PROCEEDINGS OF THE 4TH ANNUAL MEETING OF JAPANESE SOCIETY FOR ALTERNATIVES TO ANIMAL EXPERIMENTS

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SPECIAL LECTURE

THE DEVELOPMENT, VALIDATION AND REGULATORY ACCEPTANCE OF NON-ANIMAL TOXICITY TESTS : A EUROPEAN PERSPECTIVE

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The origins of the Three Rs (reduction, refinement, replacement) concept of alternatives will be reviewed, and the significance of its incorporation into national (e. g. UK) and international (e. g. EEC) legislation will be discussed. For example, with regard to replacement alternatives, the important European Directive 86／609, which is binding on all Member States of the EEC, requires that "*an experiment shall not be performed if another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available*". This type of legislation now places a moral obligation on scientists and others to actively support the development, validation, acceptance and employment of replacement alternative methods wherever possible. Emphasis will be placed on the importance of collaboration between scientisis both in academia and in industry, and in different countries, and some lessons learned from experience in the FRAME Research Programme and in ERGATT (European Research Group for Alternatives in Toxicity Testing) will be described.

Two speciffic topics will then be dicussed in detail. Firstly, the conclusions and recommendations of two ERGATT workshops held earlier in 1990 on the validation of non-animal toxicity tests (in collaboration with the CAAT, Johns Hopkins University, Baltimore, MD, USA) and on the promotion of their regulatory acceptance (in collaboration with the EEC) will be outlined. The full reports of these workshops are to be pulished in the October 1990 issue of FRAME's scientific journal, *ATLA (Alternatives to laboratory Animals)*.

Secondly, the contribution of the FRAME Research Programme to the search for alternatives to animals in acute lethal toxicity tests and in the Draize eye and skin irritancy tests will be discussed. In particular, progress regarding the integrated use of the three FRAME methods, namely, the kenacid blue (KB) cytotoxicity test, the neutral red release (NRR) test, and the fluorescein leakage (FL) test, together with the Ropak EYTEX, as components of a battery of

methods designed to replace the Draize eye test, will be outlined.

The KB method, which normally involves a 72-hour exposure period, is a cell proliferation inhibition test, in which binding of the blue dye to total cellular protein is used as an indicator of cell number. The test provides infomation on the intrinsic toxicities of chemicals, which correlates with LD50 values for certain classes of compounds. The KB ID50 value (the amount of test material which reduces the total cellular protein content to 50 % of that of appropriate control cultures) is used as a base-line for comparison with data produced in tests designed to provide information on the other kinds of toxicity. The NRR method involes exposure of cells pre-loaded with the vital dye to very high concentrations of test materials for 1 minute, and is designed to provide information on potential eye irritancy. The method has performed very well in trials organised under the auspices of the EEC and the US Cosmetic Toiletry & Fragrance Association. The FL method is a combination of the NRR method and Tchao's fluorescein permeability assay. It involves loss of the trans-epithelial impermeability of MDCK cells and is being used in studies on penetration of cellular barriers and recovery from damage. Monolayers of cells are grown Anotek filters in multi-well dishes to from a monolayer with tight junctions. If a chemical causes sufficient damage to the cellular barrier, fluorescein will pass across it. After exposure, the cells are returned to fresh medium, and retested for impermeability after a recovery period. The EYTEX method is based on the tendency of irritants to denature proteins, and is designed to provide a prediction of eye irritancy which is equivalent to the 24-hour, 0.1 ml Draize eye test score. These methods are currently being used, *inter alia*, in collaboration with a number of cosmetics manufacturers and retailers, to test new ingredients in new formulation design and to investigate the problems which sometimes arise, even after products have gone to the market.

SYMPOSIUM

"DEVELOPMENT AND VALIDATION OF ALTERNATIVES TO ANIMAL TESTING AND EXPERIMENTS"

S-1 VALIDATION PROCEDURES OF AN ALTERNATIVE METHOD FOR LD 50-TEST: ACUTE TOXICITY TESTING BY FIXED DOSE METHOD OF ITS PROTOCOL

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International collaborative study was organized by Steering Committee in UK and performed by 31 laboratories out of 12 countries to validate a fixed dose method for acute toxicity testing which was originally proposed by British Toxicology Society (BTS) in 1984 as a more humane procedure for toxicity classification of substances than traditional LD_{50} test. The procedures for validation, i. e. selection of chemicals, function of the steering group and evaluation of the results, are discussed.

S-2 VALIDATION OF ALTERNATIVES FOR TOXICOLOGY TESTING

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Validation is the process by which the credibility of an alternative test is established for a specific purpose. It consists of two components:reliability and relevance. Reliability means that test provides precise data which can be replicated both in the same laboratory over time and among laboratories. Relevance means that the data provided can be used, with confidence, in making decisions concerning the potential toxicity of chemicals and formulations. Several research programs have been conducted in the USA to evaluate existing alternative methods. Although none of these programs constitutes a full scale validation study, each one illustrates certain aspects of the validation process. These research programs will be discussed and their conclusions described. Understanding the validation process is critical to successfully transferring alternative testing methods from the research laboratory to the practical world of chemical safety testing.

S-3 DEVELOPMENT OF ALTERNATIVES FOR THE DRAIZE TESTING AND ITS VALIDATION

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The Draize occular irritancy test was compared with cytotoxicity determined from colony forming ability and neutral red uptake in cells freshly isolated from rabbit cornea and human epidermal keratinocytes. We used 52 chemiclas used in consumer products as test agents. There was a close correlation between the cytotoxicity in cultured cells *in vitro* and the Draize score *in vivo* in response to the 52 chemicals. With a few exceptions, the cationic detergents appeared to be more toxic than the anionic or nonionic compounds tested. These data suggest that the cytotoxicity test *in vitro* using primary cultured cells may be useful as a substitute for the Draize eye irritancy test. These alternatives were reviewed on the basis of scientific merit, mechanisms and the ability to represent ocular injury *in vivo* by JSAAE. These methods were also evaluated in 12 chemicals by 13 laboratories. These results of this study will be presented.

SESSION IN DEAPTH

CARCINOGENESIS ASSAY BY *IN VITRO* METHOD

SD 1 FUNDAMENTAL PROBLEMS OF *IN VITRO* TRANSFORMATION ASSAY

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So for, 2 experimental methods are available as the quantitative in vitro transformation assay. The one is the colony formation method using Syrian hamster embryo cells at low passages. The other is the focus formation method using BALB/c 3T3 cells or C3H10T½ cells. These methods are excellent for analyzing the mechanism of transformation, but not necessarily good for an alternative routine method predicting carcinogenicity of chemicals. Here, I introduce our experience on these transformation methods, and discuss about their problems and the measure for establishing as an alternative carcinogenicity test.

SD 2 DEVELOPMENT OF AN *IN VITRO* CELL TRANSFORMATION MODEL IN SYRIAN HAMSTER EMBRYO CELLS FOR CARCINOGEN DETECTION AND MECHANISTIC STUDIES

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The Procter and Gamble Co.

Research in our laboratory has focused on the development of a predictive and biologically relevant in vitro cell transformation model for assessing the carcinogenic potential of chemicals. Early passage, Syrian hamster embryo (SHE) cells were plated at a clonal density, were treated with chemical and then evaluated for preneoplastic change following seven days of clonal growth. In studies designed to optimize clonal proliferation and transformation, we found that carcinogen-induced morphological transformation (MT) occurs more reproducibly and with higher frequency when SHE cells are cultured at pH 6.70 in a modified Dulbecco's modified Eagles medium compared to the historically used pH 7.30. In addition to several structurally diverse "genotoxic" carcinogens, chemicals that are believed to be carcinogenic via "non-genotoxic" mechanisms also caused a significant increase in MT at pH 6.70 whereas non-carcinogens did not. Cells from pH 6.70 *MT* colonies were shown to progress in a step-wise manner to the neoplastic phenotype. Associated with this progression were defined phenotypes including immortality and anchorage-independent growth as well as specific karyotypic changes. These results indicate that pH 6.70 transformation of SHE cells is a promising model for both carcinogen detection and mechanistic studies.

SD 3 DETECTION OF NON-MUTAGENIC CARCINOGENS USING CULTURED SYRIAN HAMSTER EMBRYO CELLS

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The Syrian hamster embryo (SHE) cell transformation assay has been used for mechanistic studies of carcinogenesis and as a screening test for the potential carcinogenicity of chemicals. The assay has a high predictive value (90.8 %) for the detection of carcinogens, including a number of non-mutagenic carcinogens (Pienta *et al.*, 1977). To validate the assay further, we examined the abilities of 17 chemicals, which were negative in the *Salmonella*/microsome assay, to induce morphological transformation of SHE cells.

