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APPLICATION OF NORMAL HUMAN EPIDERMAL KERATINOCYTES IN SERUM-FREE MEDIUM AS AN ALTERNATIVE TO THE DRAIZE OCULAR IRRITATING TEST

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Normal human epidermal keratinocytes grown in a serum-free keratinocyte growth mediumwere evaluated as an *in vitro* alternative to the Draize ocular irritation test. The cells were culturedin96-well tissue culture plates and treated with test agents. Cytotoxicity of the agent was estimated by the uptake of neutral red by viable cells. Fifty-one chemicals which are used in cosmetics were employed as test agents.

The correlation coefficient between *in vivo* Draize score and *in vitro* test with normal human epidermal keratinocytes was 0.83. In addition, the method using normal human epidermal keratinocytes was more sensitive to a given concentration of test agents than the colony forming assayusing rabbit cornea primary cells. These results suggested that the cytotoxicity test *in vitro* usingnormal human epidermal keratinocytes is useful as an alternative to the ocular irritating test.

Introduction

While the Draize test has been the standard for judging ocular irritancy[1], it has been subjected to criticism for a number of reasons including concern for the humane treatment of animals, variability in scoring, long assay duration and relatively high cost. The inadequacies of the Draize test have led to efforts to develop alternative *in vitro* methods.

These include tests based on changes in enzyme secretion by cultured ocular cells[2], cytotoxicity in cultured cells[3, 6], and the inability of dead and damaged cells to take up eutral red[7, 8] and uridine[3].

The cells used in these studies were animal or established cell lines which might differ from human cells in their response to certain chemicals. Considering that the purpose of the Draize rabbit ocular irritation test is to determine potential irritation to the human eye by chemicals, normal human cells seem more practical than other cells.

In this study, we evaluated normal human epidermal keratinocytes (NHEK) grown in a serum-free keratinocyte growth medium (K-GM) as an *in vitro* alternative to the Draize ocular irritation test[1, 3].

Abbreviations: NHEK normal human epidermal keratinocytes. NR50 the chemical concentrations allowing 50% cell survival which were estimated using NHEK and the neutral red assay, RC rabbit cornea, LD 50 the chemical concentrationscens. allowing 50% cell survival using RC cells and colony forming assay.

Materials and Methods

Cells

NHEK[9] from humanbreast were used. Cells were cultured in K-GM (Clonetics, SanDiego CA; produced in Japan by Kurabo Industries LTD(Osaka) under license).

Cytotoxicity test using neutral red

A secondary culture of NHEK was harvested by trypsinization when the culture was between 50 to 80% confluent. The suspended cells were collected by centrifugation at 180g for 5 minutes and diluted in K-GM medium. Twentyfive hundred cells in 0.1 ml of the medium were inoculated into each well of a 96-well tissue culture plate (Coaster, Cambridge MA) and cultured for 3 days at 37℃ in a 5% CO_2 humidified atmosphere. Then, the cells were treated with the chemicals for 2 days. Test chemicals were dissolved in PBS, ethanol or dimethyl sulfoxide (DMSO) as indicated in Table 1, sterilized by filtration and diluted with K-GM medium(pH 7.4). Final concentrations of the solvents were less than 2%(V/V) and showed no effects on cell growth. After chemical treatment, 0.1ml of K-GM supplemented with neutral red (50ug/ml final concentration) was added to each well. After 3 hours, the medium was removed from each well. Cells were fixed with 1% formalin-1% $CaCl_2$ which enhanced adhesion of the cells to the plate. Neutral red incorporated into viable cells was extracted with a 1% acetic acid-50% ethanol solution. The resulting color intensity which is proportional to the number of viable cells was measured by a microplate reader (MPR A4, Tosoh, Tokyo) at 540nm. Cytotoxicity was represented as the concentration of test agent which caused a 50% reduction in neutral red uptake by a treated cell culture compared with the untreated control culture(NR-50).

Cytotoxicity test using MTT

The cells were treated with the test agents as describe above.

MTT (0.01 ml of 5mg/ml stock solution) was added to each well, and incubated at 37℃ for 4 hours. Isopropanol containing 0.04N HCl was added to each well for the dissolution of formazan to give a homogeneous blue solution suitable for absorbance measurement. The resulting color intensity was measured by a microplate reader at 570nm.

