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AN *IN VITRO* ASSAY SYSTEM FOR EYE IRRITATIONS USING ISOLATED PIG CORNEA

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Recently, many *in vitro* systems have been developed and studied vigorously to find a promising alternative to the Draize test. We developed a new *in vitro* assay system which may be useful in the screening studies on possible opacifying chemicals. The eyes were supplied by a slaughterhouse. The corneas were carefully removed and were then mounted on the perfusion apparatus.

The apparatus used in this assay system is small, light weight and can be handled easily. The combination of this apparatus and an opacitometer or photometer appears to be an effective means of accurately measuring the development of corneal opacity.

Several opacification tests on isolated pig corneas using the perfusion apparatus were carried out as the preliminary studies and the effects of distilled water and NaCl solutions of different concentrations were determined. When distilled water was applied to the intact cornea, a greater opacity developed than when a 0.9% NaCL solution was applied. When de-epithelialized cornea, de-endothelialized cornea or stroma only was exposed to a 0.9% NaCl solution,the corneal opacity increased to only a very small extent. The choice of vehicle for this assay system should, therefore, be made very carefully.

Moreover, the relation between pig corneas and bovine corneas with solutions of NaLS was studied. Although the opacity was related to the concentration of NaLS, the pig corneas were more reactive. It was thought reasonable to carry out all further experiments using pig cornea.

Based on these information, we have studied the relationship between opacity and concentration on some surfactants and industrial chemicals *in vitro*. These results have also compared to the results of *in vivo* studies. We believe that this test system is an applicable *in vitro* assay system for detecting opacifying.

USE OF PRIMARY RABBIT CORNEA CELLS AS A REPLACE OF RABBIT EYE IRRITATING TEST ; APPLICATION TO COSMETICS

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The cytotoxicity test *in vitro* using rabbit cornea cells was studied on raw materials of cosmetics. As there was a correlation between the cytotoxicity test *in vitro* and the Draize test *in vivo*, the cytotoxicity test *in vitro* was thought to be useful as a replacement of the Draize test *in vivo*. But the cytotoxicity test *in vitro* was not studied on cosmetics formulated by many raw materials. Therefore, we examined the cytotoxicity test *in vitro* using primary rabbit cornea cells as to not only detergents but also cosmetics, and compared the cytotoxicity test *in vitro* to eye irritating tests *in vivo* (Two eye irritating tests were studied, one is the Draize test the othere is the Modified Simamoto Method).

The results showed that there was a correlation between the cytotoxicity test *in vitro* and two eye irritating tests *in vivo* as to cosmetics as well as raw materials. These results suggested that, as for cosmetics as well as raw materials, the cytotoxicity test *in vitro* may be useful as a replacement of the eye irritating test.

IN VITRO ALTERNATIVES USING ESTABLISHED CELL LINES

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In recent years, a large number of *in vitro* toxicity assay systems using cells and organ cultures have been developed as alternatives to animal testing. The use of established cell lines in these tests can provide valuable informations for the prediction of chemical hazards with less time and at lower cost than *in vivo* studies. In this study, we tried to compare cell sensitivity among four cell lines: BALB 3T3 (mouse whole embryo), RC-1 (rabbit cornea), ARLJ301-3 (rat liver), FRSK (rat skin keratinocyte) in cytotoxicity tests of chemicals such as detergents, oil, and glycol. In addition, we investigated the effects of TPA in differentiation of FRSK cells. 100 ~ 500 cells were inoculated. After 24 hours, the cells were treated with chemicals for 7 ~ 10 days. The cells were fixed and stained at the termination of culture, then the colonies were scored. Keratinization of FRSK cells were detected by Rhodanile blue staining. Cationic detergents showed more sever effects than nonionic and anionic detergents. Oil and glycol were not toxic under this conditions. The sensitivity of BALB 3T3 cells was highest, followed in order by RC-1, ARLJ301-3 and FRSK cells.

TPA inhibited colony formation and keratinization of FRSK cells at the low

concentration of 0.1ng/ml. 1000 ng/ml of TPA did not inhibit colony formation of ARLJ301-3 and BALB 3T3 cells. These data suggested that the use of established cell lines had some advantages as the alternative method of Draize test, while colony formation test of FRSK cells can be available for screening of tumor promoters.

APPLICATION OF CULTURED NORMAL HUMAN CELLS FOR *IN VITRO* CYTOTOXICITY TEST AS AN ALTERNATIVE FOR DRAIZE TESTING

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Normal human differented cells, umbilical vein endothelial cells (HUV-EC) and epidermal keratinocytes (HEK), were designed to evaluate the cytotoxicity of chemicals in place of the Draize ocular irritating test. The cytotoxicity *in vitro* was determined by colony forming ability of both types of cells exposed to 11 chemicals for only 20 min after cells were incubated for 24 hours (short-term exposure) or exposed them throughout whole culture periods (long-term exposure). The cytotoxicity *in vitro* of chemicals tested for HUV-EC or HEK correlated well to the Draze score *in vivo*. The cationic detergents indicated more potent cytotoxicity than the non-ionic or anionic detergents. In particular, HEK were 10 to 1000-fold sensitive to the cationic detergents than HUV-EC. While the chemicals treated in the long-term exposure assay were more toxic those in the short-term exposure assay, close correlation between the cytotoxicity *in vitro* and the cytotoxicity *in vivo* was unchanged by exposure period of chemicals. These data suggest that the *in vitro* cytotoxicity assay systems using cultured normal human cells may be useful as substitutes for the Draize ocular irritating test.

AN *IN VITRO* CYTOTOXICITY SCREENING TEST FOR SHAMPOO PRODUCTS

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The Draize eye irritation test has been used to assess the potential eye irritation of most cosmetic products. Recently, *in vitro* cytotoxicity test has been given greater attention because of rapidity in obtaining test results, steadily growing public opinion on animal welfare and low costs. Under these circumstances, M.C.Scaife developed a new *in vitro* method asan alternative to Draize rabbiteye test. The principle of this method is measuring the cell-membrane integrity using fluorescein diacetate and ethidium bromide. We have modified this method and the

cytotoxicity of commercial shampoo products were determined. This results for 5 samples were compared with rabbit eye irritation data. The results were similar *in vitro* and *in vivo*. It was suggested that fluorescein diacetate-ethidium bromide test can reduce the rabbit eye irritation test on shampoo products.

APPLICATION OF NORMAL HUMAN EPIDERMAL KERATINOCYTES IN SERUM-FREE MEDIUM FOR *IN VITRO* TOXICOLOGY

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We evaluated normal human epidermal keratinocytes (NHEK) grown in a serumfree keratinocyte growth medium (K-GM,Kurabo) as an *in vitro* alternative method of Draize ocular irritating test. After two days of incubation in each well of 96-well tissue culture plates with test agent, growth and viability of the cells were estimated from the uptake of neutral red by the viable cells. Fifty-one chemicals including raw materials for cosmetic and medicine were used as test compounds.

The correlation coefficient between *in vivo* Drai ze score and *in vitro* test using NHEK was 0.83. In addition, the method using NHEK was more sensitive to a given concentration of test agants than the colony forming assay using rabbit cornea primary cells.

Toxicity assay using NHEK also can be done with MTT. But the color intensity developed by MTT was only one-fifth of that developed by neutral red. Thus,neutral red is more suitable than MTT for the toxicity assay with NHEK.

These results suggested that the cytotoxity test *in vitro* using NHEK is useful as substitutes for the *in vivo* Draize eye irritating tests.

USE OF RABBIT CORNEA CELLS AS AN ALTERNATIVE TO THE DRAIZE EYE IRRITATION TEST

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We studied *in vitro* procedures based on reproducidle ability of cells using cultured rabbit cornea cells as an alternative to the Draize eye irritation test. We have reported the colony forming assay using primary rabbit cornea cells would be one of the most appropriate alternatives. Therefore, this study was designed to make a more simple, econamical and objective method to measure cytotoxicity using neutral red and/or Giemsa solutions. Rabbit cornea cells cultured in a 96-well tissue-culture plate were incubared

for 48 hurts with various concentration of test-chemicals. Then, a part of plates were incubated for 3 hours with culture medium containing 50 μg/ml of neutral red. After incubation, the dye in the cells was extracted by acidic ethanol (1% acetic acid in 50% ethanol). The rest plates were stained with 5% Giemsa solutions. The day was extracted by acidic ethanol(ethanol:1N HCl=9:1). The optical density of the extracted solution from each well was determined using a microplate reader. Cytotoxic effects of test materials was expressed as the ratio of intensity of the extracted color from treated well to that from untreated control. We used 52 chemicals as test materials. The relative toxicity data determined for test chemicals by both methods generally showed a good correlation with published results obtained from Draize test *in vivo*. The results demonstrate that these methods are suitable model systems for screening chemicals without animals. Furthermore, there was difference in staining pattern between neutral red and Giemza in some chemicals. These data suggest that test-chemicals may be classified by mechanism of cytotoxic effects.

