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Neutral Red Assay Using Normal Rabbit Corneal Epithelial Cells Grown In Serum-free medium As An Alternative To The Draize Irritation Test

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Summary

Neutral red assay using normal rabbit corneal epithelial (NRCE) cells were examined as an *in vitro* alternative to the Draize ocular irritation test. The cells were cultured in serum-free medium to reduce interference with serum components, especially serum proteins.

It was confirmed that the amount of neutral red incorporated into the cells was proportional to the number of viable cells, and the incorporation of the dye was affected by the concentration of calcium ion in the medium. Seventeen detergents were compared for the NR50 values obtained by this method to Draize ocular irritation test scores. The correlation coefficient between the NR50 value and the Draize rank was −0.694, and that between the NR50 and the DS20, a Draize score equal to 20 units from a possible total of 110, was 0.644.

By using neutral red assay, the sensitivity of chemicals was compared NRCE cells with established cells (SIRC) grown in serum-containing medium. NRCE cells cultured in serum-free medium were more sensitive to the cytotoxicity of chemicals than SIRC cells in serum containing medium. The higher sensitivity of the NRCE cells was mainly due to the use of serum-free medium, and also due to the higher sensitivity of normal cells to irritants tested.

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Introduction

The Draize ocular irritation test (1) is one of the most fundamental tests for determining of the toxicity of chemicals. However, it has some serious drawbacks. This test demands a high degree of operator's skill to obtain reliable results, and, recently, it has been criticized from the viewpoint of animal welfare.

Many alteratives to the Draize test have been developed (2, 3, 4, 5), and are currently evaluated in various validation studies such as the HO/EEC program (EC-U.S.A-Japan), the validation plan of the *in vitro* alternatives to eye irritation tests for cosmetic ingredients (NIHS-Japan Cosmetic Industries Associate) and the validation program by Japanese Society of Alternatives to Animal Experiments.

We have established a culture system of normal rabbit corneal epithelial cells (NRCE), one of the targets of the Draize test,using serum-free medium (6). In the present study, we examined the difference of the sensitivity of cell types for various test chemicals on primary cultured cells isolated from normal tissue and an established cell line, and the difference of culture media for

neutral red assay as an *in vitro* alternative to the Draize test.

Materials and Methods

In vivo test

Japanese white rabbits of both sexes were used. Chemicals at least 4 different concentrations were applied directly into each eye with a micropipette. Corneal, iris and conjunctival responses were scored at 1, 3, 6, 24, 96 and 168 hr following application of each chemical according to the proposed US guidelines (1, 7, 8, 9).

Fluorescein staining was applied to assist the determination of the extent of corneal damage. The mean values of Draize score, calculated from individual scores of 3–6 rabbits at 6 different exposure times, was plotted against each chemical concentration tested to obtain a dose-response curve. A Draize score equal to 20 units from a possible total of 110 was obtained as a standard DS20 value for determining the comparative activity (7). This value was in the range of scores actually observed during evaluation of the majority of the chemicals tested.

In vitro tests

Primary NRCE cells obtained from Kurabo Industries Ltd. (Osaka, Japan) were cultured in a serum-free medium (RCGM; Kurabo Industries Ltd.).

The neutral red assay was carried out according to the method described previously (10). A secondary culture of NRCE cells was harvested by trypsinization when the culture reached to 50 to 80% confluent. The suspended cells were collected by centrifugation at 180×g for 5 min and resuspended in RCGM. Approximately 2.5×10^3 cells were inoculated in 0.1 ml of medium each in wells of a 96-well tissue culture plate (Coaster, Cambridge, MA) and cultured for 3 days at 37°C in 5% CO_2 humidified atmosphere. The cells were then treated with chemicals at least 7 different concentrations for 2 days. Test chemicals were dissolved in phosphate buffered saline (PBS), ethanol or 50% ethanol in PBS, sterilized by filtration and diluted with the medium. Final concentrations of the solvents were less than 2% (v/v) and showed no effects on cell growth. After the treatment with chemicals, 0.1 ml of RCGM supplemented with neutral red (50 μg/ml) was added to each well. After 3 hr, the medium was removed from each well, and cells were fixed in 1% formalin-1% $CaCl_2$ to enhance the adhesion of the cells to the plate. Neutral red incorporated into viable cells was extracted with 1% acetic acid-50% ethanol solution, and the color intensity was measured at 540 nm using a microplate reader. The cytotoxicity of chemicals was represented by the concentration of test chemicals which caused 50% reduction in neutral red uptake by treated cell culture compared with the untreated control cultures (NR50 value). We prepared the dose-response curves at least with one dose caused no reduction, one dose caused 100% reduction and 3 doses caused from 20% to 80% reduction in neutral red uptake for the calculation of NR50 values.