Cytotoxity test using primary RC cells and animal test

All data were taken from a previous report[6] Cytotoxity to primary RC cells was estimated by the colony forming assay. Briefly the cells were treated with chemicals for 20 min at 37℃, then washed twice with 5 ml of PBS, re-fed with Eagle's minimum essential medium with 10% fetal bovine serum and allowed to form colonies.

Results

Estimation of the number of viable NHEK cells:

Neutral red (3-amino-7-dimethylphenazine hydrochloride) passes through the intact plasma membrane and becomes concentrated in lysosomes[7, 8] and MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazoliumbromide) is cleaved by living cells to yield a dark blue formazan product[12] Therefore, these substances are frequently used to estimate the viability of cells[7, 8, 12, 13] Figure 1 shows the relationship betweenthe number of cells which were determined microscopically and color intensities developed by neutral red and MTT. Neutral red was suitable for estimating the number of NHEK because absorbance was directly proportional to

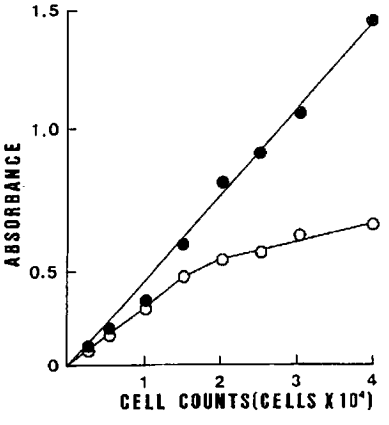

Fig. 1. Earimation of the number of NHEK cells by neutral red(● 540nm) and MTT (○ 570nm) assay. NHEK cells of known densities were seeded into 96-well tissue culture plates. Cells were incubated for a day and stained by neutral red or MTT as described in Materials and Methods.

Table 1. List of chemicals tested

Chemicals	Abbreviation	Solvent[a]
Nonionic detergents		
1. Polyoxyethylene Glycol Monolaurate (10E.O.)	POE-GML	A
2. Polyoxyethylene Lauryl Ether (9E.O.)	POE-LE	A
3. Polyoxyethylene Nonyl Phenyl Ether (10E.O)	POE-NPE	A
4. Polyoxyethylene Sorbitol Tetraoleate	POE-ST	B
5. Myristoyl Diethanoleate	MD	C
6. Sorbitan Monooleate	SM	B
7. Polyoxyethylene Sorbitan Monolaurate (20E.O.)	TWEEN20	A
8. Polyoxyethylene Sorbitan Monooleate (20E.O.)[b]	TWEEN80	C
9. Polyoxyethylene Sorbitan Monooleate (20E.O.)[c]	POE-SMO	C
10. Monoethanolamine Tallow Acid Amide	MTAA	D
11. Coconut Fatty Acid Diethanolamide	CFAD	C
12. Sucrose Fatty Acid Ester	SFAE	C
13. Polyoxyethylene Hydrogenated Castor Oil Monoisostearate	POE-COM	C
14. Sucrose Laurinic Acid Ester	SLAE	B
15. Polyethylene Glycol Monooleate (25E.O)	PEG-MO	A
Anionic detergents		
16. Sodium N-Acyl-L-Glutamate	SAGL	C
17. Sodium N-Cocoyl-L-Glutamate	SCGL	A
18. Sodium N-Lauroyl-L-Glutamate	SLGL	C
19. Sodium Hydrogenated Glyceryl Cocoate Sulphate	SGCS	C
20. Sodium N-Lauroyl Sarcosinate	SLSA	A
21. Polyoxyethylene Laurylether Phosphate (12E.O.)	POE-LP	B
22. Potassium Laurate	PL	A
23. Sodium Lauryl Sulphate	SDS	A
24. Sodium Coconut Fatty Acid Taurate	SCFAT	C
25. Sodium Tetradecenesulphonate	ST	A
26. Sodium Polyethylene Laurylether Sulphate (12E.O.)	SPLS	A
Cationic detergents		
27. Benzethonium Chloride	BC	A
28. Distearyl Dimethyl Ammonium Chloride	DMAC	C
29. dl-5-Oxopyrrolidine-2-Carboxylic Acid Salt of N α-Cocoyl Arginine Ethyl Ester	OCAE	A
30. Stearyl Trimethyl Ammonium Chloride	STAC	A
31. Stearyl Dihydroxyethoxyethyl Hydroxyethyl Ammonium Chloride	SDAC	A
32. Dimethyl Distearyl Ammonium Chloride	DSAC	B
33. Alkyl Benzyl Dimethyl Ammonium Chloride	ABAC	A
Amphoteric detergents		
34. Lauryl Dimetylaminoacetic Acid Betaine	LDAB	A
35. 2-Alkyl-N-Carboxymethyl-N-Hydroxyethyl Imidazolinium Betaine	ACIB	A
Glycol		
36. Glycerin	GLY	C
37. Polyethylene Glycol 400	PEG400	C
38. 1,3-Butylene Glycol	BG	C
39. Propylene Glycol	PG	A
40. Dipropylene Glycol	DPG	A
Oil		
41. Isopropyl Myristate	IM	B
42. Methyl Polysiloxane	MP	B
43. Tri-2-Ethyl Glycerol Hexinate	TEGH	B
44. Glycerin Triisoparmitate	GLYT	B
Antiseptic agents		
45. 2-Phonoxyethanol	2PE	C
46. Sodium Benzoate	SB	A
Others		
47. Malic Acid	MA	A
48. Sodium Malate	SM	A
49. Ethanol	ETOH	A
50. Acetone	ACET	A
51. Formalin	FORM	A