We used the replating method for the SHE cell transformation assay. Cells (2.5×10^5) in tertiary cultures were plated on 75-㎠ flasks and, after overnight incubation, treated with the chemicals for 48 hr. Following trypsinization, the cells were replated on 100-㎜ dishes at the density of 2,000-3,000 cells per dish and allowed to form colonies for 7 additional days. Table 1 shows the results of experiments. Eleven of the 17 chemicals tested are reported to be carcinogenic in rodents (no data are available for 4 chemicals). Ten of the eleven chemicals (exception was clofibrate) induced morphological transformation of SHE cells. SHE cell transformation responses for other chemicals by other investigators are listed in Table 2. All chemicals excluding phenobarbital and TCDD, showed a positive transforming activity in agreement with the rodent carcinogenicity data. These results indicate a good correlation between induction of SHE cell transformation and carcinogenicity, suggesting that the SHE cell assay system could be useful for mechanistic

Table 1 Ability of 17 chemicals, which are negative in *Salmonella* mutagenesis, to induce morphological transformation of cultured Syrian hamster embryo cells.

Chemicals	*Salmonella* mutagenesis	Morphological transformation	Carcinogenisity
Amitrole	−	+	+
Asbestos	−	+	+
Benzene	−	+	+
Clofibrate	−	−/−[a]	+
Colcemid	−	+	?
Di (2-ethylhexyl)-phthalate (DEHP)	−	+/++[a]	+
Diethylstilbestrol	−	+/++[a]	+
17β-Estradiol	−	+	+
Eugenol	−	+	±
Mono (2-ethylhexyl)-phthalate (MEHP)	−	−/+[a]	?
Progesterone	−	+	+
Na-Arsenite	−	+	+
Na-Bisulfite	−	+	?
Na-Fluoride	−	+	E[b]
Testosterone	−	+	+
Vincristine sulfate	−	+	?
Wy-14,643	−	−/+[a]	+

[a] Results from experiments without/with rat liver mitochondrial supernatant.
[b] Equivocal

Table 2. Transforming activity, which has been reported by others, in SHE cells induced by treatment with chemicals negative in *Salmonella* mutagenesis.

Chemicals	*Salmonella* mutagenesis	Morphological transformation	Carcinogenisity
5-Azacytidine	−	+	+
Bis (p-dimethylamino)-diphenylmethane	−	+	+
Diphenylhydantoin	−	+	+
Ethionine	−	+	+
Lead acetate	−	+	+
Methapyriline•HCl	−	+	+
Reserpine	−	+	+
Thioacetamide	−	+	+
Thiourea	−	+	+
O-Toluidine•HCl	−	+	+
Urethane	−	+	+
Phenobarbital	−	−	+
2, 3, 7, 8 -Tetrachloro-dibenzo-p-dioxin (TCDD)	−	−	+

studies and detection of potential carcinogenic activity of non-mutagenic carcinogens. The possible involvement of genetic mechanisms in cell transformation by these chemicals will be discussed.

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SD 4 MORPHOLOGICAL TRANSFORMATION INDUCED BY X-RAYS IN SYRIAN／GOLDEN HAMSTER EMBRYO CELLS

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We investigated the induction of morphological transformation in Syrian／golden hamster embryo cells irradiated with X-rays. At lower doses (0-2 Gy), the frequency of morphological transformation increased steeply with increasing dose. With doses above 2 Gy, the frequency increased but only slightly. Compared with morphological transformation, the expression of mutation required an expression time more than 5 days and the dynamics of expression of both phenotypes were somewhat different. It was suggested that different mechanisms might be responsible for the expression of morphological transformation and mutation.

A large fraction of morphologically transformed colonies (about 90%) could be cloned with the use of feeder layer cells. Only the progeny of these morphologically transformed clones expressed malignant phenotypes, such as the acquisition of anchorage-independence and of tumorigenicity.

From karyotypic analysis, all morphologically transformed clones showed trisomy of chromosome 11, consistently. Furthermore, chromosome changes associated with chromosome 3 was observed in all tumor-derived cells. These results suggested the involvment of the specific chromosome change in the expression of each transformed phenotypes.

PLATFORM AND POSTER PRESENTATION

ALTERNATIVE TO BREAST CARCINOGENICITY TEST USING A TISSUE CULTURE METHOD 2. *IN VITRO* MICRONUCLEUS TEST FOR A SCREEN OF BREAST CARCINOGENS

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We developed a new *in vitro* micronucleus test using cultured mouse mammary epithelial cells as a tissue-specific screen for suspected beast carcinogens.

Mammary epithelial cells from pregnant mice were cultured in serum-free Ham's F-12/ Dulbecco's modified Eagle's medium supplemented with insulin, epidermal growth factor, transferin, cholera toxin, bovine serum albumin and antibiotics. Several breast carcinogens and other chemicals were added to the cultures at day 6 of culture, and the number of micronuclei per 1,000 cells was scored at 24 hr after treatment.

In the present study, five breast carcinogens, *N*-methyl-*N*-nitro-sourea, *N*-butyl-*N*-nitrosourea, 7, 12-dimethylbenz (a) anthracene, 20-methylcholanthrene and 2-acetamidofluorene, and one direct carcinogen, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine increased the micronuclei incidence in the cultures.

Since all breast carcinogens and a direct carcinogen induced micronuclei in the culture of mouse mammary epithelial cells in a dose-related manner, but other agents generally do not, these results justify the use of micronuclei as a short-term, *in vitro* screen of suspected breast carcinogens.

DROSOPHILA WING SPOT TEST AS AN ALTERNATIVE TO MAMMALIAN MUTAGENICITY TEST.

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The mouse bone marrow micronucleus test (MNT) is widely performed *in vivo* mutagenicity test but Heddle et al. (1983) reported that 22 of 46 carcinogens were not positive in MNT. We intended to support by the *drosophila* wing spot test (WST) which is an *in vivo* alternative. Six of these 22 compound had been reported to be positive in WST. We carried out WST with the other 6 carcinogens undetected in MNT, 3-aminotriazole (3-AT), dibutylnitrosamine (DBN), ethylenethiourea (ETU), 5-iodo-2'-deoxyuridine (5-ID), maleic hydrazide (MAH) and 1-naphtylamine (1-NA). 3-AT, DBN and MAH, was positive, ETU was initially inconclusive but gave positive results by the alteration of treatment period and 5-ID and 1-NA did not gave definitive results. Thus far, 10 of 12 carcinogens which were not positive in MNT gave positive results in WST, if appropriate protocol was used. Therefore, WST is expected to detect many carcinogens which is negative in MNT.

RADIATION INDUCED CHROMOSOME ABERRATIONS DETECTED BY THE TECHNIQUE OF PREMATURE CHROMOSOME CONDENSATION

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Introduction

We reported high linear energy transfer (LET) radiations were more effective in both cell death and neoplastic cell transformation that low LET radiation[1]. However chromosome aberrations induced by high LET radiations in mitotic cells to reach mitosis for 2 hr treatment of colcemid after irradiation were less effective than that by low LET radiation. These results indicate suggestions as follows. (a) The number of chromosome aberrations per cell per unit absorbed dose induced by high LET radiations is smaller than that by low LET radiation. Biological effects, however, caused by high LET radiation-induced-chromosome aberration are more critical. (b) Because we don't take account of difference in the magnitude of cell cycle delay and interphase cell death between high LET and low LET radiations, our data of chromosome aberrations may be underestimated. It is necessary for better estimation of the risk in radiation induced chromosome aberrations to detect their in interphase not to collect mitosis after irradiation.

In this study we detected radiation induced chromosome aberrations as the induction of premature chromosome condensation (PCC).

Materials and Methods

We used primary Syrian golden hamster embryo (SHE) cells obtained by trypsinization of 13-or 14-day-old embryos[2]. The induction of PCC was performed according to the procedure for polyethylene glycol (PEG) mediated cell fusion described in detail elsewhere[3]. Briefly, mitotic Chinese hamster ovary (CHO) cells obtained by incubation in the presence of 0.1 μg/ml colcemid were mixed with equal number of irradiated SHE cells. The cell mixture was washed and after centrifugation the pellet was treated for 1 minute in 0.15 ml of PEG (MW=1540, in 75 mM Hepes 50% w/v). The fused cells were incubated at 37℃ for 1 hr and the induction of PCC was completed. Then chromosome preparations were made according to the conventional method.