The tests with SIRC, a cell line established from rabbit cornea (ATCC CCL-60), were carried out under the same conditions as described above, except for the use of Dulbecco's modified Eagle's Medium (DMEM, Life Technologies Oriental Inc., Tokyo) containing 10% fetal bovine serum (ICN Biomedicals Japan Co., Osaka, Japan) and the inoculation of half the number of cells into each well. Sodium lauryl sulfate was used as a positive control chemical in each test.

Under the conditions described above, the untreated control cultures of both cells reached to about 50% confluent in 3 days and to 100% confluent in 5 days. The incorporation of neutral red into the cells increased in proportion to the growth of the cells.

Test chemicals

All 17 test chemicals were obtained commercially and used without further purifica-

Table 1. Test chemicals in the present study

Chemicals	Abbreviation	Solvent*
Nonionic detergents		
Polyoxyethylene lauryl ether (9E. O.)	POE-LE	A
Polyoxyethylene nonyl phenyl ether (10E. O.)	POE-NPE	A
Polyoxyethylene sorbitol tetraoleate	POE-ST	B
Sorbitan monooleate	SM	B
Polyoxyethylene sorbitan monooleate (20E. O.)	TWEEN80	C
Coconut fatty acid diethanolamide	CFAD	C
Anionic detergents		
Sodium *N*-cocoyl-L-glutamate	SCGL	A
Sodium *N*-lauroyl-L-glutamate	SLGL	C
Sodium hydrogenated glyceryl cocoate sulfate	SGCS	C
Sodium lauryl sulfate	SLS	A
Sodium polyethylene laurylether sulfate	SPLS	A
Cationic detergents		
Benzethonium chloride	BC	A
Distearyl dimethyl ammonium chloride	DMAC	C
dl-5-Oxopyrrolidine-2-carboxylic acid salt of *N*-cocoyl arginine ethyl ester	OCAE	A
Stearyl trimethyl ammonium chloride	STAC	A
Amphoteric detergents		
Lauryl dimethylaminoacetic acid betaine	LDAB	A
2-Alkyl-*N*-carboxymethyl-*N*-hydroxyethyl imidazolinium betaine	ACIB	A

*Solvent used for each chemicals
A: phosphate buffered saline
B: ethanol
C: 50% ethanol in phosphate buffered saline

tion. The names and abbreviations of chemicals and solvents used for each chemical are shown in Table 1.

Binding of chemicals to serum proteins

A cellulose dialysis tube packed with 5 ml of dialyzed bovine serum, 5% bovine serum albumin or RCGM medium was put into 10 ml of RCGM medium containing detergent. After stirring overnight at room temperature, the neutral red assay with NRCE cells grown in RCGM medium was carried out, and the relative concentration of the detergent in outer solution was determined.

Results

The incorporation of neutral red into NRCE cells was shown in Fig. 1. The incorporation of the dye was proportional to the number of NRCE. The concentration of Ca^{2+} affected the amount of neutral red incorporated in cells. It was decided that RCGM containing 0.15 mM Ca^{2+} was used for the assay. The typical dose-response curve of SLS was shown in Fig. 2.

The results of both *in vivo* Draize score and *in vitro* tests with NRCE and SIRC cells for 17 test chemicals are shown in Table 2. The relationships, calculated by the least squares regression method, among the values obtained in each test are shown in Table 3. The correlation coefficients between Draize ranks and logarithmic values of *in vitro* test were −0.694 for NRCE cells and −0.689 for SIRC cells. The NR50 values obtained in the test with NRCE cells were lower than those obtained with SIRC cells, especially in anionic detergents (Table 2 and Fig. 3).

The difference in cell type and the effect of serum in medium were examined. Neutral red assay was carried out using NRCE cells and in RCGM medium containing 10% fetal bovine serum. The NR50 values of cells cultured in