a) Solvents; A=Phosphate buffered saline, B=Ethanol, C=50% ethanol in phosphate buffered saline, D=Dimethyl sulfoxide.
b) Kao Co.,LTD. c) Nikko Chemicals, Co.,LTD.

the number of cells, even up to concentrations of 40,000 cells per well. On the other hand, absorbance formed by MTT was not proportional when the cell density was above 15,000 cells per well.

Addition of the test agent to actively growing cells was required to obtain reliable results, and the growth of NHEK in K-GM medium was elucidated. When 2,500 cells in 0.1 ml of medium were seeded into each well of a 96-well plate, the cells were actively grown after a 1 to 2 day lag period and reached 30,000 cells per well after 5 days of cultivation (Fig.2), thereafter the growth rate decreased (data not shown). From the results, the test agent was added at 3 days of cultivation and viability was determined at 5 days of cultivation.

Correlation with in vivo Draize score

The cytotoxic effect of 51 test chemicals, which included detergents, glycols, antiseptic agents and oils on NHEK cells was studied. Results for 5 typical chemicals are shown in Fig. 3. Table 2 shows the NR50, i.e., the chemical concentrations allowing 50 % cell survival

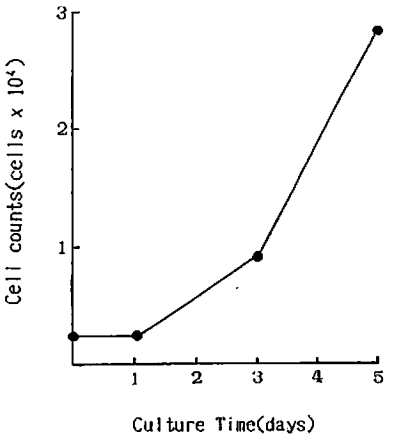

Fig. 2. Growth of NHEK in 96-well tissue culture plates. Cells were inoculated in K-GM at a density of 2.5 x 10^3 cells/ well and incubated at 37 ℃ and 5 % CO_2 in a humidified atmosphere. At the indicated times, number of cells in each well were determined microscopically.

obtained from dose response plots for each of the 51 test chemicals from the *in vitro* test using NHEK cells. The LD50 Value using RC cells and the concentration of each chemical predicted to cause a Draize score of 20 in the rabbit eye *in vivo*. were taken from a previous report[6]. Using these values in Table2 as the basis for comparison, a rank correlation analysis was performed and the results are depicted graphically in Fig. 4 and 5. In these figures, the lower toxicity was defined

