucosal irritation tests. In this study, we modified our models by reconstructing them in a Millicell for toxicological studies and investigated the effects of dental materials on keratinocytes differentiation.

Materials and Methods

Reconstruction methods of human oral mucosal models were previously described (Masuda and Sato, 1995). But in this study, slight modifications were added to the reconstruction methods. Briefly, contracted collagen gel embedding fibroblasts were fitted on the membrane of Millicell-PCF (10mm in diameter, Millipore). Keratinocytes were seeded onto the gel and cultured in KGM. After 1 day, Keratinocytes and fibroblasts were co-cultured at air-liquid interface in Reconstruction Medium composed of one part of DMEM supplemented 10% FBS and three parts of 1.5mM $CaCl_2$-added-KGM.

Denture base materials (Acron:AR), lining materials (Soft-conditioner:SC), and repair materials (Unifast:UF) were used for toxicity testing. These materials were filled to Teflon rings (7mm in diameter) as manufacturing recommendations and exposed for 24 hours to the surface of the models. Teflon rings in which noting to be filled were used as control. After 24 hours exposure, the models were fixed with 4% paraformaldehyde, and hematoxylin and eosin stained sections were investigated histopathologically. Cell viability of the models were also measured by MTT assay.

Results

After 10 days co-culture, stratified and para-keratinized epithelial layers were formed on the collagen gel in the same manner as *in vivo*.

Histopathological studies showed that loss of cell to cell contact and increased number of pyknotic cells were observed in the epithelial layer of the

model exposed to AR, SC and UF. UF affected more severely on keratinocytes and fibroblasts than other two materials.

Table 1 shows typical cell viability of the models exposed to dental materials measured by MTT assay. SC showed slightly cytotoxic effects on the models. UF was more cytotoxic than other two materials similar to histopathological damages. Although the histopathological damages were observed, AR was no effect on cell viability of the models.

Table 1 Cell viability of the methods exposed to dental materials measured by MTT assay.

	% of control
Acron	100 ± 6.9
Soft-conditioner	98.3 ± 8.5
Unifast	87.8 ± 15.4

Values are mean ± S.D..

Discussion

The models reconstructed in a Millicell posses functional properties of oral mucosa and useful for toxicity testing of dental materials in a point of reproduction of clinical use. MTT assay was not sensitive to shallow and weak cytotoxic damages of the epithelial layers because connective tissue layer were relatively thick or mitochondorial dehydrogenase acti-vity was already reduced in termi-nally differentiated keratinocytes.

Reference
Masuda, I., and Sato, A., *J. Dent. Res.* ,74: 456, 1995.

P18 The Development Of An In Vitro Screening Method To Detect Damaging Effects On Vascular Smooth Muscles. 1. Optimum Experimental Conditions And Specificity

Ikuyo KUDO, Akihiro SUZUKI, Shino MINAMIOKA, Satoko TSUKAMOTO, Seiichi WAKAMORI, Akira SONO and Youshirou KOBAYASHI

Laboratory for Toxicological Research, Institute for Life Science , Asahi Chemical Industry Co. Ltd. 632-1 Mifuku,Ohito-Cho, Tagata-gun, Shizuoka-ken 410-23, Japan

Studies designed to determine the organ specific toxicity of drugs, as well as the mechanisms for their toxic action involves the use of and the sacrificing of large numbers of experimental animals. To minimize this, an alternate *in vitro* procedure was developed for screening damaging effects on vascular smooth muscles. A7r5 cell line (purchased form Dainippon Pharmaceutical Co. Ltd) was cultured at 37°C using Dulbecco's MEM medium supplemented with 10% fetal bovine serum in an atmosphere of 5% CO_2 in air. The cells inoculated into 6-well plate were treated with test substances after a suitable preculture period. As an indicator of cell damaging effects, activities of CPK, GOT and LDH in culture medium were assayed using an automatic analyzer (model 7070, Hitachi). Protein content was measured using the MicroPCA (Pierce) method. The results showed that: (1) The number of cells increased exponentially up to 11th culture day and thereafter reached a confluent state. Protein levels paralleled this trend. (2) After reaching a confluent state, the cells exhibited the characteristic "hills and valleys growth pattern" of cultured smooth muscle cells. Electron microscopic observation indicated the development of myofilaments with increasing culture time. (3) Immuno-histochemical observation indicated that fluorescence specific to smooth muscle actine was not diminished throughout the culture period. However,

fluorescence specific to myosin diminished after 11th culture day, suggesting that the cells differentiated to a contractile type. (4) Intracellular CPK and GOT activities increased up to the 7th culture day and reached a maximum and constant level thereafter. However, LDH activity increased until day 11. In the culture medium, both CPK and LDH activities remained at a constant low level throughout the culture period but GOT activity increased even after 14th culture day. Based on the above results, the optimum preculture time was defined to be 11 days. (5) Clofibrate caused an increase in both CPK and GOT activities shortly after treatment. However, LDH activities were increased after a short lag period. No such increases were observed in cell lines which originate from tissues other than muscle, suggesting that this method is tissue-specific. Thus it suggests that this procedure may be useful for the rapid screening of substances which cause damaging effects to vascular smooth muscle.