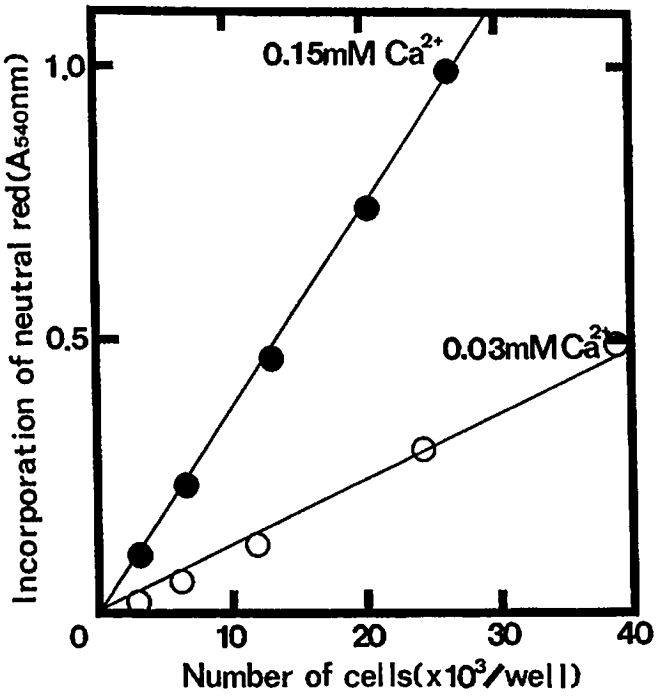

Fig. 1. Effects of calcium concentration on incorporation of neutral red into normal rabbit corneal epithelial (NRCE) cells. The cells were cultured in RCGM containing 0.03 mM or 0.15 mM Ca^{2+}, and the amount of neutral red incorporated in cells was determined.

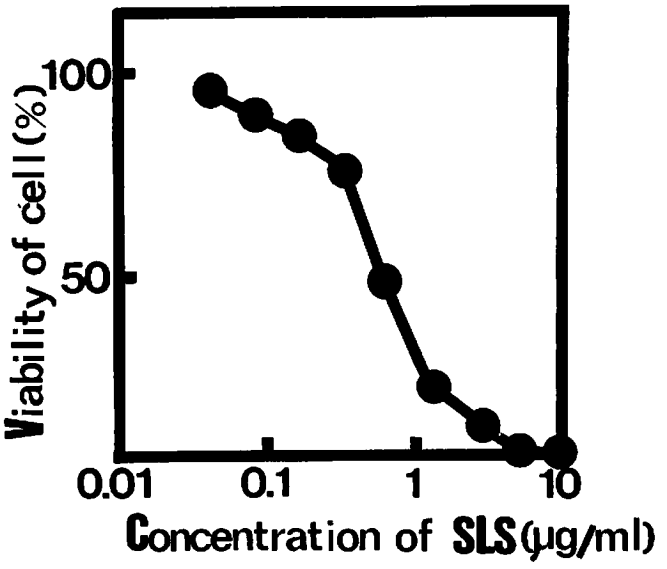

Fig. 2. Typical dose-response curve of sodium lauryl sulfate (SLS) in neutral red assay with normal rabbit corneal epithelial (NRCE) cells.

Table 2. Values determined by *in vivo* and *in vitro* tests

Detergents	DS20*	Draize rank	NR50(NRCE)**		NR50(SIRC)**	
POE-LE	4.0	severe	3.63	(0.416)a	27.7	(4.73)a
POE-NPE	5.0	moderate	4.00	(0.693)	14.7	(2.84)
POE-ST	9.0	non	377	(5.77)	590	(52.0)
SM	50	non	583	(60.3)	487	(90.2)
TWEEN80	50	non	390	(10.0)	650	(50.0)
CFAD	11	mild	5.60	(0.200)	23.8	(9.93)
SCGL	11	mild	15.8	(0.764)	453	(159)
SLGL	14	mild	143	(7.64)	493	(101)
SGCS	14	mild	46.0	(3.46)	145	(44.2)
SLS	4.5	moderate	0.893	(0.0987)	68.0	(13.9)
SPLS	8.0	moderate	2.86	(2.01)	337	(25.2)
BC	1.5	severe	2.72	(1.25)	8.67	(0.115)
DMAC	2.4	moderate	6.70	(0.557)	27.7	(4.73)
OCAE	1.5	severe	15.0	(8.55)	162	(12.6)
STAC	2.2	severe	9.13	(0.723)	7.70	(3.16)
LDAB	5.8	moderate	112	(11.5)	207	(48.6)
ACIB	30	non	20.0	(2.00)	170	(15.0)