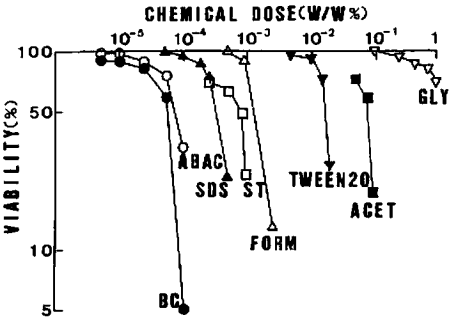

Fig. 3. Viability of NHEK treated with various chemicals (8 out of 51 chemicals tested). See Table 1 for description of chemicals. Each point shows a mean of 4 replicates.

as larger numbers of relative toxicity both *in vivo* and *in vitro*.

A significant correlation between the relative toxicity in NHEK cells *in vitro* and the relative eye irritation in the Draize test was seen in response to treatments with the 51 chemicals ($r=0.83$).

The relative toxicity in NHEK cells and the relative toxicity in RC cells also showed a good correlation($r=0.89$).

Discussion

The *in vitro* cytotoxicity test has been developed for predicting relative eye irritation by measuring cell toxicity. In these studies, investigators usually used established cells, which generally have different biological characteristics from those of the original tissues *in vivo*. In addition, the difference in their properties due to a species barrier might exist

Table 2. NHEK-NR50 and RC-LD50 in vitro testing and Draize score DS20 in vivo testing

Chemicals	[a] in vitro testing NHEK-NR50 [b] (W/W%×10[5])	RC-LD50 [c] (W/W%×10[3])	in vivo testing DS20 [d] (W/W%)
Nonionic detergents			
1.POE-GML	350	20	85
2.POE-LE	12	1.8	4
3.POE-NPE	80	4	5
4.POE-ST	2200	10.5	9
5.MD	62	5	14.5
6.SM	2050	12	50
7.TWEEN20	1700	25	>100 (200)
8.TWEEM80	1550	20	50
9.POE-SMO	1850	11	>100 (143)
10.MTAA	920	18	50
11.CFAD	78	10	11
12.SFAE	170	6	12
13.POE-COM	1300	12.5	>100 (143)
14.SLAE	1350	10	22
15.PEG-MO	460	14	70
Anionic detergents			
16.SAGL	148	5.2	4
17.SCGL	142	7.5	11
18.SLGL	850	8	13.5
19.SGCS	210	5	14
20.SLSA	180	5.5	7
21.POE-LP	31.5	7	20
22.PL	150	6	8
23.SLS	34.5	4.5	4.5
24.SCFAT	80	5	8
25.ST	69	10	12
26.SPLS	37	14	8
Cationic detergents			
27.BC	5.6	2	1.5
28.DMAC	92	3	2.4
29.OCAE	70	2	1.5
30.STAC	21.5	1.1	2.2
31.SDAC	62	2	3
32.DSAC	63	4	16
33.ABAC	7.6	1.5	2
Amphoteric detergents			
34.LDAB	390	14	5.8
35.ACIB	190	22	30
Glycol			
36.GLY	>100000	35	>100 (400)
37.PEG400	82000	45	>100 (280)
38.BG	>100000	50	>100 (224)
39.PG	>100000	70	>100 (625)
40.DPG	>100000	60	>100 (298)
Oil			
41.IM	44500	35	>100 (550)
42.MP	27000	30	>100 (490)
43.TEGH	43000	40	>100 (500)
44.GLYT	4000	30	>100 (600)
Aptiseptic			
45.2PE	25000	35	30
46.SB	16000	30	43
Others			
47.MA	10800	25	12
48.SM	53000	25	18.2
49.ETOH	>100000	60	>100 (800)
50.ACET	7900	24	45
51.FORM	160	8	8

a) See Table 1 for description of chemicals.
b) NHEK-NR50; Concentration of the chemical extrapolated from the dose-survival curve giving 50 % cell survival which was estimated using NHEK and neutral red assay.
c) RC-LD50; Concentration of the chemical extrapolated from the dose-survival curve giving 50% cell survival which was estimated using RC cells and colony forming assay.
d) DS20; Concentration of the chemical extrapolated from the dose-response curve giving a Draize test score of 20.