P19 *In Vitro* Irritancies Of Masked Compounds Under The Condition Of Metabolic Activation By S9 Mixture Using Chl Cells

Shiro YUKIYAMA, Shigeo SUGIMOTO and Osamu YAMAKITA

Drug Safety Research Laboratory, Tokushima Research Center, Taiho Pharmaceuticals, Co.,Ltd. 224-2 Ebisuno, Hiraishi, Kawauchi-cho, Tokusima,771-01, Japan

Introduction

As cell lines used in cytotoxicity test lack most of drug metabolizing pathways, it is likely that the test cannot evaluate the exact irritancies of masked compounds, which are compounds that are biologically activated with internal metabolic pathways.

Materials and methods

In this study, we compared the irritancies of ifosfamide and cyclophosphamide with or without metabolic activation by S9 mixture. Simultaneously, we confirmed the irritancies of these two drugs using *in vivo* Draize test and *in vitro* EYTEX assay.

Results and discussion

CHL cell viabilities are under 10% in 0.01% concentration of ifosfamide and cyclophosphamide with the activation, while no cytotoxicities were shown in up to 0.1% concentration of the compounds without the activation. Ifosfamide and cyclophosph-amide are also classified as moderate and severe irritants by in vivo draize test, respectively. meanwhile, these two drugs were classified as no irritants by EYTEX assay. Differencies of irritancy dependent on metabolic activation by S9 mixture were shown in CHL cytotoxicity assay. These findings suggested that the present method was useful for evaluate irritancy of a masked compound.

Reference

J. H. Draize, Published by F.D.A. Officials of U.S. Business Office, Topeka, Kansas, **46** , 1959.

P20 Evaluation Of *In Vitro* Toxicity Of 32 MEIC Compounds On Human Natural Killer Cell Function

Yoshiro KOBAYASHI[1], Kouji YAMASHIRO[1], Naoko WATANABE[1], and Tadao OHNO[2]

[1] *Laboratory of Molecular Immunology, Faculty of Science, Toho University, 2-2-1, Miyama, Funabashi, Chiba 274, Japan and* [2] *RIKEN Cell Bank, 3-1-1, Koyadai, Tsukuba, Ibaraki 305, Japan*

Introduction

In vivo toxicity is generally caused by toxic effects on cellular functions that are basic to all types of cells. However, the effects on specific functions of differentiated cells in the immune system should also be evaluated, as has been done previously for cells in the central nervous system. Recently, a simple method was reported to propagate human natural killer (NK) cells from a small number of peripheral blood mononuclear cells (PBMC). Since NK cells have been shown to play important roles in protective immune responses against viruses, parasites, bacteria, and tumor cells, cultured NK cells may provide an opportunity to evaluate an important aspect of the immunotoxicity of chemicals in humans. We have already reported in vitro toxicity of 12 multicenter evaluation of in vitro cytotoxicity (MEIC) compounds on human NK cell function(Kobayashi et al. 1995). In this study, we have extended the observation to 32 MEIC compounds.

Materials and Methods

Human PBMC were separated from the venous blood of healthy donors by the Ficoll-Urograffin density gradient method (Yamamoto et al, 1993). An NK cell-rich population was obtained by coculture with mitomycin C-treated Daudi cells in the presence of 100 units/ml of interleukin-2 for 8-10 days (Perussia et al. 1987). NK cell-mediated cytotoxicity was determined by the standard method using ^{51}Cr-labeled K562 cells as target cells.

Results and Discussion

A cultured NK cell-rich population contained over 90% CD16$^+$ and/or CD56$^+$ cells, both of which are NK cells. Killing activity toward ^{51}Cr-labeled K562 cells was augmented by about 20-fold during an 8-10 day culture period. Among 32 MEIC compounds tested, twenty compounds showed apparent inhibition on NK cell-mediated cytotoxic activity, and their IC50 values were highly correlated (r=0.98) with previously reported ED50 values for the inhibition of human squamous carcinoma cell growth. In general, inhibition of NK cell-mediated cytotoxic activity was more profound than inhibition of cell growth. Some of compounds preferentially affected NK cell function, but not target cell susceptibility, as evidenced by effects of brief exposure on effector cells and target cells. Thus a cultured human NK cell-rich population will provide a unique and sensitive method for assaying an important aspect of the immunotoxicity of chemicals *in vitro*.

This research was supported by Research Fund from JSAAE and in part by the special coordinationn fund from the Science and Technology Agency.

References

Kobayashi,Y., Fukuda,Y., Nagatake, H., Watanabe, N., Wang, X., and Ohno, T., *In Vitro Toxicol.*, 8: 31-36, 1995.

Perussia, B., Ramoni, C., Anegon, I., Cuturi, M. C., Faust,J ., and Trinchieri, G., *Nat. Immun. Cell Growth Regul.* , **6**: 171-188, 1987.
Yamamoto, T., Iwasaki, T., Watanabe, N., Oshimi, K., Naito, M., Tsuruo, T., and Kobayashi, Y., *Blood* , **81**: 1342-1346, 1993.