*: w/w %, **: μg/ml, a: mean (SD), n=3

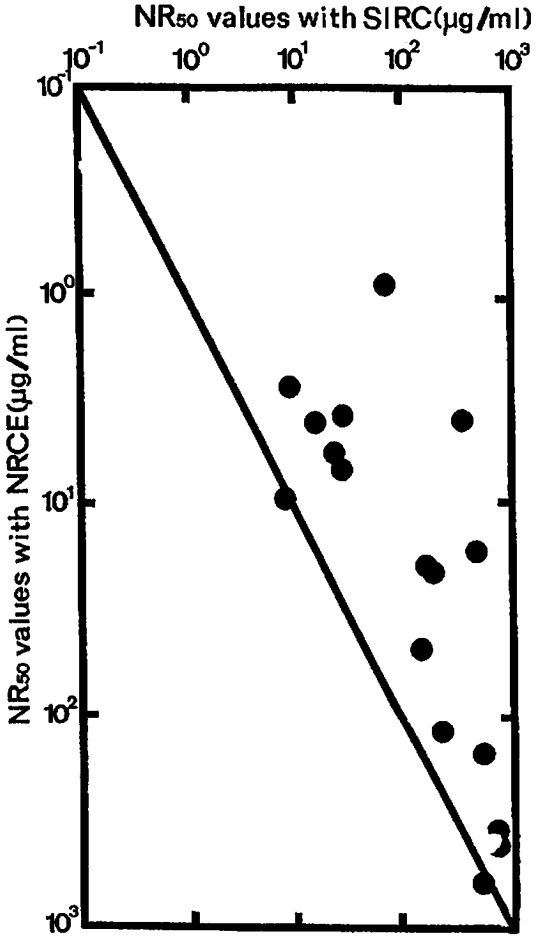

Fig. 3. Relationship between NR50 values obtained from NRCE grown in serum-free medium and those obtained from SIRC grown in serum-containing medium.

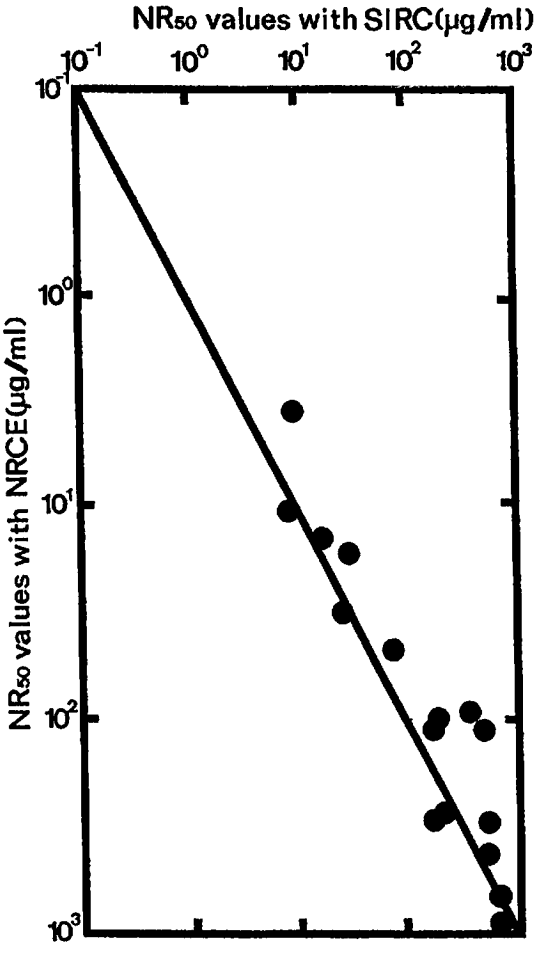

Fig. 4. Relationship between the NR50 values with NRCE cells and that of SIRC cells in media (RCGM for NRCE, and DMEM for SIRC) containing 10% FBS.

Table 3. Relationships among the values obtained in each test

Y	X	Y=aX+b (correlation coefficient)	
log(NR50:NRCE)	Draize rank*	Y=−0.531X+2.64	(−0.694)
log(NR50:SIRC)	Draize rank	Y=−0.412X+3.05	(−0.689)
log(NR50:NRCE)	log(DS20)	Y=1.17X+0.268	(0.644)
log(NR50:SIRC)	log(DS20)	Y=0.959X+1.17	(0.676)
log(NR50:NRCE)	log(NR50:SIRC)	Y=0.900X−0.513	(0.704)

*Ranks of non, mild, moderate and severe were counted as 1, 2, 3 and 4 respectively.

this condition were compared with those of SIRC grown in serum-containing medium. As shown in Fig. 4, NRCE cells were more sensitive to the cytotoxicity of chemicals than SIRC cells. Also, the values obtained with NRCE cells in serum-free RCGM medium were lower than those in the same medium containing serum (Fig. 5). The addition of bovine serum albumin (0.5%) in medium resulted in a similar effect to the addition of