Irradiation of 95 MeV N ions and 22 MeV He ions accelerated by the cyclotron was performed at the Institute of Physical and Chemical Research. Dosimetry and irradiation procedures were carried out according to methods described elsewhere[4, 5]. We estimated the beam energy and LET value at the sample position to be 3.1 MeV/n and 530 keV/μm for N ions, 4.2 MeV/n and 36 keV/μm for He ions, and 1.7 MeV/n and 77 keV/μm for He ions with a 100 μm Al absorber. The dose rate of N ions and He ions were 0.3 Gy/min and 1.0 Gy/min, respectively.

Results and Discussion

The induction rate of breaks in PCC was higher in N ions than the others. The relative biological effectiveness (RBE) to 137 Cs gamma rays was 2.4 for N ions, 1.8 for He ions with the Al absorber, and 1.4 for He ions without the absorber. On the other hand the RBE of chromosomal deletions detected by the conventional method is 0.45 for N ions. These results indicate that the PCC technique is better for the risk assesment among qualitatively different radiation-induced chromosome aberrations.

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PRELIMINARY NOTES ON THE DEVELOPMENTAL TOXICITY ASSESSMENT OF TWEEN 80 IN TISSUE RECONSTRUCTION SYSTEM.

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Fetal rat brains on day 14.5 of gestation were dissociated into single cell suspension with collagenase treatment, and cultured for 48 hours in DMEM medium containning Tween 80 in various concentrations. The number of cell aggregates was not changed in 0.01 and 0.025 %, and increased significantly in 0.05-2.0 % of Tween 80. The size of aggregates decreased around 20 % in 0.05-2.0 % in the major axis compared with that of the control specimen respectively. The shape of aggregates was abnormal in 0.05 % and more over treatment groups. Treatments with 2.5 % over of Tween 80 completely inhibited the aggregate formation.

The present results suggest that the critical dose may be between 0.025 and 0.05 % of Tween 80 in the tissue reconstruction in vitro with dissociated fetal brain cells.

IN VITRO ASSAY FOR THE SCREENING OF TERATOGENS USING EMBRYONIC CELLS.

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We can ascertained the two methods for evaluating the teratogenic activities of chemicals.

Firstly, the micromass culture using mouse and rat embryonic limb bud (LB) and midrain (MB) cells was investigated, the cells in each micro mass island differentiated to form small foci of chondrocyte in LB cultures and neurons in MB cultures. The number of individual foci was counted by use of a dissecting microscope. Validation was performed using 50 compounds. Compounds were classified according to their inhibitory activity. The sensitivity of the test was 93 % ; the spe-

cificity, 91 % ; and the final accuracy, 92 %. Further, there was a good quantitative correlation between the *in vivo* teratogenicity of the 13 arotinoids and the inhibition of LB cell differentiation.

Secondly, human embryonic palatal mesenchymal (HEPM) cell growth assay with and without metabolic activation system using microplate was investigated. Validation was performed using 26 compounds. There was also a good quantitative correlation between the in vivo teratogenicity and the IC 50 values in HEPM cell assay.

These methods are simple, allowing numerous compounds to be rapidly tested at a very low cost, further, useful for the mechanistic studies.

IN VITRO SCREENING ASSAY FOR TERATOGENS USING GROWTH INHIBITION OF HUMAN EMBRYONIC PALATAL MESENCHYMAL (HEPM) CELLS

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For establishment of the *in vitro* screening assay system for cleft plate-inducing teratogens, we have tested 24 teratogenic and 7 nonteratogenic compounds in an *in vitro* assay system which identifies the *in vivo* cleft palate-inducing ability by differentiation of their ability to inhibit growth of human embryonic palatal mesenchymal (HEPM) cells and human embryonic diploid (MRC-5) cells. When metabolic activation was required, the test compound was incubated in the cultures in the presence of the S 9 fraction of rat liver microsomes. Twenty one of 24 compounds with proven cleft palate-inductive effect in vivo preferentially inhibited the growth of HEPM cells than that of MRC-5 cells. Then, the average of relative resistant ratios (ratio of IC 50 values for HEPM cells to MRC-5 cells) of teratogens was 0.49. In contrast, almost nonteratogens identically inhibited the growth of both cell lines and the average of relative resistant ratio was 0.96. The susceptibility in this assay for teratogens was 87.5 %. These data suggested that the inhibition of cell growth *in vitro* may be useful in the prediction of teratogenic activity *in vivo*.

LIMB BUD CELL CULTURES FOR ESTIMATING THE TERATOGENIC POTENTIAL OF COMPOUNDS
– VALIDATION OF THE TEST SYSTEM WITH RETINOIDS –

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Vitamin A derivatives (Retinoids) are not only useful in the therapy of dermatological diseases like psoriasis and other keratinizing dermatoses, but also expected to develop as the anti-neoplastic drugs for possessing anti-carcinogenic activities. As some retinoids have teratogenicity in mammals, development of non or less teratogenic retinoids is desired and a new screening method is investigating as an alternative one.

In this presentation, we employed an alternative system using limb bud cell culture, and make the validation of the test system with retinoids.

Limb buds from gestation day 13 fetus of SD rats were dissociated into single cells using 0.5 % trypsin in GBSS (Gey's Balanced Salt Solution). The cell density of Limb bud cells were adjusted to 2 x10^7 viable cells/ml in CMRL medium containing 10 % Nu-serum. High cell density cultures were set up by dispensing the cell suspension as a discrete drop in the center of each well of a dish. Retinoids were added on day 1 of culture. The accumulation of cartilage proteoglycans was estimated by alcian blue staining. The bound dye was extracted from the cultures with guanidine hydrochloride and the absorbance was determined spectrophotometrically. Inhibition activity of chondrogenesis by retinoids was represented as the inhibition concentration 50 % (IC_{50}).

All-*trans*-retinoic acid, 13-*cis*-retinoic acid and etretin dose-dependently showed the inhibition of chondrogenesis and their IC_{50} was 7x10^{-8}M, 1.6x 10^{-7}M and 7x10^{-8}M, respectively.

Etretinate did not show the inhibition of chondrogenesis, however showed inhibitory activity by the addition of esterase into culture medium.

The plasma concentration of dams treated with the minimum dosage which caused teratological changes in fetus is higher than IC_{50} value of the results using *in vitro* limb bud cell culture, and is enough concentration to develop fetal malformation. Intensity of teratogenic potential of compounds is well correlated to the inhibition activity of chondrogenesis. Therefore, the limb bud cell culture system may be useful for the estimation of teratogenicity of compounds as an alternative method for reproduction studies.

EFFECTS OF DIMETHYL SULFOXIDE (DMSO), POLYETHYLENE GLYCOL (PEG) AND ETHANOL (EtOH) ON CHONDROGENESIS AND ASSESSMENTS USING THE IMAGE ANALYSIS SYSTEM IN RAT LIMB BUD CELL CULTURE.

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Effects of DMSO, PEG and EtOH on chondrogenesis were examined in rat limb bud cell culture. Fore limb buds were taken from embryos on day 12 of pregnancy in Jcl:SD rats and dissociated into a single cell. The single cell suspensions were incubated for 5 days at 37℃ in an atmosphere of 5 % CO_2 and 95 % air. Effects on differentiation of the mesenchymal cells into chondrocytes were assessed by measuring absorbance values of alcian blue which stained proteoglycans. Cytotoxicity was assessed by the MTT assay. Moreover, alcian blue-stained regions were analyzed for the number of spots, area and brightness level using the computerized image analysis system.

Differentiation was suppressed at 0.5～2 % of EtOH, but was not at 0.25～1 % of DMSO nor 0.1 ～0.5 % of PEG. Cell survival was suppressed at 0.8～2 % of DMSO. Therefore, the final concentrations of these vehicles in culture media are recommended to be 0.25～5 % of DMSO, 0.1～0.5 of PEG, or less than 0.5 % of EtOH, respectively.

There were good correlations between absorbance values and area of brightness levels of alcian blue-stained regions. These results suggest that area or brightness levels are good parameters for estimation of chondrogenesis in rat limb bud cell culture.