P21 A New Functional Toxicity Tests Using Cortical Neuron Networks With Bundant Synapses In Culture

Yoichiro KURODA, Kazuo KOBAYASHI, Masahiro KAWAHARA

Deptment of Molecular and Cellular Neurobiology, Tokyo Metroporitan Institute for Neuroscience

Dissociated and cerebral cortical neurons from rat embryonic brain make many synapses in culture. The neuronal networks in culture fire spontaneously in synchronous oscillation and can be maintained for long period, up to 6 months. For an application of these long-term exposure of aluminum, a risk factor of Alzheimer disease on the cultured cortical neurons were investigated.

P22 Pharmacological And Toxicological Studies Using Chick Embryos(7)-Effect Of Cardiovascular Drugs On Electrocardiograms In Chick Embryos(2)

Kazuru SAITO[1], Takasi SUGIYAMA, Hiroyuki MIYAZAKI, Miyuki FUKUSHI and Hideyo SHIMADA

Department of Clinical Pharmacology, School of Pharmaceutical Sciences, Kitasato University and [1]Kitasato Institute Hospital, 5-9-1, Shirokan, Minatoku, Tokyo 108, Japan

Introduction

We have studied the toxicity and pharmacological effect of caldiobascular drugs using physiological techniques including ECG of chick embryos in order to push ahead the alternative methods to mammals (Sato et al., 1992). ECG recordings often failed mainly because of activation of embryos in egg shell. We have already reported that good ECG recordings can be obtained after treatment of pentobarbital(PB)(Sugiyama et al., 1994). In the present study, we compared the anesthetic effect of pentobarbital(PB), and mixture of urethane and a-chloralose(UC). Furthermore, we observed the pharmacological effect of nicorandil (NCR) on anesthetized chick embryos.

Materials and Methods

The 16th day embryos of White Leghorns were used in the series of these experiments. After a single injection of PB or UC was injected into the air sac of eggs and ECG tracings were recorded 0 to 60 min. NCR was injected into the air sac of eggs on the 16th day of incubation 20 min after injection of anesthetic. PQ, QRS and QT intervals were measured and HRs were calculated from R-R intervals.

Results and Conclusion

The stable ECG patterns were obtained from 10 to 60 min after 1mg/egg of PB or 0.1 ml/egg injection of [U(45mg)+C(450mg)] (Fig. 1) without any

changes on heat rates. However, over doses of anesthetics induced bradycardia and arrhythmia with A-V block etc. The pharmacological effect of NCR on anesthetized chick embryos did not show any differences between these two anesthesics. These result may suggest that small amount of anesthetic premedication into the air sac of fertile eggs is a very useful technic to evaluate the pharmacological effects of cardiovascular drugs.

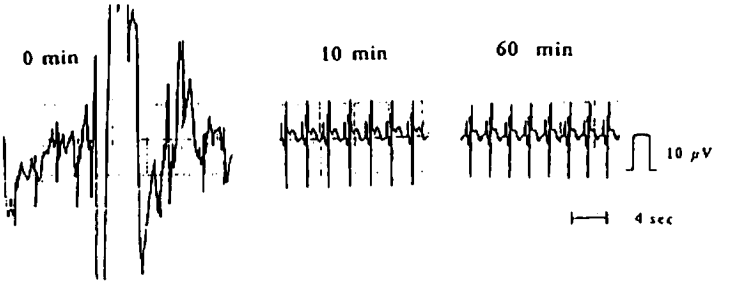

Figure 1 ECG tracings after injection of UC anestheic into the 16th-day fertile egg. The UC anesthetic at a 0.1 ml was injected into the air *sac* of egg.

References
Saito, K. et. al., *Alternative Research, Proceeding of the 6th annual Meeting of JSAAE*, pp. 90-91, 1992.
Sugiyama, T. et. al., *Can. J. Physiol. harmacol.* , **72**: 22-36, 1994.

P23 Pharmacological And Toxicological Studies Using Chick Embryos (8) - Effects Of Cardiovascular Drugs On Electrocardiograms In Chick Embryos (3)

Hiroyuki MIYAZAKI[1], Takashi SUGIYAMA[1], Kazuru SAITO[2], Miyuki HUKUSHI[1] and Hideyo SHIMADA[1]

[1]Department of Clinical Pharmacology, School of Pharmaceutical Sciences, Kitasato University, 5-9-1, Shirokane, Minato-ku, Tokyo 108, Japan. and [2]Kitasato Institute Hospital

Introduction

In order to push on the alternative method to mammals, we have been used chick embryos and got good results (Miyazaki et al., 1994a). In this study, we investigated the pharmacological effects of cardio-vascular drugs, mainly focused on acetylcholine (ACh) and atropine (ATR), using the electrocardiograms (ECGs) obtained from chick embryos.

Materials and Methods

A single injection of ACh or ATR was made into the air sac of fertile White Leghorn eggs on the 16th day of incubation. Furthermore, in order to evaluate the antagonistic effects between ACh and ATR, ATR was injected 5 min later ACh injection. ECGs were recorded using our original ECG systems(Miyazaki et al., 1994b). R-R, PQ, QRS and QT intervals of ECGs were measured, and heart rates (HR) were calculated from R-R intervals.

Results

HR decreased after ACh injection till 5 min, and after that increased gradually. The prolongation of R-R intervals were observed accompanied by prolongation of PQ, QRS and QT intervals. HR increased after ATR injection without any changes of PQ, QRS or QT intervals. After injection of ACh with 5 min later injection of ATR, HR decreased initially by the effects of ACh and then increased quite rapidly by the effects of ATR. In this group, QT intervals were shortened paralled to the HR.