PREDICTION OF HUMAN OCULAR IRRITANCY BY THE LOW-VOLUME EYE TEST, A REFINED ALTERNATIVE TO THE DRAIZE TEST

John F. Griffith

The Procter & Gamble Company

Thank you for your gracious invitation to Dr. Yam and mtself to speak at this Third Symposium of the Japan Alternate Animal Research Association. It is an honor to be here.

The purpose of my talk today is to describe research carried out at the Procter & Gamble Company, Cincinnati, Ohio, U.S.A., in developing an alternative procedure to the Draize rabbit eye irritation test. The test to be described is the Low-Volume Eye Irritation Test.

The Draize test is the procedure most widely used in the world today. It was originally developed by Dr.John Draized and colleagues at the United States Food and Drug Administration (USFDA) in the early 1940's for evaluating the safety of drugs and other products used in or around the eyes[1]. This procedure, with slight modifications,is today part of the testing codes and requirements of many countries and international agencies.

The Draize test has been criticized for having several deficiencies[2, 3], among them being high interlaboratory variability, large animal-to-animal variability within tests,much greater sensitivity of the rabbit eye than the human eye due to differences in anatomy and physiology, a specified dose of 100 μl that probably greatly exceeds typical human accidental exposures, at least of consumer products, and a dosing procedure that unrealistically prolongs contact of the test substance with the eye.These factors tend to greatly exaggerate the irritant potential of substances for the human eye and reduce the sensitivity of the test for making comparisons of moderate to strong irritants.

Added to these and other technical criticisms of the test has been the growing opposition in Europe and the U.S. to any use of the test on the grounds that it is inhumane as well. This has led to efforts in many countries by animal 'rights' advocates to have the test banned. Unfortunately this movement is in direct opposition to regulatory requirements to use the test and the fact that there is as yet no validated or widely

accepted alternative to the Draize test.

Many proposals have been made on how to improve the Draize test, most of which relate to more precise or objective techniques for evaluating irritant responses. None of these suggested changes that would make the test more humane or more predictable of human experience. Howevere, in 1977 a review of the subject in the U.S. by a National Academy of Sciences (NAS) committee[4] suggested that a smaller dose than the 0.1g or 0.1ml specifiedin the Draize procedure could reduce the response of eyes of test animals to a range more consis tent with available information on human experience. At Procter & Gamble we acted on this suggestion by cayying out a study of dose response characteristics of 21 chemicals, solutions and mixtures with known degrees of irritant potential to the human eyes[5]. We studied dose volumes in the range of 3μl to 100μl andconcluded that 10μl applied to the corneal surface most consistently identified substantial and severe irritants, using NAS criteria.

We then developed a test protocol based on the 10μl does. We called it the low-volume eye test and began to use it in parallel with the conventional Draize procedure. This provided us with comparable data bases. After a few years testing experience, we dropped further use of the classical Draize test and now used only the low-volume test for making safety assesments.

Our basis for relying exclusively on the lowvolume test for ocular safety assessments comes from human experience we have documented with detergent products that have been tested by both the Draize and low-volume test. This human experience is primarily from consumers who have splashed the products in their eyes and have then contacted us by telephone. In many such cases, through prompt follow-up, we have been able to establish the number of hours or days for complete recovery from the accident. When a sufficient number of such cases have been accumulated for a given product, the median recovery time can be compared with those for Draize and low-volume test.

From 1979-81 we followed up on 171 consumer eye accidents with 10laundry,dishwashing and household cleaning products that had been tests by both animal methods[6]. Correlation coefficients and p values for human vs. low-volume test date and human vs. Draize test data are shown in Table 1. We did another such followup study in 1983-84, of 218 eye accidents involving 12 products[7]. As in the earlier study, there was a stronger correlation between human recovery time and low-volume test recovery rimes than between the human and Draize testdate. Figure 1 shows the distribution of recovery times for the 12 products surveyed in 1983-84, the 10 for 1979-81, the low volume and the Draize rabbit test

Encouraged by the above results, but desiring more controlled human exposure data, we next conducted a clinical study of the ocular irritancy of four diluted products on human volunteers and compared the results with results obtained with the same dose volumes and product

Table 1. *Comparison of Median Days for Recovery from Eye Exposures Consumers vs. Low-Volume and Draize Test Results*

Comparison		*Correlation Coefficient*	*pValue*[a]
1979-81	human data vs. low-volume[b]	0.82	0.004
1979-81	human data vs. Draize[b]	0.52	>0.1
1983-84	human data vs. low-volume[c]	0.89	<0.001
1983-84	human data vs. Draize[c]	0.48	>0.1

a Probability that relationship is due to chance.

b 10 products; Freeberg, *et al. J. Toxicol. - Cut to Ocular Toxicol.* 1(3), 53-64(1984).

c 12 products; Freeberg, *et al.* ibid. 5(2), 115-123 (1986).

Table 2. *Comparisons of Statistical Parameters for Mean Recovery Time Animal vs. Human*

Comparison	*Correlation Coefficient*	*pValue*
10 μl. human vs. 10 μl. animal	0.66	< 0.037
100 μl. human vs. 100 μl. animal	0.35	>0.316
10 μl. human vs. 100 μl. animal	0.40	>0.256
100 μl. human vs. 10 μl. animal	0.72	<0.018

Freeberg *et al. Fundamental Appl. Toxicol.* 7, 626-634 (1986).

concentrations using the low-volume and Draize test methods[8]. Human subjects' eyes and animal eyes were all graded by the same trained individuals. Although human recovery times were always less than those of rabbits in comparable exposures, we once again found much better correlation between the low-volume test (10 μl.) and human exposure, in terms of recovery time, than between the Draize test (100 μl.) and human exposure. This was true whether the human subjects were exposed to 10 μl. or 100 μl. volumes of the test materials (Table 2).

In order that one may appreciate the magnitude and the difference between 10 μl. and 10 μl., Figure 2 shows photographic simulations of these amounts of powdered detergent on the surface the human eye. We estimate that 10 μl. well exceed the amount of material that contacts the eye in most accidental exposures.