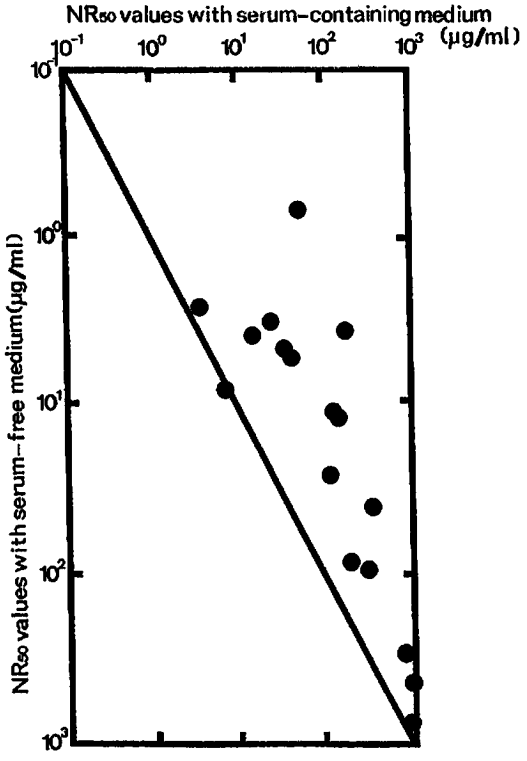

Fig. 5. Difference in the NR50 values in NRCE cells cultured in RCGM with or without 10% FBS.

Table 4. Effects of serum and albumin in medium on NR50 value of NRCE and SIRC cells

Detergents	NR50 (μg/ml)			
	NRCE			SIRC
	RCGM	RCGM+FBS	RCGM+BSA	DME+FBS
SLS(anionic)	0.893	44.6	50.9	68.0
BC(cationic)	2.72	3.21	3.95	8.67
LDAB(amphoteric)	112	256	195	207

FBS: 10% fetal bovine serum
BSA: 0.5% bovine serum albumin

serum. As shown in Table 4, the effects of serum and albumin on the NR50 values were most remarkable in anionic detergents.

The binding of detergents to serum components was examined. The results are shown in Table 5. The relative concentration of detergents in outer RCGM medium decreased after dialysis against serum or albumin solution. The concentration of SLS was most effectively decreased when serum or albumin was present in the inner medium. BC was less effective in the binding to serum components.

Discussion

A good correlation was obtained between the relative cytotoxicity of 17 detergents for NRCE and SIRC cells in the *in vitro* test and the Draize rank obtained in *in vivo* test. When two *in vitro* tests, normal cells grown in

Table 5. Bindings of detergents to serum components and albumin

Detergents	Relative concentration		
	dialyzed against		
	RCGM	FBS	BSA
SLS	1.0	0.090	0.12
BC	1.0	0.60	0.56
LDAB	1.0	0.47	0.42

10 ml of RCGM medium containing detergent (300 μg/ml for SLS and BC, 3 mg/ml for LDAB) was dialyzed against 5 ml of dialyzed fetal bovine serum (FBS), 5% bovine serum albumin (BSA) or RCGM medium.

serum-free medium and established cells grown in serum-containing medium were compared, the former was more sensitive to detergents. This difference in sensitivity was mainly due to serum proteins in the medium. The difference in sensitivity among assay systems varied with the chemicals applied. For example, the NR50 values of sodium lauryl sulfate (SLS), which is known to bind to proteins, were seriously affected by the present of serum and serum albumin. On the otehr hand, benzethonium chloride (BC), an cationic detergent, was little affected. The degree of the effect influenced by serum proteins was directly proportional to the affinity of each detergent to serum components or albumin as shown in Table 5.

Although the highest cytotoxicity of detergents was obtained in cells cultured in serum-free medium, the difference in cell type also had a significant effect on the cytotoxicity of detergents.

In the Draize irritation test in rabbits, test chemicals were directly applied to the eye, and the chemicals do not contact with serum at least on the first step. Therefore, normal cells grown in serum-free medium may be a good model of the *in vivo* test. We determined the difference in the NR50 value between non-toxic and mildly toxic detergents by Student's t-test. This should be mentioned in the methods section tee. The P value in NRCE-RCGM system was 0.05, and that in SIRC-DMEM-10% FBS system was 0.15. This shows that normal cells grown in serum-free meium are more sensitive to weekly toxic chemicals than established ells grown in serum-containing medium.

In conclusion, this neutral red assay using normal rabbit corneal epithelial cells cultured in serum-free medium can be successfully used for preliminary screening for cytotoxicity of chemicals, because it is more sensitive to mildly toxic chemicals and easy to be carried out.

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