Conclusion

These results suggested that ECGs obtained from chick embryos may offer the fairly good pharmacological informations in the field of cardiovascular drugs, almost equally to those of mammals.

References

Miyazaki, H. et. al. , *In vitro Toxicology*, 7: 243-246, 1994a.

Miyazaki,H.et al.,*Altenative Research*, Proc. of the 6th Annual Meeting of JSAAE),pp.131-132, 1994b.

P24 Pharmacological And Toxicological Studies Using Chick Embryos (9) - Effects Of Cardiovascular Drugs On Electrocardiograms In Chick Embryos(4)

Takashi SUGIYAMA[1], Hiroyuki MIYAZAKI[1], Kazuru SAITO[2], and Hideyo SHIMADA[1]

Department of Clinical Pharmacology, School of Pharmaceutical Sciences, Kitasato University and Kitasato Institute Hospital, Tokyo 108 Japan

We have obtained many useful information from chick embryos using physiological techniques as for screening tests of cardiovascular drugs (Sugiyama et al, 1992, 1994). Recently, proarrhythmic actions induced by anitiarrhythmic drugs have been widely noticed. It is well known that the pharmacological action of antiarrhythmic drugs, i.e. Ia, Ib, Ic, II and IV which were classified by Vaughn Williams.

Materials and Methods

Different doses of procainamide (Ia), lidocaine (Ib), aprindine(Ic), propranolol (II) and verapamil(IV) were injected into the air sac of fertile eggs on the 16th day of incubation, respectably. ECGs were recorded from 0 to 60 min after treatment of drugs. PQ, QRS and QT intervals were measured and HRs were calculated from R-R intervals.

Results

Decrease of HRs was observed dose-despondently in all drugs. When the extremely high dosage of drugs was injected, all drugs induced arrhythmias, i.e. A-V block et al. QT intervals show different values drugs to drug. Effects of various kinds of antiarrhythmic drugs to the chick embryos were discussed using ECG systems.

References

Sugiyama, T. et.al., *ΑΛΤΕΧ*, **2**: 84-85, 1992

Sugiyama, T. et.al., *J.J.P.(suppl.)*, **64**: 228, 1994

Drugs	Class	Qualitative changes in ECG parameters				
		HR	RR	PQ	QRS	QT
Procainamide	I a	⇩⇩	↑↑	↑↑	↑↑	↑↑
Lidocaine	I b	⇩⇩	↑↑	↑	→	↑
Flecainide	I c	⇩⇩	↑↑	↑↑	↑↑	↑
Propranolol	II	⇩⇩	↑↑	→	→	↑
Verapamil	IV	⇩	↑	→	→	→

↑↑:significant prolongation ↑:prolongation, ↓:shorting, →:no change
⇩⇩:decrease

Table I Changes in ECG parameters in chick embryos treated with antiarrhytmic drugs

P25 Detection Of Phototoxic Compounds Using Cultured Cell- Expression Of Genotoxicity And Phototoxicity

Shinobu WAKURI, Yuzuki NAKAGAWA, and Noriho TANAKA

Hatano Research Institute, Food and Drug Safety Center, Hatano, Kanagawa, 257 Japan

The neutral red(NR) assay using BALB/3T3 cells with UV irradiation is a good *in vivo* alternative method to the phototoxicity test in animals. It is considered that some phototoxic compounds may generate genotoxicity when the cells are irradiated with UVA. In order to establish the assay systems for photogenotoxic compounds, we studied the phototoxicity and the expression and enhancement of genotoxicity with quinolone compounds which have been suggested to induce phototoxicity and generation of reactive oxygens.

Seven quinolone compounds were used in this study. The NR assay was employed for detecting phototoxicity. The mouse lymphoma, L5178Y $tk^{+/-}$-3.7.2C assay (MLA) and the comet assay were used to detect genotoxicity in the presence and absence of UVA.

The quinolone compounds except naldixic acid showed cytotoxicity at concentrations producing precipitate in the NR assay under UV non-irradiation. However, they showed cytotoxicity under UV irradiation, and IC50 values at 0.025-0.075 mg/ml. Ofloxacin also showed cytotoxicity (20% surviving) just after treatment at MLA under UV irradiation. The results of MLA will be presented. In comet assay, the interaction between the treatment with quinolone compounds and UV irradiation are under investigation.

P26 Chemical Models For Cytochrome P450 As An Alternative Of Metabolic Activation System In Mutation Assay

Keiko INAMI, Eriko OKOCHI and Masataka MOCHIZUKI
Kyoritsu College of Pharmacy, Shibakoen 1-5-30, Minato-ku, Tokyo 105, Japan

Introduction

Many carcinogens damage DNA and then show mutagenicity and carcinogenicity. Ames test is a short term screening of carcinogens, and many carcinogens require enzymatic activation through oxidation by cytochrome P450, which metabolize promutagen to active form in mutation assay. Iron(III)porphyrin and oxidant catalyzed the oxidation mimicking cytochrome P450.The activation of N-nitrosodialkylamines with a chemical model, tetrakis (penta-fluorophenyl)porphyrinatoiron (III) chloride (Fe(F5P)Cl) and tert-butyl hydroperoxide (t-BuOOH) reproduced the enzymatic meta-bolic pathway of N-nitrosodialkylamines giving the corresponding alcohols and aldehydes as their products(Okochi et al., 1995a).