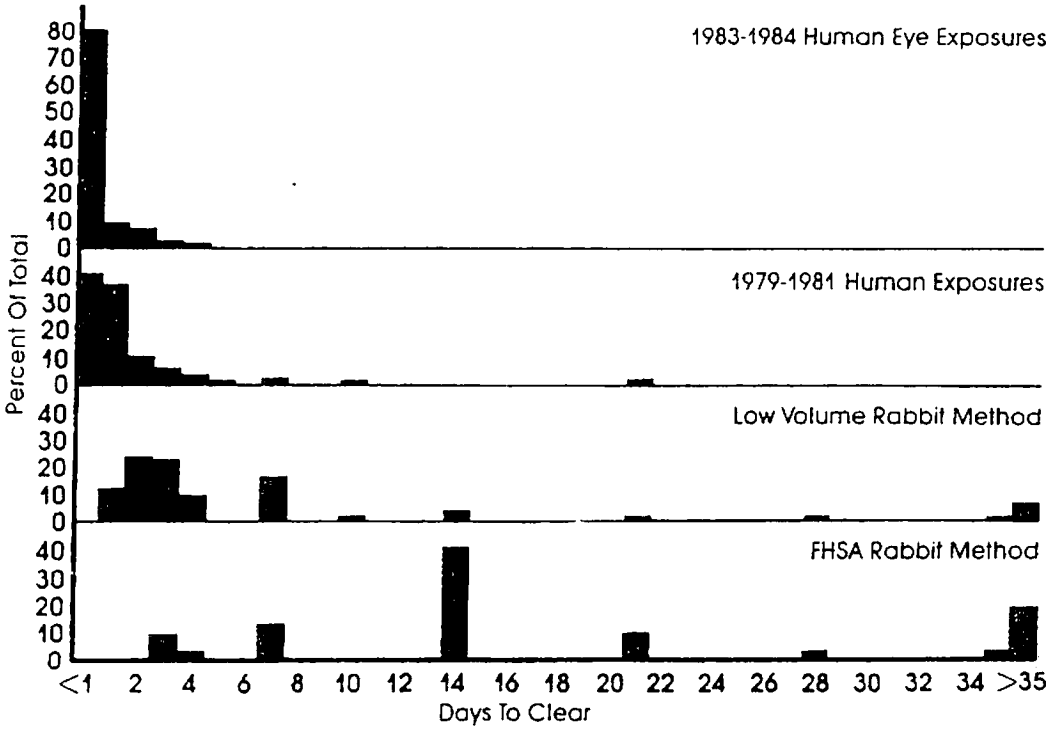
Other companies beside Procter & Gamble have evaluated the use of a 10 μl dose volume in eye irritation testing[9]. In 1987 Frazier et. al., in a random sampling of U.S. contract pharmaceutical and chemical company labs found that 10% were using a 10 μl test volume and otherswere evaluating it[10]. Two industry associations in the U.S., the Soap and Detergent Association and the Cosmetic Toiletries and Fragrance Association are presently carrying out programs to evaluate nonanimal alternatives to the Draize test. Because of growing interest in the low volume test as an immediately available, more humans procedures, it is being included in portions of these programs. Procter & Gamble is fully supportive of such activities, for we believe that ultimately the more accurate low volume test will prove to be a better validation standard for evaluating alternative eye tests than the Draize test itself[11].

In France, OPAL, an organization for the preservation of animal laboratories, is conducting an experimental program to evaluate the low-volume method along with two *in vitro* alternatives to the Draize eye test.

In 1986, Switzerland and the United Kinfdom sponsored a proposal before the Organization for Economic Cooperation and Development for consideration the low-volume method as an alternative to the Draize test, which was then and is still the official procedure of the OECD. The expert panel who reviewed the proposal concouded that the method had merit, but that there was insufficient world experience with it involving a wide variety of substances for them to recommend its adoption as a guideline procedure. They did urge member countries, in cluding Japan, which was represented on the expert panel by Mr. S. Yamane of CITI, to try the method in order to gain experience with it.

To data, no international or national regulatory body has adopted the method, although a few, including the Environmental Protection Agency and the Food and Drug Administration the U.S. are evaluating it. If the method is eventually accepted, we expect at first its use will be for testing limited classes of materials. We feel confident, however, that greater familiarity with this less stressful and more accurate procedure will gain broader support by both industry and regulatory toxicologists.

Fig. 1. Distribution of exposures, time for eye to clear (1984 human consumer date compared to 1980 humam date and animals tesss)



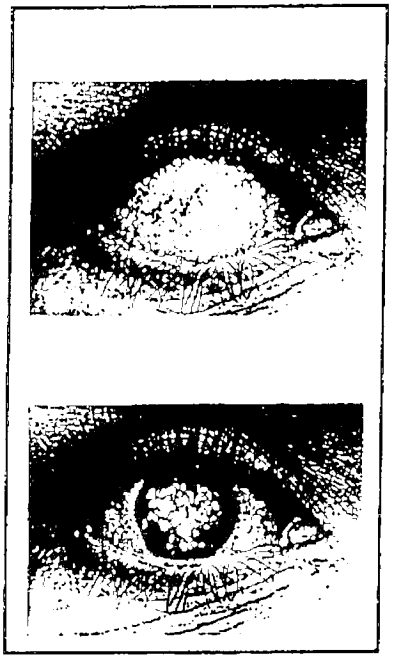


Fig. 2. Simulation of 100 μl (above) and 10 μl solid material in human eye.

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AN ALTERNATIVE METHOD TO THE DRAIZE EYE IRRITATION TEST-USE OF LIPOSOMES PREPARED FROM CONEAL LIPID

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Various *in vitro* methods have been proposed for predicting the ability of chemicals to cause ocular irritation, These methods use isolated eyes or established cell lines derived from the cornea of rabbit eyes. The principle of these methods lies in the determination of the toxic effects of the test materials on the cells under defined conditions.

In the study reported here, liposomes prepared from the lipid extracts of the cornea of bovine eyes were used as a model of corner cells. Liposomes containing 4-methylumbelliferyl phosphate (Um-P) were incubated with 12 surface-active agents. The Um-P released from the liposomes by each test agent was hydrolyzed with alkaline phosphatase and the resultant 4-methlumbelliferone was assayed spectrofluorometrically. The values for Um-P_{50} (the concentration of test material at which 50% of Um-P is released) showed a good inverse correlation with the irritation scores obtained by the Draize eye test. The correlation coefficients between Um-P_{50} values and corneal scores and b etween Um-P_{50} values and total sclres were -0.737 and -0.734, respectively. The lipid compositions of cornea of bovine eyes were decided by thc the analyses of thin-layer chromatography and gas-chromatography. And the liposomes were reconstituted by the results of both chromatographical analyses.

The results suggest that the liposomal assay reported here may be useful as an *in vitro* model for predicting eye irritancy of chemicals.

AN ALTERNATIVE METHOD TO THE DRAIYE EYE IRRITATION TEST-USE OF CHORIALLANTOIC MEMBRANE OF THE CHICK EMBRYO

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The applicability of the chorioallantoic membrane (CAM) test using the fertile eggs as an alternative method to the Draize eye irritation test was examined. Twelve surfactants were applied onto the CAM on day 10 of incubation. Each CAM was observed macroscopically for hyperaemia, haemorrhages and coagulation at 0.5, 2 and 5 minutes post treatment. The score was assigned based on the time of onset of each hazardous effect. There was good correlation between the scores obtained by CAM test and those by Draize test ($r=0.86$), suggesting that the CAM test be useful as an alternarive model. However, the scoring system has disadvantage as for its objectivity and quantitativeness. To overcome these defects, trypan blue method was evaluated in which injured cells would be stained and quantitatively measured. Five minutes after the application of surfactants onto the CAM, the CAM was treated with 0.1% trypan blue in phosphate buffered saline (pH 7.4) for 1 minute. Trypan blue adsorbed in the lesion was extracted with formamide and measured spectrophotometrically. The amount of the pigment extracted showed a good correlation with the Draize test scores ($r=0.89$). The results suggest that trypan blue staining method be more suitable than the original CAM test for predicting eye irritancy of chemicals.

ESTABLISHMENT OF SPONTANEOUSLY TRANSFORMED HUMAN UMBILICAL VEIN ENDOTHELIAL CELL LINE AND ITS ABILITY OF PLASMINOGEN ACTIVATOR PRODUCTION

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A new cell line from human umbilical vein has been established and maintained for more than 5 years.This strain designated ECV304 is characterized by a cobblestone monolayer growth pattern, high proliferative potential without any specific growth factor requirement, anchorage dependency with contact inhibition. karyotype analysis of this cell line revealed to be human chromosomal constitution with a high trisomic karyotype. Ultrastructurally, endothelium - specific Weibel - Palade bodies were identified. Immunocytochemical staining

for the von willebrand factor, Ulex europaeus I lectin, and human large vessel endothelium antigen was positive. And angiotensin converting enzyme activity was also demonstrated. From these observations described above, ECV304 were shown to be of an immortalized endothelial cell line of human origin.

The serum-free conditioned medium of ECV304 has fibrinolytic activity at the molecular weight 50 KD by fibrin autography. It also has fibrinolysis inhibiting band at 45KD by reverse fibrin autography. They seem to be urokinase type plaminogen activator (uPA) and plasminogen activator inhibitor-1 (PAI-1). By enzyme immunoassay, ECV304 conditioned medium contained the same level of uPA antigen as in primary-cultured human umbilical vein endothelial cells, although tissue type plasminogen activator(tPA) and PAI-1 antigens were both much decreased.

Furthermore, the activity of the uPA increased by plasmin treatment and the activity was not influenced by phenyl methyl sulfonyl fluoride treatment. These observation suggest that the uPA of ECV304 issingle chain pro-urokinase type PA.