EFFECTS OF METHOTREXATE ON MOUSE EMBRYOS CULTURED IN CHEMICALLY DEFINED MEDIUM, ASF 301

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We have developed the screening system for drug and chemical agents by using whole embryo cultures. The advantages of the whole embryo culture are to examine the direct effect of drugs on embryos. The toxicity of methotrexate (MTX) was investigated in mouse embryos cultured for 24 hours from day 11 of gestation with rat serum or serum-free chemically defined medium, ASF 301. Previous reports have indicated that MTX, an anticancer drug, caused a severe toxicity to liver in cultured mouse embryos. Exposure to MTX at concentrations of 500 or 1000 μg/ml caused cell death or degeneated surface epithelia of liver in cultured mouse embryos. On the other hand, in regard to embryonic growth parameters (the crown-rump length and the total number of somite), there were no differences between treated and control groups. In the present study, the embryonic protein contents and the crown-rump length in cultured mouse embryos were significantly decreased in MTX-treated groups with ASF 301 medium. Mouse embryos cultured in MTX-containing medium of ASF 301 were severely affected by the toxicity of MTX. These results suggest that the drug binding protein in rat serum medium used for whole embryo culture may reduce the MTX-induced toxicity.

INFLUENCES OF DRUG BINDING PROTEIN ON SALICYLIC ACID-INDUCED TOXICITY IN CULTURED RAT EMBRYOS

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Previous studies reported that the drug binding protein in rat serum of rat embryo culture medium reduced salicylic acid-induced toxicity on cultured embryos. The result might be due to the decrease of free type of salicylic acid in embryo culture medium. We measured the salicylic acid concentration in the rat serum or the cosmedium 001 of culture medium. Salicylic acid contents were 454±13 μg/ml in cosmedium 001 and 184±46 μg/ml in rat serum. Rat cultured embryos in cosmedium 001 significantly increased the incidence of salicylic acid-induced abnormalities. Thus, the results of the present study indicated that the rat serum of embryo culture medium disturbed the toxic effects of saliclyic acid. This effect mighs be due to drug binding protein in rat serum of culture medium.

AN ALTERNATIVE OF DRAIZE EYE IRRITATION TEST : NEUTRAL RED CYTOTOXICITY ASSAY

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In this study, toxicity of 24 chemicals, which included detergent, oil and solvents, were evaluated by *in vitro* neutral red cytotoxicity assay. We compared sensitivity of three kinds of cells, V 79 established cell line, rabbit cornea cells (RE), and normal human epidermal keratinocytes (NHEK). V 79 cells were grown in Eagle's minimum essential medium supplemented 5 % fetal calf serum (FCS-MEM), RE cells were maintained in 10 % FCS-MEM. NHEK were grown in a serum-free keratinocyte grown medium (K-GM). V 79 or RE cells (9×10^3) in 200 μl of each mediul were seeded to each well of the 96-well tissue culture plates, and were allowed to attach over a 24 hours period. NHEK (2.5×10^3) were incubated for 72 hr. The medium was removed from each well and the cells were treated with test chemicals for 24 hr. The test solution was removed and the medium containing neutral red was added to each well.After 3 hr-incubation at 37℃, the medium was taken off and the cells were fixed and washed with formaldehyde-$CaCl_2$ solution. Then, the dye in the cells was extracted with acetic acid-ethanol solution. The absorbances of the extracts were measured at 540 nm using microplate reader. The cytotoxic potential of test coemicals was expressed as IC 50 (μg/ml). IC 50 values were the concentration of test chemicals that induced a 50 % inhibition of the absorbance observed with the controls. The IC 50 value of test chemicals using NHEK was the lowest among three cells. However, the order of the cytotoxicity potentials of test chemicals were similar in these kinds of cells. Cationic detergent showed strong toxicity, while anionic and non-ionic detergents were weak. The cytotoxic potentials of the chemicals using these cells correlated well with the results obtained from in vivo Draize test. It was suggested that the neutral red cytotoxicity assay was useful to evaluate eye irritancy of chemicals as an alternative of Draize test.

AN EPERIENCE IN USING EYTEX™ *IN VITRO* OCULAR SAFETY TEST – COMPARISON WITH *IN VIVO* DRAIZE –

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There are, recently, many alternative methods for the rabbit eye irritation test developed and reported. In the present study, we used the EYTEX method, one of the potential alternatives, for assessment of eye irritancies of marketed anti-tumor drugs and compared the outcomes with those of the Draize test. We also discussed a few problems that occurred in the EYTEX assay.

In the EYTEX method, all agents were the injective form for clinical use were classified as "NON" to "MILD", while the oral agents were determined to be "MILD" or "MODERATE".

In the comparison between the results of the EYTEX and Draize methods, 90.9 % equivalence

and 85.7 % predictive value of the EYTEX method to the Draize data were confirmed. But, there occurred a few problems as follows. One was that, in the EYTEX procedure, the addition of activator caused the background yellow color of reagent to disappear, but then, in mixing the reagent with some test agents, the color reappeared. The other was that some test agents with deep color might affect the data measured.

In conclusion, the EYTEX method was easier and a more valuable alternative to the *in vivo* rabbit eye irritation test of pharmaceuticals. While a few problems did occur in some agents, they were not serious and may be overcome.

INDUCTION OF ORNITHINE DECARBOXYLASE IN CULTURED HUMAN SKIN CELLS BY 12-0-TETRADECANOYLPHORBOL-13-ACETATE (TPA)

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Teikoku Seiyaku Co., Ltd. 2) Tokushima Univ.

As a simple test for tumor promoters screening, we studied an ability of ornithine decarboxylase (ODC) induction by TPA in third-cultured human breast skin cells, purchased commercially.

We measured the ODC protein in cultured skin cells by enzyme immunoassay (EIA) method, because this assay could measure with slight cellular extract as compared with the assay of enzyme activity. An anti-ODC antibody used for EIA method was made from rabbits immunized by ODC antigen purified from mice kidney.

In third-cultured skin cells, a relationship between the ODC protein and activity was $Y = 0.09X + 0.34$, and the correlation efficient, r, was 0.990.

The ODC protein in third-cultured skin cells was the most increase at 6 hours after treatment with TPA of 16 μM in final concentration, and then was 45.0 ng/mg total protein. Now the ODC protein induced by TPA was about 2 times of TPA untreatment.

Usually, these skin cells showed long shape, but shrinked at 2 hours with TPA treatment. But after the fifth-culture, these skin cells losed an ability of ODC induction by TPA, although the shape changed.

These findings suggest that an ability of ODC induction in third-cultured skin cells derived from human breast can use as a simple test for tumor promoter screening.

EVALUATION OF INTRACELLULAR ADENO SINE TRIPHOSPHATE LEVEL AS AN INDICATOR FOR CYTOTOXICITY *IN VITRO* — EFFECT OF INORGANIC METALS ON RABBIT ERYTHROCYTES —

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In order to establish changes in cellular adenosine triphosphate (ATP) levels as an indicator for cytotoxicity *in vitro*, we studied the effect of inorganic metal salts on rabbit erythrocytes using the luciferin/luciferase bioluminescent system, emitted light being measured in an automated

Lumac luminometer (M 1500). At first, the inhibitory effect of the test substances on the bioluminescent system was investigated. At the concentrations of more than 100 μM, both aluminium chloride and iron chloride showed the inhibititory effect. Mercuric chloride, copper sulfate and lead acetate were inhibitory at 1,000 μM, but not at 100 μM. However, the other test substance did not show the inhibititory effect even at 1,000 μM. Therefore, the incubation mediums were removed from the incubated cells by centrifugation for 5 min at 1,000 $\times$ g three times before determination of ATP concentrations. Cell suspensions in saline were incubated with the different inorganic metal salts for 1 hr at room temperature and then the ATP levels of the washed cells were determined. The intracellular ATP concentrations of cells treated with 10 μM mercuric chloride were decreased to less than 1 % as compared with controls. Other heavy metal salts such as copper sulfate, potasium dichromate and lead acetate decreased the intracellular ATP by 10-20 % at 100 μM and by 2-4 % at 1,000 μM. Although aluminium chloride and iron chloride showed significant decreasing effects at more than 100 μM, this might be partly due to formation of a complex of ATP with these metal ions. However, the other tested inorganic metal salts including cadmium chloride, manganese chloride, sodium arsenate, zinc acetate magnesium chloridd and calcium chloride did not show any effect even at 1,000 μM. Although it is further needed to study the correlation between ATP reduction and established cytotoxic indices such as an osmotic fragility of red cell membrane, direct cytotoxic effect for other isolated or cultured cells may be adequately studied by this simple, rapid and inexpensive method.