Materials and Methods

A bacterial mutation assay was based on the Ames test, and an iron (III) porphyrin and an oxidant were used for metabolic activation as a substitute for S9 mix. The bacterial strain used were Salmonella typhimurium TA1535, a sensitive strain to base-pair change mutagens, Salmonella typhimurium YG7108, a more sensitive strain to alkylating agents because of its deficiency of O6-alkylguanine-DNA alkyltransferase, and Salmonella typhimuri- um TA1538, a sensitive strain to frameshift mutagens. The mutagens used were N-nitrosodialkylamines (alkyl = methyl ; NDM, ethyl ; NDE, propyl ; NDP, butyl ; NDB), 2-aminofluorene (2-AF), benzo(a)pyrene (B(a)P) and a tryptophane pyrolysate (Trp-P-2).

Results and Discussions

N-Nitrosodialkylamines appeared mutagenic with Fe(F5P)Cl/t-BuOOH as a substitute for S9mix. NDP and NDB were mutagenic in Salmonella typhimurium TA1535, and the mutagenicity increased by pre-incubation and a higher concentration of mutagens. All the N-nitrosodialkylamines were mutagenic using the chemical model in Salmonella typhimurium YG7108(Okochi et al, 1995b) Other carcinogens, 2-AF, B(a)P and Trp-P-2 were mutagenic with Fe(F5P)Cl/t-BuOOH in Salmonella typhimurium TA1538, and their mutagenicity was easier to detect than that of N-nitrosodialkylamines. The effect of solvent was investigated and the mutagenicity was in the order ; CH3CN, 1,4-dioxane, DMF, H_2O and CH_3OH. meta-Chloroperoxybenzoic acid (mCPBA) was also effective as an oxidant to activate the mutagens. Thus, the chemical model oxidized and activated carcinogens in mutation assay as an alternative of metabolic activation enzymatic system.

References

Okochi, E. and Mochizuki, M., Dealkylation of N-Nitrosodialkylamine by Metalloporphyrin/Oxidant Model System for Cytochrome P450, *Chem. Pharm. Bull.*, **43**: in press, 1995a.

Okochi,E.,Namai,E.,Ito,K.,and Mochizuki,M., Activation of N-Nitrosodialkyl-amines to Mutagens by a Metalloporphyrin/Oxidant Model System for Cytochrome P450, *Biol. Pharm. Bull.*, **18**: 49-52, 1995b.

P27 Prediction Of *In Vitro* Drug Metabolism In Human From *In Vitro* Data Using Expression System Of Human P-450 Isozymes

Takafumi IWATSUBO[1], Kan CHIBA[2], Takashi ISHIZAKI[2], Noriaki SHIMADA[3], Carol E. GREEN[4], Charles A. TYSON[4], and Yuichi SUGIYAMA[5]

[1]*Yamanouchi Pharmaceutical Co., Ltd.*, [2]*International Medical Center of Japan*, [3]*Daiichi Pure Chemicals Co., Ltd.*, [4]*SRI International*, [5]*Department of Phamaceuticals, Faculty of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokto 113, Japan*

Interspecies differences in hepatic drug metabolism make it difficult to predict *in vivo* drug metabolism in human from animal data. As an alternative way, we have proposed the method for predicting *in vivo* drug metabolism from *in vitro* data using isolated hepatocytes or hepatic microsomes in humans, and reported that this method may be possible based on literature data. However, it is difficult to get human liver samples timely when *in vitro* experiments should be made. Then we examines the possibility for applying the expression system of human P-450 isozymes to predicting *in vivo* drug metabolism in human. The drug metabolic clearance obtained from in vivo expression system is comparable to tha obtained *in vivo* when the contents of each P-450 isozyme in both systems were taken into consideration, suggesting that the expression system of human P-450 isozymes can be a useful tool to predict *in vivo* drug metabolism in human.

P28 Toxicological Study Of The Three-Dimensional Cultured Skin Model By Surfactant

Eiji MORI[1], Masagaru OHBAYASHI[2], Hajime KOJIMA[1] and Hiroaki KONISHI[1]

[1]*Biochemical Research Institute, Nippon Menard Cosmetic Co., Ltd., and* [2]*Institute of Applied Biochemistry*

Introduction

Results with 3-dimensional cultured skin model (3-D model) have shown a high correlation with Draize primary skin irritation test finding. However, there are seen chemicals that did not exhibit correspondence between the results of 3-D model and *in vivo* animal testing (Miyamoto et al., 1994). We therefore compared the s-D model with an in vivo animal test of surfactants, applying a histological approach.

Materials and Methods

We performed MTT assays and a histological study (H and E staining) using the 3D-model "3D-Histoculture". We tested sodium lauryl sulfate (SLS) as an anionic surfactant, benzethonium chloride (BC) as a cationic surfactant and t-octylohenoxypoly-etoxyethanol (Triton X-100) as a nonionic surfactant, and compared the results with of Draize primary skin irritation testing (Appraisal of the Safety of Chemicals in Foods, 1959) in rabbits and guinea pigs.