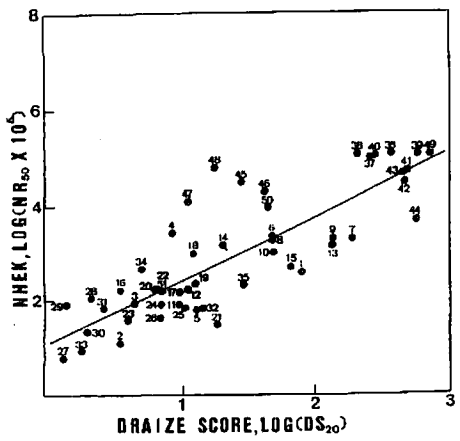

Fig. 4. Rank correlation of the cytotoxicity in vitro to NHEK (Neutral red assay) and in vivo eye irritation by Draize test. Logarithmic values of the concentration (w/w%;) of each chemical predicted to cause a Draize score in vivo and logarithmic values of the concentration multiplied by 10^5 (w/w%) of each chemical allowing 50% survival was used as a measurement of cytotoxicity to NHEK in vitro. The regression equation was $y = 1.32x + 1.02$ and the regression coefficient was $r = 0.83$. The numbers correspond to those in Table 1.

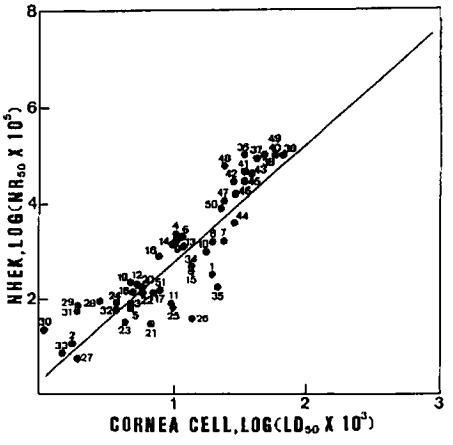

Fig. 5. Rank correlation of the cytotoxicity to NHEK (Neutral red assay) and to primary Rabbit Cornea cells (colony forming assay). Logarithmic values of the concentration (w/w%) multiplied by 10^3 (w/w%) of each chemical allowing 50% survival was used to determine cytotoxicity to primary Rabbit Cornea cells, and logarithmic values of the concentration multi plied by 10^5 (w/w%) of each chemical allowing 50% survival was used as a measure-ment of cytotoxicity to NHEK. The regression equation was $y = 2.43x + 0.36$ and the regression coefficient was $r = 0.89$. The numbers correspond to those in Table 1.

between human and the other animal cells. Therefore, we tried to use primary cells of normal human epidermal keratinocytes(NHEK). A significant correlation between the relative toxicity in NHEK cells and both the relative toxicity in RC cells and the relative toxicity *in vivo* was observed. Thus NHEK can be successfully used for preliminary screening and to determine the degree of toxicity of chemicals.

When compared with the colony forming assay using RC cells, the neutral red assay using NHEK cells was more sensitive. Strong toxic compounds *in vivo*, for example, benzethonium chloride (BC, chemical 27 in Table 2.) gave a 50 times a lower NR50 value ($5.6X10^{-5}$, w/w%) than LD50($200X10^{-5}$, w/w%). On the other hand, weak toxic compounds such as ethanol (ETOH, chemical 49 in Table 2) showed a higher NR50 value (1, w/w%) than LD50 (0.06, w/w%). Trerefore cytotoxicity range analysis using NHEK and the neutral red assay can be performed more easily than those using RC cells and the colony forming assay. Since these two systems are different in assay method (colony forming assay and uptake of neutral red), culture medium(serum free medium in NR assay and serum medium in RC assay), origin of cells and exposure time to test chemicals, it is not obvious which cells are more suitable as an alternative to the Draize ocular irritation test. It is expected, however, that NHEK correlate better with *in vivo* human eye irritancy than any other animal cells.

In addition, neutral red assay using NHEK is easy to perform, is objective, and can be applied to an automatic assay.

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