This work is a report on novel case of phenotypic alteration of normal venous endothelial cells of human origin *in vitro* and qeneration of a transformant with indefinite life spans. This line may be useful in studies of various functions of blood vessele.

AN EFFICIENT METHOD OF LONG TERM PRIMARY TISSUE CULTURES OF MOUSE DORSAL ROOT GANGLION (DRG)

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The long term primary tissue cultures of DRG obtained from 18-day fetal mice were efficiently achieved with high concentration of 2.5sNGF in addition to the medium containing of horse serum and chicken embryo extracts.

A preliminary experiment of chronic ethanol effect of the culture was observed by Laser Scan Microscopes.

ESTABLISHMENT OF WILD-TYPE AND ACATALASEMIC MOUSE CELL LINES

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Permanent fibroblast cell lines have been established from wild-type C3H / Cs[a] a and mutant C3H / Cs[b] mouse. The latter have lower levels of catalase enzyme activity than the former. When compared to wild - type C3H cells, the catalase-deficient cells are more sensitive to the toxicity of hydrogen peroxide. Furthermore, They are more sensitive to X - rays and near ulraviolet ligt. Contrary to our expectation, they are more resistant to bleomycin. These cell lines will be of use in the study whether active oxygens play in the several physical or chemical drugs.

SUSCEPTIBILITY OF INSECTS AND THEIR CULTURED CELLS TO CHEMICALS

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Effects of rotenone on the growth of several continuous cell lines of insects and on mortality of the insects when applied topically to whole body insects were examined. The susceptibility was different between cell lines derived from different insect species. Within a species the susceptibility was also different between cell lines derived from different tissues. The susceptibility decreased in the order of cabbage armyworm ovarial cell lines, fat body cell lines of the same species, swallowtail butter fly ovarial cell line and cabbage armyworm hemocyte cell lines. The LD_{50} value of rotenone for the 3rd instar larvae of cabbage armyworm was 5 mg/g. The 3rd instar larvae of swallowtail butter fly was found to be insensitive to rotenone when topically applied.

INVESTIGATION OF MASS TESTING METHOD FOR NEURONAL ANESTETIZATION USING HOUSEFLY

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We investigated the neuronal anesthetization of housefly by administration of glutamic acid, a transmitter of excitatory neuromuscularinjection. The anesthetized rate (M) was followed the function of $M = \alpha_0 t\ EXP(-kt)$rather than that of Probit method. The similar results were given by glutamine and arginine,and also taurine which has an effect of inhibitory neuromuscular transmitter. The result suggests that it is better method than Probit analysis for assay of neuroactive compounds.

THE USE OF MOUSE WHOLE EMBRYO CULTURE AS THE ALTERNATIES IN EMBRYOTOXICITY AND TERATOGENICITY TEST

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Mouse whole embryo culture requires much amount of rat serum although having many advantages as the alternative for animal tests *in vitro* screening system. In the present study,

in order to decrease a lot of using exprimental animals, ICR mouse embryos were explanted at day 11 of gestation and embryos were cultured one per 25 ml bottle containing 5 ml of heatinactivated 100% rat serum or synthetic medium (ASF-301) for 24 hours at 38℃ with the oxgenating gas mixture of 95% O_2 5% CO_2 using the rotator.

As regards heart rate, crown-rump length, total protein contents and total somite number, embryos cultured in ASF-301 showed almost same as those cultured in 100% rat serum. These results indicated that moues embryos could be without rat serum by ASF-301.

PRELIMINARY NOTES ON THE DEVELOPMENTAL TOXICITY ASSESSMENT OF ETHYL ALCOHOL IN WHOLE EMBRYO CULTURE AND TISSUE RECONSTRUCTION SYSTEMS

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Rat embryos of Slc:SD strain on day 9.5 of gestation were cultured for 48 hours in 100% rat serum containing different concentrations of ethyl alcohol(EtOH)by New's method[1]. The embryos were examined on the morphological development using the system by Brown and Fabro. A few embryos treated with 0.5% of EtOH had abnormalities including growth retardation, reduction of somite formation and open neural tube. On the contrary, these abnormalities were produced in almost all embryos treated with 0.75% of EtOH and the severity was increased with the increasing concentration of EtOH.

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 containing EtOH in various concentrations. The number of cell aggregates increased at 2 times in 1.5% and 50 times in 2.0% of EtOH snd the size decreased at 53 % in 1.5% and 15% in 2.0% in the major axis compared with that of the control specimen respectively. Treatments with 3% over of EtOH completely inhibited the aggreagte formation.

These results suggest that the critical dose in the present system may be around 0.5% of EtOH in developing rat embryos and also around 1.5% in the tissue reconstruction with dissociated fetal brain cells.

USE OF CHICK EMBRYO IN SCREENING FOR EMBRYOTOXICITY. 1. EFFECTS OF CYCROPHOSPHAMIDE ON CHICK EMBRYOS AND RAT FETUSES

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Cycrophosphamide (CA) an alkyrating agent which developed as an anticancer drug were used in this study to compare the toxicity of this durg on the developing chick embryos and rat fetuses. A single dose of CA (0.05 ㎎／0.04 ml／egg) was injected into the yolk sac of chick embryos at day 5 of incubation.The eggs were opened at day 19 of incubation. In rat, pregnant mothers received a single intraperitoneal injection of CA (12 ㎎／1 ml／Kg) at day 13 of pregnancy. They were sacrificed at day 20 of pregnancy.

Death rate in the chick embryo was 16.2% in CA injected group, 12.3% in saline injected group and 6.5% in intact group. Malformation rate was 7.0% in CA injected group, 3.8% in saline injected group and 0 % in intact group. In histologically, the germ cells in the testes and ovaries were not or slightly observed in CA injected group. Accordingly, in the testes, thr seminiferous tubules were not developed well, and in the ovaries the cortex was strikingly thin.

In the rat,dead and absorped fetuses were not observed in both CA injected and saline injected groups. Malformation rate was 69.2% in CA injected group. In histological observation, the germ cells in overies were fewer in CA injected group than that in saline injected group. However in the testes histological difference was not observed in both CA injected and saline injected groups.

In present study, incidence of the external malformation induced by CA was higher in chick embryos than that in rat fetuses. However, abnormality of the gonads was clearly defined in chick embryos compared with that in rat fetuses. In this result, the chick gonad seems to have a sensitivity to drugs. Thus, it suggests that the use of chick embryo may have some advantage to evaluate the embryo toxity of the drugs.

HEN'S FERTILE EGG SCREENING TEST (HEST): DETERMINATION OF ANDROGENIC ACTIVITY OF STEROIDS AND LD 50 OF DRUGS

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Regarding the actual discussion of refinement of animal handling, reduction of number of animals and replacement of animal experimentation, hen's fertile egg screening test (HEST) is attractive for determination of activities and toxicities of drugs. For a toxicity

test,15-day-old developing chick embryos were administered different doses of drug through air chamber and numbers of dead animals were counted at 48 h after the treatment. LD50s were determined and their values were compared to the values listed in the Registry of Toxic Effects of Chemical Substance (1985 - 1986 Ed.) . Androgenic activity was determined by weghing wet weight of comb. As a result, it was demonstrated that HEST could be used for determination of LD50 of drugs and androgenic activities of steroids.

AN ATTEMPT TO DEVELOP COMPUTER - SIMULATED ALTERNATIVES TO *IN VIVO* LD_{50} ASSAY

Yonehara, Y., Aoyama, T., Kimura, H., Ikebuchi, M., Terado, T., and Sugahara, T[1].

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To investigate possibility of application of a computer simulation for alternatives to *in vivo* LD_{50} assay, we carried out the calculation of LD_{50} with the computer simulation using data of *in vitro* cell sensitivities to various drugs which were mainly anticancer agents. The method of the computer simulation was based on that for radiation-induced late effects by " System Dynamics "which was developed by Aoyama *et al.*.

The LD_{50} values calculated with the data from cultured cells of mice were in better agreements with the values obtained experimentally than with the data from Chinese hamster cells. Especially close agreement was found in the value for bleomycin. The reasons for the good agreement may be that the drug has similar mechanism of action for cell killing to that of X-ray and that the two sets of parameters of cell survival curve for 2 phases were used for the simulation.