Results and Discussion

In the *in vivo* irritation tests, SLS and BC demonstrated the high irritation, whereas Triton X-100 was without effect. In the MTT assay using the high cell toxicity. The results of Triton X-100 did not correspond with the results between the *in vivo* irritation tests and the 3-D model.

Histological study in the *in vivo* animal test revealed marked degeneration and pronounced cellular infiltration and necrosis in the SLS and BC cases,

while Triton X-100 produced slight cellular degradation. In the 3-D model, SLS and BC caused marked cellular infiltration and necrosis, and Triton X-100 produced slight cellular infiltration. Thus in the case of histological finding, a good correspondence between the *in vivo* irritation test and the 3-D model was found for all three surfactants.

In conclusion, prediction of Draize primary skin irritation test results may require histological study using a 3-D cultured skin model.

References

Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics, by the Staff of the Division of Pharmacology Food and Drug Administration, pp. 46, Department of Health Education and Welfare, F.D.A. officials of U.S. Business Office, Topeka, Kansas, ed. by Draisze, J.H., 1959.

Miyamoto, S., Mori, E., Kojima, H., Sasaki, T., Manabe, S., Tannno, K., and Konishi, H., Alternatives Research, Proceeding of the 8th Annual Meeting of the Japanese Society for Alternatives to Animal Experiments, pp.97-98, 1994.

P29 Correlation Between Fluidity Of Human Epidermal Lipids And Irritation Potential Of Anionic Surfactants - Investigation With Electron Resonance Spectroscopic (ESR) Method

Yoshiaki KAWASAKI[1,2], Danyi QUAN[2], Yoshinobu TAKINO[1], Miyuki OHNUMA[1], Kazutami SAKAMOTO[1], and Howard I. MAIBACH[2]

[1]Central Research Laboratories, Ajinomoto Co., Inc., Japan and [2]Department of Dermatology, University of California, San Francisco, USA

An electron spin resonance (ESR: 5-doxyl strearic acid (5-DSA) as the spin labels) study was performed to investigate the effect of anionic surfactants on the lipid bilayers of human stratum corneum. An increase of fluidity of skin lipid bilayers suggested that a decrease of skin barrier function according to the ESR spectral data and human patch test. ESR technique may provide a facile and robust method to define not only subclinical irritancy potential of anionic surfactants and other materials but also interaction between cell and chemical materials.

P30 Analysis Of Scoring-Variance In Draize Test As An Evaluation Standard For The Alternative Method

Junko KUMAKAWA, Toyozo KANEKO, Junko MOMMA, Akihiko HIROSE, Yasushi KAWASAKI, Sachiko SUZUKI, Tohru INOUE and Yuji KUROKAWA

Division of Toxicology, National Institute of Health Sciences, 1-18-1 Kami-Yoga, Setagaya-ku, Tokyo 158, Japan

We have analyzed the variance of Draize Score for establishing the standardization of the database for the alternative method. Three strains were used ; Std:JW/CSK, Std:NZW and Fb:JW/CSK. A question as to which has been more contributing the scoring-variance either strain differences, or personal differences of each testing individual, were analyzed. The coefficient variance (after 6 days), possible due to different observers, were 1.31, 1.17 and 0.57 for the concentration of Sodium Lauryl Sulfate (SLS) at 30%, 10% and 3%, respectively. On the other hand, individual differences seen in each

testing animal were prominent at a high concentration group in each testing substance ; namely the coefficient of variance after 6 days, were 0.75 in 1% Benzalkonium chloride, 0.81 in Cetyl Pyridinium chloride, and 1.22 in 10% SLS. We conclude that scoring difference among the testing animals. Also, it is of importance that, because of lesser variances, the substances scored as moderately irritating (more than score 25) are required to be focused on the score at 24 hours after testing, although, the mean maximum score was appeared to be maximum at 48 hours.

P31 Difference Of Toxicity Result The Material Extraction Condition In Cytotoxic Test Of Biomedical Material

Minoru TAKIZAWA, Masuo KOYAMA, and Toshihiko TAKAHASHI

Medical Products Plant, Hakka Electric Machine Works, Co., Ltd.
3055 Togura, Togura-machi, Hanishina-gun, Nagano-pref., 389-08, Japan

Introduction

The guideline of the biological assay of the medical devices and the biomedical material was formally provided for in July this year in Japan. The cytotoxic test is indispensable to all medical devices and important for considering the safety of the biomedical material. It is provided to use colony formation assay and use the medium to which the serum is added in the guideline. However, the serum is an uncertain element of the toxicity test. Then, we tested with a medium with the serum and a medium not so to clarify the importance of the serum on the material extracting condition and the results were compared.

Materials and Methods

Test materials are the PTFE tube, the PVC tube, the PU tube and the Wire Solder (Sn-Pb). The materials are extracted with medium (1g: 10ml) for 24 hour at 37° in CO_2 incubator. Three kinds of medium are used for extraction. (1) MEM+5%FBS (MO5), (2) MEM (without serum), (3) Serum Free Media (ASF301, Aji-no-moto). 5% FBS is added in No.2 MEM (without serum) after extraction. The extraction medium is diluted several concentration and used for experiment by colony formation assay (V79 cells were used).