SCREENING OF DERMAL IRRITANTS BY SKINTEX™

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Food and Drug Safety Center

The SKINTEX™ system was developed to predict dermal safety and chemical irritation by ROPAK Lab. The assay system are non-animal tests based on a physicochemical model of dermal irritation. The principle of SKINTEX™ method is that testing chemicals are absorbed through permeability barrier and react directly with a protein matrix, then are quantitated spectrophotometrically.

We evaluated the SKINTEX™ system for the dermal irritation of chemicals by comparing with the Primary Dermal Irritation Indices (PDII) obtained from the standard Draize skin test using rabbits. The Skintex Class of non-ionic detergents (polyoxyethylene nonyl phenyl ether and coconut fatty acid diethanolamide), anionic detergents (sodium hydrogenated glyceryl cocoate sulphate and sodium coconut fatty acid taurate), cationic detergents (benzethonium chloride and stearyl trimethyl ammonium chloride), amphoteric detergents (2-alkyl-*N*-carboxymethyl-*N*-hydroxy-ethyl imidazolinium betaine), glycol (1, 3 - Butylene glycol), oil (tri - 2 - ethyl glycerol hexinate) and seven cosmetic products were similar as *In Vivo* Dermal Irritation Class. These data suggested that the SKINTEX™ kit was easy dermal safety evaluation system with saving time and money.

NATIVE-STATE THREE-DIMENSIONAL *IN VITRO* HISTOCULTURE TO ASSAY OCULAR TOXICITY

Li. Linnga, and Hoffman, R. M.

AntiCancer, Inc. and University of California, San Diego

Federal regulations require an *in vivo* test in rabbits modified from the method described by Draize et al. in 1944 to test toxicity of chemicals, pharmaceuticals and household products. There are major negative factors concerning this test, including subjectivity, variability, time, and high cost. There is also strong public opinion favoring elimination of animal experimentation. The above facts emphasize the need for *in vitro* methods to assess ocular toxicity. Recent studies have proposed *in vitro* methods to estimate ocular irritancy utilizing protozoan cells, or mammalian cells or tissues in culture or a macromolecular solution termed EYETEX. Here we decribe a new native-state *in vitro* ocular safety assay. Human normal conjunctiva has been histocultured for 24 hours with a 3-dimensional gel-supported native-state in vitro culture system which we have developed. The visualization of the 3-dimensional components of the histocultured conjunctiva *in vitro* is greatly enhanced by the use of confocal laser scanning microscpy. The fluorescent dyes BCECF and propidium iodide (PI) were used to identify living and dead cells, respectively, in the cultured human normal conjunctiva. 70 % and 5 % ethanol were tested as the toxins. The histocultured conjunctiva was doubulestained with BCECF and PI before and after treatment. The PI-positive dead cells were counted before and after treatment, such that the ratio of killed cells in treated cultures to untreated controls was obtained. We have observed a dose response of ethanol toxicity on the in vitro cultured human normal conjunctiva. *In vitro/in vivo* correlative studies are in process. The data obtained indicate that the nativestate model is highly representative of the *in vivo* state for toxicity testing of nomal conjunctiva.

NATIVE-STATE *IN VITRO* THREE-DIMENSIONAL HISTOCULTURE OF SKIN TO ASSAY TOXIC SUBSTANCES

Li, Lingna, Margolis, L. B., Hoffman, R. M.

AntiCancer, Inc. and University of California, San Diego

There is an important need for an in vivo-like-*in vitro* system to stuy toxicology of topically applied, ingested and environmental agents on skin. The *in vitro* system should have the benefit of allowing the study of a large variety of substances on an individual specimen over relatively long time periods. The Native-State *in vitro* Three-Dimensional Histoculture System cultures all the components of skin for upwards of 10 days and allows the determination of the effects of agents on the various component cell types and their interactions. The fuluorescent dyes BCECF and propidum iodide (PI) readily identify living and dead cells, respectively, in the histocultured skin and are therefore used to measure cytotoxicity. The visualizatiom of the three-dimensional components and all cell types of the histocultured skin *in vitro* is greatly enhanced by the use of confocal

laser scanning microscopy. Inhibition by toxins of the various cell types of the histocultured skin is readily measured by histological autoradiography after the histocultures are exposed to toxins and then to [^{3}H] thymidine for 3 days. Model toxins such as ethanol and adriamycin when applied to the histocultured skin demonstrate that toxicity is measured in a facile, quantitative and dose-responsive manner. Results in vitro reflect the *in vivo* situation in that hair follicle cells, being one of the most rapidly proliferationg, epithelial cell types in the human body and one of the most sensitive to toxic agents, are also most sensitive in the Native-State *in vitro* Histoculture System. Results thus far obtained indicate that the Native-State *in vitro* Three-Dimensional His-toculture System should be highly useful in the replacement of animal systems on the one hand, and in the replacement of more simple cell culture systems on the other hand for the study of the effects of various products, drugs and pollutants on skin.

NATIVE-STATE THREE-DIMENSIONAL *IN VITRO* HISTOCULTUTE TO ASSAY COLON-SPECIFIC CARCINOGENS.

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AntiCancer, Inc. and University of California, San Diego

Colon cancer is the leading form of cancer in the United States and is rapidly becoming a leading form in Japan and in other areas of the world. The incidence in epidemiology of colon cancer consistent with an etiology that involves dietary components. There is evidence that colon carcinogens and promoters may initially be cytotoxic and exert their effect via subsequent compensatory hyperplasia leading to the neoplastic state. There is, therefore, an important need to screen dietary components for their potential carcinogenic effects on the colon. Toward this end, we have further developed the Native-state *in vitro* Three-Dimenisional Histoculture Technology to culture normal and malignant colon for relatively long periods of time. Colon tumors in Native-State Histoculture demonstrate good morphological and functional integrity allowing the mucosal respones to carcinogens to be studied in an isolated environment removed form various physiological influences prevailing *in vivo*. Using the fluorescent dyez BCECF and propidium iodide (PI) which indicate cell viability and death respectively, we have been able to design a quantiative toxicity assay on the histocultured colon. Confocal microscopy has been used, which allows the three-dimensional observation of the histocultures in a way previously not possible, greatly enhancing the ability to assess the various agents applied to the specimens, in particular to the colonic crypt. Inhibition of cell proliferationg the crypt cells by toxins of the histocultured colon is readily measured by histological autoradiography after the histocultures are exposed to toxins and then to [^{3}H] thymidine foy 3 days. Validation of the in vitro cytotoxicity observations as a means of assessing carcinogens will be done particularly with experiments focusing on the *in vitro* cytotoxic effects of various free-fatty acids compared to their carcinogenic or tumor promoting effects *in vivo*.

AN ALTERNATIVE METHOD FOR DRAIZE TEST ··· SIMPLIFIED ASSAY FOR LIVE/DEAD CELLS BY MEASURING LDH ACTIVITY

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Alternative methods for Draize eye irritant test have been proposed, for example colony formation of cultured cells, MTT assay or Neutral red intake assay. These methods depend on determining surviving cells after appropriate treatment with detergents. We tried to quantify dead cells after the treatment by measuring lactate dehydrogenase (LDH) released. This method gave us advantage to determine not only highly sensitive determination of dead cells but residual live cells of the same sample by dissolving with a high concentration of a detergent. Thus we can determine ED_{50} and ED_1 simultaneously in a 96-well culture plate.

The human carcinoma cell line, SQ-5, and the normal human fibroblast, NB1RGB, were submitted for the assay. Cells in a 96-well culture plate were treated with varied concentrations of 10 detergents, i. e. 3 non-ionic, 2 anionic, 3 cationic, and 2 amphoteric detergents. ED_{50} of these detergents well corelated with the Draize score (DS_{20}) previously reported by Watanabe, *et al.* (3). Direct measurement of ED_1 of each detergent revealed that, although ED_{50} of alkyl imidazolin was higher than that of Polyoxyethylene (10) lauric acid, ED_1 of the former was lower than that of the latter. These results suggest us that some of commonly used detergents may show relatively higher cytotoxicity in low concentration region.

AN *IN VITRO* STUDY TO PREDICT OCULAR IRRITATION:USE OF RABBIT CORNEA PRIMARY CELLS

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Cytotoxicity study using rabbit primary cornea cells was performed with the 94 various chemicals for an approach to alternative methods for Draize test. A good correlation was obtained between the *in vivo* and *in vitro* in detergents but not in acids and bases with high dissociation potential. Eye irritation caused by acids and bases correlated with their dissociation constants (pka). Cytotoxicity of acids and bases correlated with their octanol/water partition coefficients (logP) but not with their pKa values.