We also calculated D_0 values for the target cells using the data of LD_{50} and analyzed relationship between estimated values and those derived from experiments with cultured cells of mice. On these calculation, the target cells of the drugs were assumed to be in the hematopoietic and digestive organs. From the analysis, there were almost linear relationships between the values. Further incorporation of the *in vitro - in vivo* D_0 relationships into this simulation system using more data on the drugs with different action mechanisms would be needed in order to improve the computer-simulated alternative system.

CURRENT STATUS OF CELL BANKING AND CELL LINE DATA BASE IN JAPAN

Ohno, T[1]., Mizusawa, H[2]., and Takeuch, M[3].

1 *Cell Bank, RIKEN GENE BANK.*, 2 *Cell Bank, Nat. Inst. Hygene.*, 3 Cell bank, Inst. Ferment.-Osaka.

Cultured animal cell lines are expected to be the best alternative for animal testing. We here descrive about 7 CELL BANKs recently opened to public in Japan. Although scale of each CELL BANK is rather small if you compare to those in US, over 2,600 distributions of cultured animal cells have been made last year.

We are now developing "Inter-Bank Data Base System" to utilize efficiently data of cultured cell lines hold in each CELL BANK.

INTRODUCING ALTDBASE : DATA BASE ON THE REFERENCES OF ALTERNATIVES OF ANIMAL TESTING

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The Research Group Japan Alternatives to Animal Testing have eatablished ALTDBASE, a unique data base on the references of alternative methods to animal experiments. During the last two decades,there has been growing interest in the use of alternative techniques for animal experimentation. Although this is to be welcomed it should be recognized that this rapid rate of expansion has led to a number of difficulties for scientists working in the field. One major problem is the lack of availability of relevant and reliable up-to-date information on the techniques currently in use and under development.

Although a large number of publication are now in existence, it is relatively difficult for research scientists to survey the published literatures as they appear in great diversity of literatures. The principal aim of ALTDBASE to provide an easy-to-access information data base for references of altenative methods to animal experimentation. The data base now contains about 500 literatures, and based on a commercially available relational data base software "RBase Pro" being operated on NEC personal computer PC-9801 UV.

The data base can be easily accessed via public telecommunicatin system, either domestic or international.

STUDIES ON THE MECHANISM OF EYE IRRITATION INDUCED BY CHEMICALS IN RABBITS : USE OF AN *IN VITRO* SYSREM USING THE RED BLOOD CELLS OF RAT FOR PREDICTING CHEMICAL EYE IRRITANTS

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The red blood cells of rat were used as *in vitro* system to study the mechanism of eye irritation induced by chemicals in rabbits.

One hundred and twenty chemicals including drugs, reagents, surfactants and solvents were used in this study. The primary eye irritation in rabbit was evaluated according to Draize's method. The *in vitro* system consists of two methods, effects of chemical on lipid membrane and protein could be detected by induction of hemolysis and production of methemoglobin from eluted hemoglobin, and effects on precipitation of protein could be detected by induction of maximum production of precipitation of γ -globulin, respectively.

In primary eye irritation test, acids, bases and surfactants induced severe irritation on rabbit eye. In the *in vitro* system, bases and surfactants with strong effects on eye induced hemolysis. The minimum concentrations of surfactants for hemolysis were lower than that of bases. Bases induced production of methemoglobin, and acids induced aggregation of red blood cells and precipitation of methemoglobin. The chemicals with slight effects on eye induced slight or no effect on red blood cell and hemoglobin.

The adaptive least-squares method was used for analysis of correlation between eye irritation classes and the results from the *in vitro* system. In this study, a three-classes discrimination was made for eye irritation. Three parameters were used for the discrimination of chemicals. Minimum chemical concentration for induction of hemolysis and maximum production of methemoglobin were used as parameters which indicate the effects chemicals on lipid membrane and protein, respectively. Maximum production of precipitation of γ -globulin was used as a parameter which indicates the preciptation effect of chemical on protein. The accuracy in classifying the chemicals was 80 % in the recognition.

These results suggest that the *in vitro* system using red blood cells of rat can contribute to classify the mechanism of eye irritation and predict eye irritation of chemicals.

EYTEX, AN *IN VITRO* TEST TO PREDICT OCULAR IRRITATION

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The EYTEX method is an *in vitro* test to predict ocular irritation of chemicals and formulations based on alterations in a protein matrix. Results of a multi-laboratory study of one hundred and

thirty samples have been published (Kelly, C. P. et al, Pharmacoperial Forum, Jan-Fed, 1989). Four independent studies were conducted to further evaluate the accuracy, reproducibility, and applicability of the EYTEX method. This method was performed according to the previously published procedures.

Dose-response curves were produced at concentrations of samples similar to the Draize test. Spectrophotometric data was used to establish irritancy classifications, and an EYTEX / Draize Equivalent was calculated. These studues included a wide range of chemicals and formulations with varying degrees of irritation and mechanisms of ocular toxicity. Nine hundred and twenty ond cosmetics, household, petroleum, and agricultural products were analyzed by the EYTEX method. Seven hundred and thirty six EYTEX results were within one classification of the Draize result. The EYTEX results exhibited an overall substantial equivalence of 91% to the Draize results with a range of 89% to 93% depending on the particular product group. The inter-assay coefficient of variation was 10.7%. These studies indicate that the EYTEX method exhibits a high correlation with the Draize test for the four diverse product groups studied and validates the predictive potential of the EYTEX method as an *in vitro* screen for ocular irritation.

DETERMINATION OF INTRACELLULAR FREE CALCIUM ION DISTRIBUTION WITH FLUORESCENT CALCIUM INDICATOR USING VIDEO IMAGE PROCESSING

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We constructed an instrument for determining the distribution of intracellular free Ca^{2+} concentration ($[Ca^{2+}]i$) using fluorescent Ca indicator fura-2. This instrument consists of an inverted vertical light fluorescence microscope attached to a xenon lamp and automatic changer of excitation filters, and image processor.

Fura-2 may interact with intracellular proteins. We used a new Ca concentration buffer containing 80mg/ml bovine serum albumin.

In the case of stimulation with 80mM KCl, the distribution image of $[Ca^{2+}]$ in NG108-15 cells increased around the cell membrane of peripherary fast, and then in interior. On the contrary, The change of $[Ca^{2+}]i$ with stimulation of bradykininbegun from within cells, due to the release of Ca^{2+} from intra-cellular calcium store site. We also measured of intracellular pH distribution on the same instrument using fluorescent pH indicator BCECF.

CHEMILUMINESCENT ASSAY FOR RAPID DETERMINATION OF VIABLE CELL NUMBER AND ACTIVITY OF CELLS

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The production of H_2O_2 by intact cells is promoted on the presence of menadione and is proportionsl to the density of viable cells. The concentration of produced is determined by measurement of chemiluminescence which is generated in the mixture of H_2O_2, pyrene, and bis(2, 4, 6-trichlorophenyl) oxalate. Viable yeast cell number above 10^4 cells/ml is determined for 2 min, and mammalian cell density and the activity of plant tissues are determined for 10 and 5 min, respectively. This method is applied to the measurement of viable cell number after the addition of antibiotics, anticancer drug, cell growth factor and various stress.

ALTERNATIVE METHODS FOR ACUTE ORAL TOXICITY TESTING

John Yam, D. A. B. T.

The Procter & Gamble Company

It is my honor and pleasure to speak to such a distinguished group today.The title of my talk is "Alternative Methods for Acute Oral Toxicity Testing". But before I get into this topic, I'd like to spend a few minutes introducing you to the philosophy and approach taken by The Procter & Gamble Company (P&G) on the development of alternative testing methods.

The Procter & Gemble Company is a multinational company with its headquarters in Cincinnati, Ohio, U.S.A. It markets a wide range of consumer products including laundry and cleaning products, personal care products, food and beverage products and pharmaceutical products.

Since so many homes use one or more of P&G's products, it should be of no surprise that we place very high regard on product safety. In fact, it is written in our Company policy that "P&G products shall be safe for humans and the environment under conditions of intended use and foreseeable misuse". And, "P&G products shall meet the requirements of all relevant laws and regulations".

At the same time, P&G is also fully committed to the responsible use of animals. The Company has an Animal Use Policy which states P&G's commitment to:

- The use of animals in testing only when necessary and only when no acceptable alternatives exist;
- The highest ethical and professional standards in animal care and treatment;and
- The continued development, validation and adoption of new test methods which eliminate the need for animals (REPLACEMENT), reduce the number of animals used (REDUCTION), or which are less stressful (REFINEMENT).