Result and Discussion

In the experiment which used PVC, it was shown a stronger cytotoxicity when extracting with the MO5 medium which the serum entered than the other medium without serum. On the other hand, the Wire Solder (Sn-Pb) showed a stronger cytotoxicity when extracting with the ASF301 medium than the other medium. As a result, it was able to be guessed that the existence of the serum in the material extraction condition was important. Especially it was suggested that the serum be necessary to elute monomer of the organic system and a poisonous material of the additive etc. from the high molecule material. On the contrary, the metallic ion combine with protein of the serum. So it was suggested that the cytotoxicity become lower than the original toxicity on the inorganic material by existence of serum. Therefore, it was concluded now that the Serum Free Media was not able to be used in this experimentation system.

P32 Studying Usefulness Of Anti-Cancer Drug Sensitivity Test Using Collagen Gel Droplet Embedded Culture And Image Analysis (CD-DST)

Keizo TANISAKA[1], Hisayuki KOBAYASHI[1], Masahiro KOEZUKA[1], Osamu DOI[2], Toshihiko TAKI[3], Satoshi HARA[4]

[1]Res. Lab., Nitta Gelatin Inc.,2-22 Futamata, Yao, Osaka 581, [2]Dept. Surg.,Centr. Adult Dis., Osaka, [3]Dept. Breast Surg., Kitano Hosp., [4]1st Dept. Surg., kinki Univ. Sch. Med.

We developed a new *in vitro* assay for chemosensitivity testing using collagen gel droplet embedded culture and image analysis (CD-DST method). This method has the characteristics of being able to assay with small numbers of cells, suppression of the growth of contaminated fibroblast cells with sufficient growth of cancer cells by using a serum free medium, and determination of only the cancer cells as result of eliminating the fibroblast cells on image by an image analyzing technique.

In comparison with *in vivo* nude mouse assay using human cancer cell lines, this method showed a complete correlation both in true positive and true negative rates. The in vitro accumulation response rates of MMC, CDDP, VDS and VP-16 for primary lung cancers and of MMC, 5-FU and ADR for primary breast cancers were similar respectively to the clinical response rates.

These results suggest the new method (CD-DST) would be able to contribute to cancer chemotherapy studies.

References

Kobayashi, H., Tanisaka, K., Kondo, N. et al., Development of new *in vitro* chemosensitivity test using collagen gel droplet embedded culture and its clinical usefulness, *Jpn. J. Cancer Chemother*, **22**: 1933-1939, 1995.

Koezuka, M., Kondo,N., Kobayashi , H. et al., Drug sensitivity test for primary culture of human cancer cells using collagen gel embedded culture and image analysis, *International Journal of Oncology*, **2**: 953-959, 1993.

P33 Development Of Drug Metabolism Simulator For Alternatives To Animal Experimentation Using 3-Dimensional Culture Of Hepatocytes

Kohji NAKAZAWA, Taku MATSUSHITA and Kazumori FUNATSU

Dept. of Chem. Eng., Kyushu Univ. 6-10-1 Hakozaki, Higashi-ku, Fukuoka 812, Japan

Introduction

As an alternative to animals experimentation for development of new drugs, primary culture of hepatocytes are currently used to study various aspects of the metabolism of new drugs, because the liver is a principal organ for drug metabolism. For development of a drug metabolism simulator using primary hepatocytes, it is important to maintain the function of hepatocytes *in vitro*. But the functions of the usual monolayer (2-dimensional) cultured hepatocytes rapidly decrease in a week. We have already reported that by 3-dimensional culture of rat hepatocytes (spheroid) culture using polyurethane foam (PUF) as a culture substratum, hepatocyte specific functions such as albumin production and ammonia detoxification can be maintained for more than 2 weeks *in vivo* (Ijima et al., 1993). In this study, we investigated about the development of a drug metabolism simulator using PUF/spheroid culture system.

Materials and Methods

We used lidocaine (anesthetic drug) as a model drug. Primary rat hepatocytes were prepared from male Wister rats (7 weeks age) by the collagenase liver perfusion method. Lidocaine metabolic reaction were performed by monolayer and PUF/spheroid stationary culture systems. The drug metabolites were analyzed by GC/MS, and viable cells and nuclei numbers were measured by the MTT method and the nuclei counting method, respectively. We developed the simulator as *in vitro* liver model for continuous measurement of drug metabolism using PUF/spheroid packed-bed, and continuous lidocaine metabolism were measured.

Results and Discussion

The rat hepatocytes converted lidocaine to monoethyglycinexylidide (MEGX) which was N-deethylation of lidocaine. The metabolic activity of hepatocytes/spheroid was 1.5- to 2.0-fold higher than that of monolayer in stationary culture for 10 days.

The activity of albumin production and viability of cultured hepatocytes in monolayer and spheroid decreased by lidocaine treatment with depending on the lidocaine concentration. But the activity and viability in PUF/spheroid culture system were maintained at higher level than that in monolayer culture under the lidocaine treatment which seems to be resulted from the high metabolic activity of spheroid.

The metabolic activity of the simulator using PUF/spheroid packed-bed culture module was about 10-fold higher than that of PUF/spheroid stationary culture. This phenomena seems to be resulted from better mass transfer of oxygen and nutrient between cell and medium in the device than that of stationary culture.