The results suggested that eye irritation caused by acids and bases mainly related to their dissociation. and that the mechanism of ocular irritaion of acids and bases was different from that of detergents.

OPACITOMETER METHOD AS AN ALTERNATIVE IN DRAIZE METHOD

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The purpose of our work is establishment of an *in vitro* opacitometer method as an alternative of Draize method. Opacitometer method consists in the objective assessment of irritant-induced opacity in the isolated porcine cornea which is clamped between two halves of holder to measure transmitted light through cornea to photocell arranged in it.

We studied a relation between Draize method and opacitomenter method using chlorophenol isomer, then tried to clarify the mechanism of chlorophenol isomer to produce porcine corneal opacification. We found a good correlation between these opacities of porcine corneas and the score of Draize method. It was suggested that the opacity may be mainly due to a reaction between chlorophenol isomer and porcine corneal epithelial proteins.

The measurement of irritant-induced opacity of isolated porcine cornea *in vitro* may be a technique which can be developed into an objective, cost-effective alternative to many of the *in vivo* ocular irritancy tests currently carried out.

AN *IN VITRO* METHOD, SOLATEX™, TO PREDICT PHOTOTOXIC POTENTIAL

V. C. Gorden and C. P. KELLY

Ropak Laboratories

A new test *in vitro* methodology, SOLATEX™, has been developed to predict the phototoxic potential of compounds. A phototoxic effect is considered when a significant enhancement of an in vitro SKINTEX/PDII Equivalent is shown for the UV light with the compound. The test system has been evaluated with 20 materials classified as strong, idiosyncratic, and negative based on the human response. No compounds in the negative group produced significant photo induced irritation in this system. Some compounds and formulations produced protective effects against UV light which can be quantitated and compared to SPF factors for a series of formulations with SPF from 1 to 15.

EVALUATION OF AN *IN VITRO* DERMAL IRRITATION METHOD

Soto, R. J [1] and Gorden,V. C [2].

[1]*S. C. Johnson & Son, Inc.* [2]*Ropak Laboratories*

An *in vitro* assay was evaluated by comparing *in vivo* Draize scores for twenty five experimental consumer product formulations. The SKINTEX *in vitro* assay (SIA) was chosen for in-house evaluation because it is objective, easy to learn and economical. Results from previous published studies have shown good performance. Double-blind formulas that were tested consisted of furniture, laundry, air freshener, repellent, insecticide, floor and personal care products. Primary dermal irritation index (PDII) scores for SIA and retrospective *in vivo* scores were used for comparison. PDII scores were classified into the following classes:minimal, mild, moderate and severe. Based on these results, SIA has demonstrated value as a screening tool for a broad variety of consumer products. Highly viscous materials did not qualify to run suitably in the SIA. SIA provide adequate equivalence and correlation for screening purposes. This method can not evaluate physical irri-tation, allergic, nor recovery mechanisms. Advantages of SIA include the capability to test undiluted products, reduce animal use, and lower costs.

RECONSTRUCTION OF GRANDS OR DUCTS FROM HUMAN AND MOUSE CELLS IN COLLAGEN GEL CULTURL

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The normal and tumor cells were cultivated in three dimensional collagen gel culture and the possibilities of reconstruction of original glands or ducts were investigated *in vitro*. Cells used were from mouse normal salivary glands, mammary gland, human normal parotic glands, submandibular glands, mammary glands. For tumor cells, pleomorphic adenoma, adenoid cystic carcinoma of salivary glands and papillotubular carcinoma, mucinous carcinoma of breast were used. They were treated with collagenase respectively.

Matured glandular formations were clealy observed in the collagen gel culture for about 30 days of cells from each normal mouse and human tissues. It is suggested that the normal cells reconstructed their glands or ducts *in vitro* collagen gel cultures as *in vivo*. On the other hand, in the collagen ges cultures of adenoid cystic carcinoma and pleomorphic adenoma cells, glandular formations were not observed. Although the former was grown in round colony like patterns differed from *in vivo*, the latter was cultivated as *in vivo*. In the culture of papillotubular carcinoma and mucinous carcinoma of breast, glandular formations were observed. Therefore, collagen gel culture method is useful to reconstruct the normal and some tumor tissues as *in vivo*.

EXPRLSSION OF STRESS PROTEINS IN MAMMALIAN CELLS TREATED WITH TOXICIC AGENT, AS AN INDICATOR OF CYTOTOXICITY

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We studied expression of stress proteins in HeLa cells treated with toxic, mutagenic and carcinogenic agents, as an indicator of cytotoxicity. HeLa cells were treated with As, Cd, 4-nitroquinoline 1-oxide (4NQO) and Ethyl methane sulphonate (EMS) for 2 h. and, then were labeled for 1 h. with ^{35}S-Methionine. Stress proteins (hsp72) in labeled cells were detected by SDS-PAGE and western blot analysis. Cytotoxicity was determined by MTT assay. Dose of 50% survival in cells treated with As, Cd, 4 NQO and EMS were 110 μM, 18 μM, 200 nM and 35 mM, respectively. Expression of hsp 72 in cells treated with various agent was reached to maximum level at about 50% survival. At level of less 10% survival, however, hsp72 in cells was slightly expressed except for it in EMS treated cells.

APPLICATION OF BIOLUMINESCENCE ATP ASSAY FOR CYTOTOXICITY TEST

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The applicability of aednosine triphosphate (ATP) assays was studied for evaluating cytotoxic agents *in vitro*. BALB 3T3 (mouse whole embryo), FRSK (foetal rat skin keratinocytes) cells and rabbit erythrocytes were used. Many chemicals including surfactants, glycols and others were applied in this assay. Intracellular ATP content was measured by the luciferin-luciferase method using LUMAC luminometer (M 1500).

The sensitivity limit was 100 cells in the final simple. ATP content was decreased at 6 hours after exposure of cytotoxic agents in BALB 3T3 cells and erythrocytes. Catinic surfactants showed severe effects than non-ionic and anionic surfactants. These results of ATP assay showed a good accordance with the results of colony formation assay in almost agents applied.

Our findings suggest that ATP-bioluminescence method is an powerful altermative cytotoxicity assay because of its simplicity, sensitivity and universal applicable to any type of cells.

MEASUREMENT OF INTRACELLULAR MAGNESIUM ION DISTRIBUTION IN SINGLE CULTURE CELLS

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We have constructed an apparatus for measurement of distribution of intracellular magnesium ion concentration ([Mg^{2+}] i) combining the fluorescent magnesium indicator dye Mag-fura-2 with fluorescence microscopy. The apparatus and data processing were the same as the measurement for the intracellular calcium ion concentration distribution. Observation of [Mg^{2+}] i was measured by the dual wavelength fluorometry, excitation of Mag-fura-2 was performed at 340 nm and 380nm, and emission at 500nm was measured.

Levels of [Mg^{2+}] i in the cytoplasm and the ncleus were clearly different. However, the image of [Mg^{2+}] i in the cytoplasm was observed homogeneously distribution. The average [Mg^{2+}] i in the cytoplasm was the range of 1-2 mM, and the nucleus was 0.1 mM below.

LONG-TERM CHICK EMBRYO CULTURE AND HATCH MARKING USE OF DUCK EGG SHELL

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[1)]*National Institute of Animal Health,* [2)]*National Institute of Animal Industry*

In order to establish a method for the long-term culture of chick embryo, duck egg shell (DES) was tested for its suitability as a vessel to sustain chick-embryo development. 35 chick embryos of fertile laid eggs incubated from 3 days before were transferred to DESs with all egg contents. As a shell-less control experiment, 37 chick embryos were transferred to the vessels of polyethylene cling film. Approximately 60% of chick embryos transferred to DESs were developed until 18 days of incubation (15 days after the transfer) and 3 birds were hatched at 22 days of incubation. Two male chicks had grown up to 6 months of age showing normal body weight and sperm fertility. All the chick embryos transferred to polyethylene vessels died until 18 days of incubation. It was suggested that DES culture system may be useful for the experiment using chick embryo culture.

ASSESSMENT OF SENSITIVITY OF TUMORS TO ANTITUMOR AGENTS BY ESTIMATING TUMOR GRAFT VASCULARIZATION ON THE CHICK CHORIOALLANTOIC MEMBRANE (CAM)

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[1]*Azabu University.* [2]*Practitioner*

Introduction

In the treatment of human tumors, a practical test to evaluate the relative efficacies of different antitumor drugs needs to be conducted prior to the initiation of clinical chemotherapy. Although some authors have suggested assays using cultured tissue[4], nude mice[1], and mouse subrenal capsule, a predictive role in chemosensitivity in clinical oncology has not yet been established. Blood vessel growth, or angiogenesis, is a requirement for tumor growth. Tumor grafts have been shown able to elicit centripetal angiogenesis with a star-shaped or spoke-wheel capillary arrangement in the CAM[2]. After considerable study, we found the method of estimating tumor graft's vascularization to be the most reliable.