I'm delighted to find out your association also

Table 1. Major Criticisms of the classical LD_{50} test

A precise LD_{50} is not necessary.
The test neglects morbidity or toxicity.
The animals are subjected to unnecessary pain and distress.

defines "alternatives" the same way. That is, "Replacement, Reduction and Refinement," or the 3Rs. While replacement should be our ultimate goal, it is only practical and scientifically sound to invest part of our research efforts on reduction and refinement which are more likely to bring success in the short term.

At Procter & Gamble, we have taken this misson to search for alternative methods to animal testing seriously. To date, a number of scientists at our Miami Valley Laboratories in Cincinnati are devoted to alternative research. Our in-house programs are broad and cover a number of focus areas, including acute oral toxicity, eye irritation, skin irritancy, contact sensitization and photoallergy, genetic toxicology, respiratory toxicology, and noninvasive techniques.

In addition, we also support a number of research programs at universities. In June of this yeay, we announced a new program, called The Universety Animal Alternatives Research Program, to further encourage development of alternative methods. The program will fund up to $50,000 U.S dollars for three years perresearch program, and three research programs each year.

Now I would like to return to the topic of alternative methods for acute oral toxicity. The classical LD_{50} test, which was first introduced in 1927 by Treven, a British toxicologist, has been for many years the standard test for acute oral toxicity[2]. As many of you are aware, the classsical LD_{50} test has come under increasing criticism from the animal rights community and some scientists[3] (See Table 1). In the U.S., legislation has been introduced at the federal and state levels to ban the LD_{50} test.

To date, regulatory agencies in the United States, including the Food and Drug Administration (FDA), the Environmental Protection Agency (EPA), and the Consumer Product Safety Commission (CPSC), discourage the use of the classical LD_{50} test, and accept the submissoin of data from alternative methods[4-5]. The 1985 report on acute oral toxicity, prepared by the European Chemical Industry Ecology & Toxicology Center (ECETOC), also took a similar position[6].

While it is generally accepted that the determination of a precise LD_{50} is not necessary, the scientific community generally agrees that acute oral toxicity testing is still necessary to determine the toxic properties of a chemical[3-6].

Table 2. Desired Information From an Acute Oral Toxicity Study

• Signs of toxicity	• Dose-response relationship
- body weight changes	- estimate of median lethal dose
- clinical observations	- slope of the dose-responese curve
- gross pathology	- highest nontoxic dose
• Onset, duration and reversibility of toxic effects.	• Target organ(s) of toxicity

A few years ago, P&G discontinued the of the classical LD_{50} test and adopted two alternative methods--The Limit Test and the Up-and-Down Method--for use in acute oral toxicity studies in rodents.

I'm sure most of you are familiar with the Limit Test. It uses a single "limit" dose which is often 2000 mg／kg body weight. Usually 10 animals (5 males, 5 females) are used. The test provides information on LD_{50} relative to the selected dose, as well as some information on signs of toxicity and the mode of action. However, the test provides no information on does-response.

The Limit Test is most suitable for chemicals that are relatively nontoxic, and compounds that have some information on their acute toxicity from which an appropriate limit dose can be selected. It is also useful in positioning a chemical relative to an arbitrary category or level of toxicity. The test is accepted by a majority of regulatory agencies, including those in the U.S., Europe and Japan.

For chemicals for which there is little or no information on acute oral toxicity, the Up-and-Down Method is preferred. This is a statistical procedure described by Dixon and Mood[7], Which was adapted for acute oral toxicity testing in rodents by Bruce of P&G[8-9]. In this method, animals are dosed one at a time, starting the first animal at the toxicologist's best estimate of the LD_{50}. If this animal survives at the end of 24 hours, the next animal receives a higher dose. If the first animal dies, the next animal receives a lower dose. The dosing is repeated until four animals have been dosed after reversal of the initial outcome. If only one sex, e.g., the female, is used, which is usually adequate, the test requires only 6-10 animals. In addition to signs of toxicity, the test provides an estimated LD_{50} value using the method of maximum likelihood.

The LD_{50} values obtained from the Up-and-Down Method were found to correlate very well with values obtained from the classecal LD_{50} test, both in simulation studies using historical data, and in actual parallel testing[8-9]. The Up-and-Down Method is acceptable to the U.S. regulatory agencies, and has been published as a standard test method by the American Society for Testing and Materials (ASTM-E1163-87)[10].

The British Toxicology Society recently proposed another approach to evaluate acute oral toxicity, called the Fixed-Dose Procedure. The procedure focuses on toxicity rather than lethality as an end point. Recently, mare than 26 laboratories, including several laboratories from P&G and Japan, participated in an international validation study of the Fixed-Dose Procedure. The results were presented last month in Brussels at the Commission of the European Communities-sponsored workshop on LD_{50} testing. The results suggest that the Fixed-Dose Procedure can be used as an alternative test method for acute oral toxicity.

Table 3 provides an overall comparison of the three alternative methods that can be used in

Table 3. Summary of Alternative Methods to Acute Oral Toxicity Testing

	Classical LD_{50} test	Limit Test	Up-and-Down Method	Fixed-Dose Procedure
Number of Animals	40-60	10	6-10 (F only)	10-20
Information obtained				
- LD_{50}	value	range	estimated value	range
- dose response	+	−	±	±
-signs of toxicity	+	±	+	+
Animal Welfare advantages		Reduction	Reduction	Reduction Refinement
Regulatory acceptance	All regulatory agencies	Most regulatory agencies	U.S. regulatory agencies	New,not yet accepted

place of the classical LD_{50} test. All three methods offer advantages in reduction and refinement. The choice of which method to use depends on the availanble information on the toxicity of the compound, the type of toxicity information required, and the need to fulfill certain regulatory requirements.

At the present, there are no acceptable nonanimal tests for acute oral toxicity. Much work remains to be done before replacement tests such as those besed on quantitative structure activity relationship or in vitro cell cultures can be used as substitutes for acute oral toxicity testing.

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A FLOURESCENT *IN VITRO* TOXICITY ASSAY WHICH MONITORS ACUTE PLASMA METBRANE DAMAGE OF EPITHELIAL CELLS

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Introduction

Increased public awareness of the use of animals in toxicit testing has expanded the search for new tools and novel modol systems for *in vitro* testing. We have have developed an *in vitro* toxicity assay which employs an analog of 6-carboxyfluorescein diacetate (BCECF-AM, Molecular Probes) to monitor acute plasma membrane damage to Madin-Darby canine

kidney (MDCK) cells in response to various detergens. MDCK cells were seeded at 5 x 10^5 cells / cm^2 on a collagen-coated (rat tail Type 1, Collaborative Research) transparent microporous membrane (Millicell-CM, Millipore Corporation). MDCK cells grown on microporous membranes exhibit a differentiated *in vivo*-like ultrastructure characterized by cuboidal morphology, basal nucleus, desmosomes, tight junctions, functional polarity, apical microvilli and basement membrane deposition. After five days, the cells were loaded with the vital fluorescent dye, BCECF-AM. BCECF-AM is initially nonfluorescent but is cleaved by non-specific esterases intracellularly to yied at fluorescent signal. There is a dose response efflux of the dye when the apical membrane is exposed to the test sample. Sodium dodecil sulfate, Tween-20, Tween-60, Triton X-100 and benzalkonim chloride were among the detergents tested. Apical and basal dye release and residual intracellular fluorescence were quantified with a CytoFluor™ 2300 fluorescent plate reader (Millipore Corporation) with excitation at 485 nm, and emission at 538 nm. Our studies show that BCECF-AM is a sensitive, quantifiable fluorescent indicator of acute epithelial plasma membrane damage and irritancy.

Materials and Methods

Cell Culture: MDCK cells (ATCC No.34) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). NHEK cells (Clonetics Corporation) were cultured in KGM (Clonetics Corporation) with 10% FBS and 1.7 mM calcium. Both cell types were grown on collagen-coated (rat tail type 1, Collaborative Research) mciroporous Millicell-CM™ culture plate inserts with Bioporetm memrbane (Millipore Cprporation). Both cell types were seeded at $5x10^5$ cells/cm^2. MDCK cells were used at confluence, 4 days after seeded. NHEK cells were used at 8 days after seeding.