Consequently, PUF/spheroid 3-dimensional culture seems to provide a promising culture system for development of drug metabolism simulator.

Acknowledgment

This study was partly supported by Japanese Society of Alternatives to Animal Experiments (1994).

Reference

Ijima, H., Matsushima , T., and K Funatsu, K., *Japanese Journal of Artificial Organs*, **22** : 171-176, 1993.

P34 The Study Of Cytotoxicity Of Amino Acid Type Amphoteric Surfactants

Takako CHIKUMA, Masahiko SHIMADA, Noriko TAKE, Kyu TAKEUCHI, Hitoshi KOYASHI, Motoo KOYAMA

NOF CORPORATION, Olcochemical Research Laboratory

In this study, three types of alternative methods, NR, WST-1, and LDH, were applied for the evaluation of cytotoxicity of several surfactants including amino acid type amphoterics which are utilized in personal care products such as shampoo. Membrane fluidity change of human epidermal keratinocytes by the surfactants was also detected by the polarized light measurement of probe molecule in the cell membrane. LIDA (lauryl imino diacetate) showed the lowest cytotoxicity in each of three methods. The membrane fluidity was

scarcely changed for LIDA, whereas the other surfactants showed obvious increase of the fluidity. The little change of fluidity may be due to LIDA's low permiating character into the cell membrane. This low permiating character could be caused by LIDA's chemical structure having two polar groups; carboxy ethyl groups attached to nitrogen atom.

P35 Development And Application Of Simulation Program For Prediction Of Tissue Distributions Based On Physiological Pharmacokinetics (2)

Shunichi FUKANO

Department Toxicology The Tokyo Metropolitan Research laboratory of Public Health, 3-24-1, Hyakunincho, Shinjuku-ku, Tokyo 169, Japan.

Computer simulation as means of alternatives to animal experiments and testings is not still generalized, because of the complications to get the additional knowledge of computer and mathematics. Then, the simulation program for the prediction of tissue distribution of xenobiotics (*PPP*) was developed using a physiologically based pharmacokinetic model. *PPP* works on Microsoft Windows 3.1 and the simulation curves are readily obtainable without any knowledge of programming and mathematics. *PPP* has the following features: Various physiological models are displayed according to the physiological data (partition coefficient, blood flow-rate and volume of each tissue) and other parameters (dose-position, dose-method, free fraction of blood, clearance, Km Vm,etc.). The simulation curves are calculated automatically. Users need not input differential equations. The graphical section displays the time-courses of the contents, the concentrations, the experimental data and the half lives, and Area Under the Curve (AUC) of each tissue.

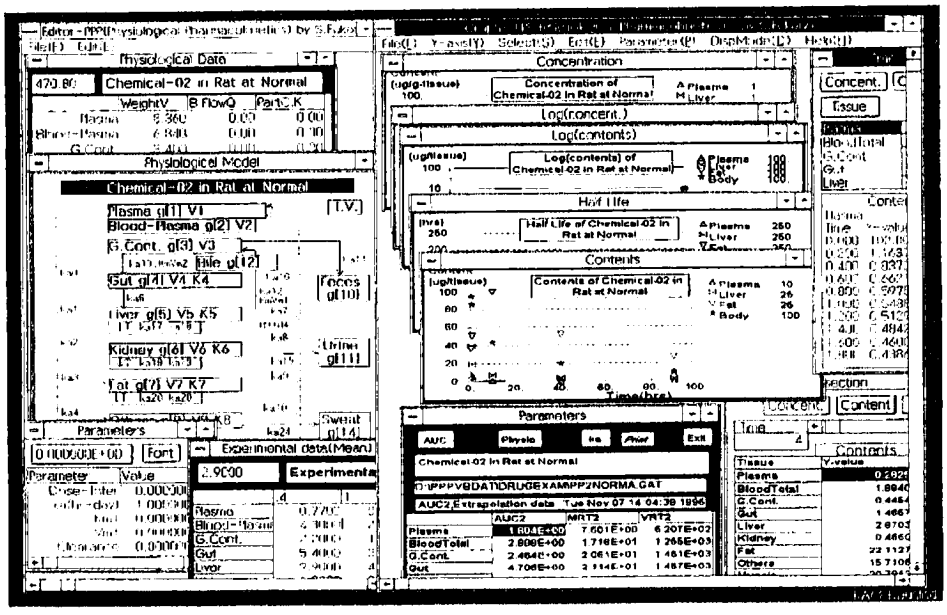

Figure 1 View of *PPP*.

The experimental planning using the simulation is effective for animal testing, because this can save time and money. *PPP* is easy to use and useful tool for pharmacokinetic simulations and would be able to reduce the number of animals for experiments.

A part of this study was supported by the Research Grant from the Japanese Society of Alternatives to Animal Experiments.

References

Pharmacokinetics (Introduction and Application volumes), Ed. by Hanano, M., Nanzando, Japan, ,1987 and 1988.

Yamaoka, K., and Tanigawara,Y., Introduction to pharmacokinetics by micro computer, Nankoudou, Japan, 1983.

Mills, J. J. , and Andersen, M. E., Toxicokinetics of Dioxin and Related Compounds, Proceeding of the Workshop on Risk Assessment and Risk Management of Toxic Chemicals , National Institute for Environment Studies (Japan), pp. 102-110, 1992.