Materials and Methods

Tumor preparation: Dog squamous epithelial cell carcinoma, canine transmissible veneral tumor (CTVT), rat fibrosarcoma and mouse adenocarcinoma were used for this assay. Solid tumor tissue samples were cut into 0.5 ㎜ with scalpels.

The CAM-assesy protocol: At day 10 of incubation, artificial air spaces were formed. The small piece of solid tumor tissue was placed directly onto the CAM. The windows were closed with aluminum sheets and sealed with tape and returned to the incubator. Three days after implantation, on day 13 of incubation, when vascularization of a successful graft was essentially complete, the aluminum covers were removed, opening the windows. The test substance, Oncovin (Sionogi Pharmacy Co., Ltd.), was injected into the yolk sacs. The windows were closed again and the eggs were put into the incubator. Four days later, on day 17 of incubation, the CAM were observed under a binocular microscope and removed from the embryo. The CAM were extended, fixed with 10 % formalin, dehydrated and mounted onto a glass slide with balsam.

Quantification of vascular responses: A thin transparent plastic ring (20 ㎜ in internal diameter) was placed on the slide in such a way that the central reaction focus of the CAM response was located in the center of the ring. We assessed the rate of vascular response by counting vessels which crossed the inner edge of the ring under a low-power microscope.

We defined a positive reaction as one that lost more than 30 % of the radially arranged vessels, a negative reaction as one that showed a decrease of less than 30 % of the vessels. The rate of vascular response in treated eggs was determined by dividing the number of the spoke-wheel vessels by the mean number of untreated group. The average number of the spoke-wheel vessels of untreated eggs was determined by dividing the total number of vessels by the total number of eggs. A rate of 0.7 or more, which means less than 30 % loss of the vessels, is 1+; vascular response between 0.7 and 0.4, which means 30 to 60 % loss of the vessels, is 2+, vascular response less than 0.4, which means more than 60 % loss, is 3+. The evaluations were made on individual eggs. The test for each preparation required 40 eggs (20 for control, 20 for treatment). After the reading of the 20 eggs, the percentage of positive eggs was determined by adding up the numbers of eggs with scores of 2+ and 3+, and dividing this number of positives

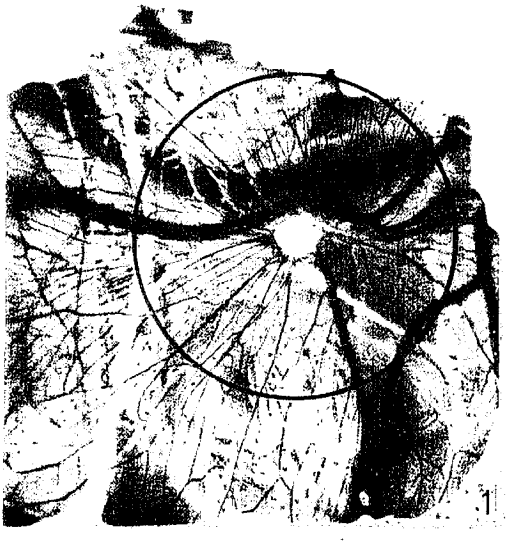

Fig. 1. Non-treated:Spoke-wheel capillary growth directed towards the neoplastic nodule is seen in a scanning circle on the CAM.

Fig. 2. Treated with anticancer drug:The spoke-wheel arrangement is loss and the number of capillaries wpthin the ciycle is obviously reduced, showing 3+ positive.

by the total number of viable eggs. For example, in a series of 18 surviving eggs, 4 with a 3+ score and 6 with a 2+ score, gives 10 positives. The remainding 1+ or 0, are all negatives. The resulting 10 positives divided by the total of 18 gives 55.5 % positives.

Results

Untreated eggs : The surviving tumor tissue grown and spread beyond its original margin. The grew of the tumor was followed by the centripetal angiogenesis with a star-shaped or spoke-wheel formation (Fig. 1). All sprouts originating from the radially arranged vessels can be observed to continue elongation and anastomose to form the capillary network. The graft was free of degenerating:In severe rezponse, the spoke-wheel arrangement disappeared and the number of vessels reduced drastically (Fig. 2). When the numbers of radial vessels in the treated group we re averaged for each tumor, and expressed as a ratio of the average vascularization in untreated group, a decrease was seen. The percentage of positives were 78, 90, 68, and 89 for squamous cell carcinoma, CTVT, fibrosarcoma, and adenocarcinoma, respectively. The technique is thus capable of demonstrating sensitivity of tumors to an anticancer agent. Histomorphology of the grafts also showed extensive degeneration and/or necrotic lesions. The nuclei of the cells were pyknotic, and the cytoplasm were stained deeply with eosin and vacuolated. The degree of degeneration varied in the different lesions, both within the same CAM and in differlnt eggs. The rate of vascular response showed a strong correlation with the severity of degeneration confirmed by histological observation.

Discussion

When compared to the previously proposed studies, this CAM-assay may have centain unique advantages. The assay is easier and less expensive to perform and interprete than the other assaies using mammals and does not require esaborate animal room faclities and tissue culturl system.

Recently, Uchida[1] introduced a chick-embryo chorioallantoic membrane (CAM) assay for use in determining the chemosensitivity of tumors. His study involves measuring the volume of tumor grafts onto the CAM. Although promising in terms of its prldictive acuracy, it may be difficult to obtain reliable and reproducible results on the

LDH RELEASE ASSAY FOR CYTOTOXICITY OF ANTITUMOR AGENTS

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Cytotoxicity of antitumor agents was monitored by measuring lactate dehydrogenase (LDH) activity released from cultured human tumor cells. The assay provpdes a sensitive, rapid, inexpensive, and quantititive meastrement of cell death.

Human lung squamous cell carcinoma, SQ-5, and human lung adenocarcinoma, HCL-1, were seeded in 96-well culture plates at a cell density of $2x10^3$/well and $4x10^3$/well, respectively. After 3-day preculture, doxorubicin hydrochloride (DNR) or 5 fluorouracpl (5 FU) was added with final concentrations of 0.1, 1.0, 10 mcg/ml. Portions of culture sup were sampled after 24, 48, and 96 hr incubation. LDH activities of these culture sup were proportional to dead cells. Remaining cells in the well were dirsolved with 0.3 % lauryl dimethylaminoacetic acid betaine. LDH activity in this solution reflected number of live cells.

When SQ-5 cells were treated with DNR, the cells released LDH dependeng on the concentration of the drug within 24 hr. However, HCL-1 showed resistance to the drug at the concentration, 0.1 and 1.0 mcg/ml. LDH activity of the cell lysate increased by 48 hr at these concentrations. At 10 mcg /ml, the LDH activity of the cell lysate decreased unidirectionally from 24 hr.

Treatment of the tumor cells with 5 FU resulted in no essential release of LDH into culture sup by 48 hr. By 96 hr, however, half of the cells died at the concentration of 10 mcg/ml of 5 FU. As has been assumed, 5 FU showed cysotoxicity to both of the tumor cells in time dependent manner. At lower concentations oe 5 FU, HLC-1 seemed relatively resistant to 5 FU action when compared to SQ-5.

MUTICELLULAR SPHEROID OF HUMAN EMBRYO CELLS

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We have developed a new cell culture technology by using a themo-responsive polymer, poly-N-isopropyl acrylamide (PNIPAAm) that is in a solid state at culture tetperature oe 37°C and becomes roluble in culture medium at room temperature for a cell culture substratum. We succeeded in preparation of multicellular spheroid of human dermal fibroblasts by detaching the cell sheet cultured on this subssratum without use of conventional cell detaching agents[1)]. In this study we attempted to make multicellular spheroid of human embryo cells which are considered to have high activity of differentiation and also sensitive drug-susceptibility by using this technology. The human embryo cells werl cultured on the PNIPA-Am substratum conjugated with collagen at 37°C until the cell-cell contact was formed and then detached from the substratum by lowering the temperature. When the detached cell sheet was washed with PBS and transfered to a hydrophobic

sensitivity of tumors to antineoplastic agents by only mlasuring tumor volume. The same volume of a tumor does not necessarily represent same tumor growth activity, as the tumor may have degenerating and/or necrotic tissue in its neoplastic tissue. The new methvd presented here may potentially overcome the above disadvantage. This assay is reliable, reproducible and easy to standardize, so that data from different labs can be reproduced readily. The test requires 7 days. This shorter period of time (7 days) may have obvious advantages in a clinical situation. Permanent slides are made so that the results of the assay may be interpreted, rechecked, or compared to other assays at any convenient future time. The assay data revealed a strong correlation between the CAM chemosensitivity of different types of tumors. This suggests that the assay could prove useful in new drug screening. Further work will be requjred to determine the accuracy and limitations of the assay.