Cell Staining: BCECF-AM was reconsituted in DMSO and diluted in serum-free, phenol red-free DMEM (40 $\mu g/mL$) and cells were stained 35 minutes at room temperature. Cells were rinsed prior to cytotoxicity testing.

Cytotoxicity testing: Dilutions of the various detergents were made in seurm-free, phenol red-free DMEM and applied only to the apical membrane inside the Millicell-CM™.

Ditection: Dye release from MDCK cells as an indicator of cytotoxicity was measured using the CytoFlour™ fluorescent plate reader from Millipore Corporation. Dye release from the NHEK cells were measured using the Fluoroskan II from Flow Labs. With each instrument, supernatant containing released fluorescent dye was read directly using an excitation wavelength of 485 nm and an emission 538 nm.

Results and Discussions

In an attempt to simulate one aspect of the Draize eye irritancy test *in vitro* we have used the epithelial cell line MDCK cultured on a porous substrate. Since epithelial cells cultured on a porous substrate develop differentiated morphology and fuction; we have access to both the apical and basolteral side of the cells and are able to apply the test sample to the apical side of the cell which is the same as *in vivo*.

Cellular damage was assessed after intracellular staining with BCECF-AM. Sodium dodecyl suflate (SDS) was apically applied in concentrations of 2, 0.4, 0.08 and 0.02 gram per cent. There was a dose response release of dye to the apical compartment corresponding to the degree of damage to the apical membrance. Results are expressed as percent of control. Since BCECF-AM is pH sensitive, the values of the test samples above 100% may be caused by a pH shift or by continued hydrolysis of the BCECF-AM once released into the medium through the damaged apical membrance. Cellular damage hy SDS was also assessed in a complimetary fashion by looking at the fluorescence retained by the cells after exposure to the test sample.

BCECF-AM stained by the cells was qualified after being released by Triton X-100. Therefore, at lower concentrations of SDS the fluorescent signal increases indicating less damage to the plasma membrane.

Subsequent assays tested a panel of detergents

consisting of Tween-60, Tween-20, SDS, Berzalkonium chloride, a commercial shampoo, and Triton X-100. Controls were cells stained with BCECF-AM in order to monitor the spontaneous release of the dye over time and also cells that were never stained with BCECF-AM. Once again, the concentrations applied to the MDCK epithelial monolayer were 2, 0.4, 0.08, and 0.02 gram percent. Plasma membrane damage was indicate by dye release to the apical compartment and also by monitoring dye retained by the cells after exposure to the test sample. Benzalkonium chioride was the most severe irritant tested as measured by this assay. SDS and Triton X-100 were determined to be moderate irritants, the shampoo to be a mild irritant, and Tween-20 and Tween-60 to be mild to non-irritants.

This test may be a useful *in vitro* assay measuring plasma membrane damage caused bt detergents. The next step is to statistically compare this assay with the *in vivo* eye irritancy test.

In the future, it may be possible to establish a toxicity profile of a compound or a product formulation by using a matrix of in vitro tests linked by a common fluorescent detection system.

THERMAL INDUCED-CELL KILLING AND EXPRESSION OF HEAT SHOCK PROTEINS

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We examined the correlation of thermal induced-cell killing and synthesis of heat shock proteins (hsps) in Sarcoma 180 (S-180) cells *in vitro* and *in vivo*. After 43℃/30 min shock, cells were returned to 37℃ or room temp. for 3-24h and, thereafter pulse-ladeled for 1h with ^{35}S-methionine (Met.), ^{3}H-thymidine (TdR) and 5-brmodeoxyuridine (BrdU). In cultured cells, cellular proteins were analyzed by SDS-PAGE and Western blot with human hsp72-specific an-tibody. Hsps were detected at 110, 92, 80 and 74/73/72 kDa., respectivly. Cells incubated at 37℃ for 6h post heat, reached the maximum rate of synthesized major hsps and, thereafter reduced that. Then, cells which were stressed by 43℃/15, 30, 60 and 90 min shock and followed by a 37℃ recovery time(6h) were labeled, too. The cell proliferation was analyzed by cell survival assay and measurement of ^{3}H-TdR activity incorporated into DNA. The synthesis is of DNA and cellular proteins correlated with thermal induced-cell killing in heat-shocked cells. The rate of synthesized hsps in 43℃/30, 60 and 90 min shocked cells also, correlated with that. At tumor tissue, the major hsp in heat-shocked S-180 cells were similar to that in culture cells. The cell proliferation, however an-alyzed by the tumor growth time and immunocytochmical observation unclearly corresponded to that in cultured cells. These results showed that thermal induced-cell killing in tumor tissue may depend on host physiolosical mechanisms.

CORRELATION OF *IN VITRO* AND *IN VIVO* INDUCTION OF DRUG METABOLIZING ACTIVITIES BY METHYLCHOLANTHRENE TYPE-INDUCERS

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Methylcholanthrene (MC) at 0.1 μM induced ethoxycoumarine O-deethylation (EC-DE), Propoxycoumarine O-depropylation, and benzo (a) pyrene hydroxylation (AHH) in primary culture of rat hepatocytes. Increases in P-450 c and 450 d were also observed by MC. On the other hand, methoxycoumarine O-demethylation was not induced. Similar results were also obtained by the other MC type-inducers (1, 2, 5, 6, -dibenzanthracene, 1, 2, 3, 4 - dibenzanthracene, crysene, methylcholanthrene, 1, 2 -benzanthracene, 2, 3 - benzofluorene, 1, 6 - dinitrobenzene, α - naphthoflavone). Phenobarbital, SKF-525 A, pyrene, aminopyrine, and aniline did not induce those activity. The inductions of EC-DE and AHH by these chemicals *in vitro* correlated rather well with those *in vivo*.

DEVELOPMENT OF *IN VITRO* SYSTEMS TO ASSESS TOXICNEFFECTS OF CHEMICALS BY INDUCING POTEMCY FOR XENOBIOTIC METABOLIZING ACTIVITY IN MAMMALIAN CELL LINES

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Cultures of established cell lines derived from (H4) and human (Chang liver) hepatoma were treated with monochlorodibenzofurans (mDBFs), chlorinated ethylenes (Cl-Es) or aldehydes (Alds) to examine inducing potency for benzo (a) pyrene hydroxylase (AHH) activity. Treatment with 4 isomers of mDBF for 20 h induced AHH activity in the order of 3-Cl- > 2-Cl- = 4-Cl > 1-Cl-DBF, showing a maximum 14-fold increase. Cl-Es and Alds too induced this activity after 48h but to lesser extents (2~3 fold of the control).

The order of lethel effects on rats or mice was the same as that of AHH induction in these three kinds of chemicals except 1,1-dichloroethylene. In addition, Alds stimulated the nasal mucosa of rats in the same order as that of AHH induction.

Thess results suggest that the toxicity of chemicals may be assessed by activation of a few genes in cultured cell systems.

ASSESSMENT OF CYTOTOXICITY FOLLOWING CELL RECOVERY

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Conventional *in vitro* cytotoxicity tests are based on the degree of inhibition of cell growth by added to the medium and use the data obtained immediately after cessation of the factor's effects. We have proposed a new *in vitro* cytotoxicity test, that allows the affected cells to recover. In the present study, the influence of serum was investigated. The test media were of 16 kinds of metal ions (Ag, Al, Au, Cd, Co, Cr, Cu, Fe, Hg, In, Ni, Pd, Pt, Ti, V, Zn; 5 ppm) and 3 kinds of dental monomers (HEMA, 3 G, MMA; 10 μ l/10ml). Three kinds of cells (L-929, L-P3, Gin-1) were cultured in 96 well multi-dishes, followed by treatment with test medium for 24 hours in a CO_2 incubator. The cells were replaced to a normal culture environment for subsequent cultivation. L-P 3 cells grown in serum-free medium showed significantly lower cell recovery compared with L-929 cells, while Gin-1 cells showed little defference between both condetions. Our findings suggest that cell recovery tests, are important for testing cytotoxicity.

DIURETICS MODIFY ARGININE VASOPRESSIN (AVP)-STIMULATED cAMP BUT NOT NATRIURETIC PEPTIDE (ANP)-STIMULATED cGMP FORMATION IN CULTURED RENAL CELLS

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Sulfonamide-derived diuretics suppressed AVP-stimulated cAMP formation in cultured rat and dog renal cells. Suppressive effect of diuretics on AVP-stimulated cAMP formation *in vitro* paralleled with the reported diuretic potency *in vivo*. The difference in effective doses between rats and dogs seems to accord with those observed *in vivo*. It is suggested that most sulfonamide-related diuretics act at least partly through inhibition of AVP actions.

CYTOTOXIC EFFECT OF FEEDER LAYER

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Recenly, Okada et al. proposed that critical cell number (the minimum number of clonogenic cells per spheroid to repopulate) might be very important as a determinant of radiosensetivity of tumors.

As the size of spheroid (clonogenic cell number per spheroid) increased, the critical cell number increased markedly. That is, according to critical cell number, larger spheroids became more radiosensitive than smaller ones. They supposed that the viable or clonogenic cells may be killed by the surrounking dead or dying cells. In order to test the critical cell number in monolayer cells, we examined the cytotoxicity of feeder layer. So far, feeder layer (F.L.) has been used as a growth stimulating conditioner for cultured cells. 25 Gy or more of X-irradiated cells adhere to the bottom of petri dishes even 5 days after irradiation, without cell division. These cells may help landing of overlayed intact or treated cells after plating.

Cultured AOI cells derived from human lung cancer (adenosquamousca., given from Dr. M. Akiyama (Hiroshima)) were maintained with alpha-MEM + 10 % FBS in 95 % air + 5 % CO_2. At 35.0 - 37.0 ℃,25 Gy of X-irradiated F. L. did show potent cytotoxicity, but at 39.0 ℃, only slight cytotoxicity was observed. The cytotoxicity of conditioned media from F.L. was more apparent. Thus, in the cell culture system in which such cytotoxic substances are secreted, critical cell number may act a role even in monolayer cells.

THE MULTICELL SPHEROID MODEL FOR CANCER THERAPY STUDY

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HMV-1 cells, derived from human malignant melanoma, were cultured as multicellular spheroids in spinner flasks. Cell survival curve of spheroids of approximately 400 μ m diameters after X-irradiation consisted of an initial steeper component and a shallower one, suggesting that they include hypoxic cells which were located in the inner region.

We also studied the development and decay of thermotolerance after hyperthermic treatment at 44 ℃ for 15 min in both monolayer and spheroids. The thermotolerance of cells in spheroids was more strongly developed after priming heat than those in monolayer. The kinetics of the thermotolerance decay, on the other hand, was almost the same in spheroids and monolayer cells. Thus, the characteristics of cells in spheroids are different from the intrinsic cellular

properties observed after growth of cells in standard cell culture systems. The use of spheroids model for the study of cancer therapy gives further insight into the action of radiation and chemotherapy agents in solid tumor.

ALTERNATIVE TO BREAST CARCINOGENICITY TEST USING A TISSUE CULTURE METHOD

1. APPEARANCE OF MICRONUCLEI IN THE MOUSE MAMMARY EPITHELIAL CELLS CULTURED WITH CHEMICAL CARCINOGENS

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In an attempt to establish an *in vitro* short-term test for breast carcinnogens, we studied several known carcinogens tested by micronucleus assay in the primaty cultures of mouse mammary epithelial cells. Mammary epithelial cells from 2 month old ICR virgin mice were cultured on collagen gel in serum-free Ham's F-12/ Dulbecco's modified Eagle's medium supplemented with insulin, bovine serum albumin, epidermal growth factor, transferrin and cholera-toxin.

At day 6 of culture, known chemical carcinogens were added to the cultures, and the number of micronuclei per 1,000 cells was scored at 24 hr after treatment. Two breast carcinogens, N-methyl-N-nitrosourea (MNU) and 7, 12-dimethylbe [a] anthracene (DMBA), increased the micronuclei incidence in the cultures. The cells grown in this serum-free medium metabolized DMBA to the activated form. These results suggest that the quatitation of micronuclei in the mouse mammary epithelial cells cultured under serum-free conditions might be used as an *in vitro* short-term screen for breast carcinogens.

DRUG SENSITIVITY TEST USING COLLAGEN GEL CULTURE METHOD

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In vivo dryg sensitivity test such as nude mouse assay and SRC assay has proved to be a useful system for screening new chemical compounds for anticancer activity and for predicting clinical response to cytotoxic therapy in cancer patients. However, these assay methods have not been fully established as practical assay systems because of many disadvantages. As an alternative to these *in vivo* methods, we examined advantage for assay using collagen gel matrix culture to develop a new *in vitro* drug sensitivity assay system.

Human cancer cells ovtained from 4 human cancers serially trans-planted into nude mice and 27 human cancer patients were embedded in collagen gels. The cells cultured in collagen gels showed high cloning efficiencies (0.092 ~ 2.563), easy diserimination of tumor and contaminating fibroblastic cells, and *in vivo*-like three dimen- correlation between the anti-cancer effect of drugs in the *in vivo* nude mouse assay and in the *in vitro* collagen gel assay was also observed. These data suggest that collagen gel assay system is useful for drug sensitivity test on diverse human cancer cells.

THE GROWTH AND DIFFERENTIATION OF HUMAN ENDOMETRIAL CARCINOMA CELLS (Human Endo-metrial Carcinoma Cell Line (NTK-1)) – NEW CULTURE METHODS USING AN EXTRACELLULAR MATRIX –

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Introduction

The incidence rate for endometrial carcinoma tends to rise with increased longevity of population and more attention has been given to the diagnosis and treatment of endometrial carcinoma. The endometrial carcinoma cells have several cell biological characteristics which are closely related to the management.

In ordinaty culture, carcinoma cell of established cell line shows the homogeneous and flat cell shape and can not make a three dimensional structure in culture dish. Furthermore in general, the pattern of tumor markers secreted form cultured carcinoma cell line show the distinct trend. There is disagreement among concerning the comparison results from characteristics of cultured cells and that of *in vivo*. For example, we always used these established cells for the sensitivity test of anti-cancer drugs and irradiation, but in many cases, results from these assays have plagued clinicians in conflicting therapeytic outcome.

In this study we employed the several coating materials, basement membrane extracts (BME) and various types of collagen, as substrates of NTK-1 cells culture system and investigated the morphological and functional differences on phenotypes and characteristics between *in vivo* and *in vitro*. Furthermore the application of this culture model for the study of endometrial cell carcinoma has discussed briefly.

Materials and Methods

The NTK-1 cell line was established from endometrial carcinoma[1]. BME was prepared form mouse chondrosarcoma[2] and various types of collagen were obtained from a commercial source. NTK-1 cells were cultured on these substrates and examined the cell attachment rate, cell growth, cell morphology and interaction between NTK-1 cell and these substrates[3]. The tumor markers from patient and cultured medium were assayed by RIA kits.

Results and Disussion

The tumor was composed of atypical endometrial cells growing in an abortive tubular and partially cribriform pattern *in vivo*. The *in vitro* epithelial-like cell shapes were acen on type I, III and IV collagens. The growth ingibition of NTK-1 was observed on type II and V collagen. The NTK-1 cell on BME reconstructed the three dimensional morphology. The study of semi-thin

section of NTK-1 cell on BME cleatly indicated that there were at least two types of cells; the first cell reconstituted the gland in BME and the other cells formed multi layer cell sheet on BME. But no three dimensional structure was seen on collagens. Scanning electron microscopy indicated the difference between the feature in ordinary cell culture system of plastic culture dish (flat shape) and on BME coated culture dish (round shape and rich in microvilli). The growth pattern of NTK-1 on BME is characteristic in that colony development and branch formation.

NTK-1 produced the large amount of TPA but did not CA 125 on ordinaty culture and very small amount on collagen coated dishes. Cultured on BME, NTK1 Cells became to produce the CA 125 antigen in spent media.

Ultrastrustual studies of the NTK-1 cells on BME indicated that the mononuclear cells with high muclear-cytoplasmic ratio had a number of vacules,lipid drops and rough endoplasmic reticulum. These structures resembled that of the original tumor and nude mice[1].

These data may indicate that this new culture system is very useful for the investigation of the growth of carcinoma and invasion system in culture dish. Furthermore, it may be useful for the sensitivity test of anti-cancer drugs and irradiation because the carcinoma cells on BME represent the same phenotypes and characteristics of original tumor.

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