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ROOM TEMPERATURE CYTOTOXICITY TEST OF ANTI-TUMOR DRUGS USING INSECT CELL LINES

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In vitro cytotoxicity of anti-tumor drugs is usually tested with mammalian cell lines. We performed this test using insect cell lines which can grow rapidly at room temperature and require no CO_2-incubator. Minimum inhibitory concentration (MIC) of cell growth was measured for (1) mitomycin C, (2) actinomycin D, (3) chromomycin A_3, (4) adriamycin, (5) daunomycin, and (6) neocarzinostatin. NIH-SaPe-4 derived from fleshfly showed higher sensitivity to 2, 3, 5, and 6 than mammalian cells, i. e. HeLa, KB, HEp #2, FL, and Vero. For example, MIC of actinomycin D was 0.01 mcg/ml. SES-MaBr-1 derived from cabbage armyworm, however, showed resistance to the growth inhibition by 1, 4, and 5. Minimum inhibitory concetration of 4 was 100 mcg/ml. It is, thus, possible to replace mammalian cell lines to suitably-selected insect cell lines in cytotoxicity test of anti-tumor drugs, that brings us convenience to handle cell lines at room temperature without gas phase control. By utilizing insect cell lines, simplified fully automatic test system will be constructed for anti-tumor drug screening.

dish, the cell sheet gradually folded and finally changed to a spheroid in 2 days. The study on a drug-susceptibility and specific expression of proteins of the spheroid is in progress.

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RAT WHOLE EMBRYO CULTURE ON ASF 301 — EFFECTS OF OPEN YOLK SAC METHOD —

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Rat whole embryo culture is suitable method to examind the chemical compound effect on embryo without maternal factor. The purpose of the present study was done to elucidate the effect of the synthetic medium (ASF 301) on cultured rat embryos. Rat embryos were investigated in culture period for 48 hrs from day 11 of gestation. ASF 301 treated embryos were decreased in yolk sac blood circulation at culture period of 4 hours and in the time point of 48 hours in culture, these embryo were dped. On the other hand, culture rat embryos with rat serum for 2 hourr from the biginning in culture were not observed on the effect of ASF 301 in culture period of 24 hours. These results suggested that ASF 301 was disturbed the roles of yolk sac in cultured rat embryos. These results indicated that rat serum developed on the growth or function of yolk sac of culturued embryos.

RAT WHOLE EMBRYO CULTURE IN CHEMICALLY DEFINED MEDIUM, ASF 301 — EFFECTS OF ASF 301 ON THE BLOOD VESSEL OF YOLK SAC —

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Previous experiment indicated that the yolk sac function of cultured rat embryos was disturbed when embryos were cultured in serum free medium, ASF 301 and rat serum recovered the damage of yolk sac in cultured embryos. One of the yolk sac function was observed as the blood circulation in embryos cultured as whole embryo.

Rat embryos cultured in ASF 301 were significantly decreased in the diameter of blood vessels in yolk sac compared with embryos cultured in rat serum.

However, embryos cultured in ASF 301 showed a decrease in incidence of ASF 301-induced toxic effect when embryos were culture in rat serum for 2 hours.

This report suggested that ASF 301-induced toxic effect may be due to the decrease of blood circulation in yolk sac of cultured rat embryos.

EFFECTS OF PIG SLRUM ON CULTURED RAT EMBRYOS

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We have developed the whole embryo culture system by the use of pig serum. Present study was investigated in rat embryos cultured for 48 hours from day 11 or day 12 of gestation with rat serum or pig serum.

(1) THE EXPERIMENT OF CULTURE PERIOD OF 48 HOURS FROM DAY 11 OF GESTATION IN RATS.

The embryonic total somite, crown-rump length and protein contents in 100 % pig serum cultured group were found to be significantly reduced *in vitro* embryos as compared to the comtrol, on the other hand 50% pig serul +50% rat serum cultured embryos did not changed from these parameters in control embryos.

(2) THE EXPERIMENT OF CULTURE PERIOD OF 48 HOURS FROM DAY 12 OF GESTATION IN RATS.

100% pig serum or 50% pig serum cultured embryos were died for 24 hours from day 12 of gestation in rats. These restlts suggested that cultured rat embryos with 50% pig serum were possible to the whole embryo culture of 48 hours from day 11 of gestation.

SPECIES-SPECIFIC FACTORS REQUIRED FOR THE CULTURE OF POSTIMPLANTATION RAT EMBRYOS

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Objective

Postimplantation rat embryo culture method[1] is widely used in developmental toxicology. This method requires rat serum as a culture medium, because of unidentified species-specific factors for embryonic development. To do this method, accordingly, it is necessary to use extra animals for the preparation of serum. We have been studying the species-specfic factors, to replace rat serum by easily available media, and subsequently, to reduce the number of animals to be used. We previously showed that a species-specfic factor (s) is contained in rat serum globulin fraction, using rabbit serum as a basal medium[2]. In the present study, we investigated the species-spcific factors in the globulin fraction.

Materials and Methods

Rat embryos at 9.5 days p. c. were cultured for 48 h. Rabbit serum was used as a medium and 25 % rat serum fractions were added. At the end of the culture period, embryonic development was assessed by the presence or aisence of heart-beat, axial rotation, yolj-sac diameter, crown-rump length, somite number, morphological score[3] and protein content. Rat serum globulin (GRS) was isolated by $(NH_4)_2 SO_4$ precipitation. GRS was divided into two fractions, unbound (UGR) and bound (BGR), by DEAE ion-exchange chromatography with stepwise elution. Prior to culture, these fractions were dialyzed against Hanks' balanced salt solution (HBSS). These fractions were analyzed by native-polyacrylamide gel elec-

trophoresis.

Results

In rabbit serum with 25% HBSS, no embryos showed heart-beat nor axial rotation. With 25% UGR, some embryos showed heart-beat and/or axial rotation, and crown-rump length, somite number, embryonic protein and morphological score increased. The embryos showed blood islands in the yolk sac. With 25% BGR, embryonic development was more advanced than that with UGR, but blood islands were not observed. With 25% UGR and 25% BGR, embryonic development was about the same as with BGR, and blood islands were observed. Electrophoretic analysis showed that there were no obvious protein bands common to UGR and BGR.

Discussion

The present results indicate that each of UGR a nd BGR contains species-specific factors. It is considered that these factors in UGR and BGR are distinct each other, since the features of embryonic development with these fractions were different and no additive effects were observed when these fractions were added together. This is supported by the results of electrophoretic analysis of the fractions. Thus there are multiple spcies-specific factors for rat embryonic development. For the use of alternative media in the embryo culture, these multiple factors will be essential.

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SOAKING OF NEWLY LAID SILKWORM EGGS : EXPLOIT OF A SUBSTITUTIVE TERATOGENICITY TEST SYSTEM.

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Great efforts have been concentrated to develop and exploit a sensitive system in teratogenesis studies to substitute for the pregnant mammalian system. The domesticated silkworm, *Bombyx mori*, is a promising organism, we believe, to lay open fresh avenues of knowledge towards the chemical induction of malformation. Since time immemorial, the silkworm has been under the patronage of man differentiated into a number of characteristic races and strains. The silkworm has moderate body size convenient to deal with, basic informations are available pertaining to the use of this insect as an experimental animal. The egg shell is very porous having many spiracles on the surface. Trials have therefore been attempted to find an effective method of mutation induction by egg treatment, either by painting with or soaking in chemical solution and by treating with gas where appropriate. If a given chemical can pass through the spiracles and penetrate into egg plasm, experiments on chemical teratogenesis as well as chemical mutagenesis would be conducted with ease. The effectiveness of the egg treatment was shown through the killing effect and/or the mutagenicity by using of some chemicals. Our results described here below will show that the soaking of newly laid eggs in aqueous solution of N-methyl-N-nitrosourea (MNE), a known muta-