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IN VITRO CYTOTOXICITY TEST USING RABBIT CONJUNCTIVA, RABBIT CORNEA AND HELA CELLS AS ALTERNATIVES FOR THE DRAIZE EYE IRRITATION TEST

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In vitro cytotoxicities of 7 detergents, 5 shampoos and 3 rinses were determined on the basis of the colony forming abilities of three types of cells, primary rabbit conjunctival (RCN) cells, corneal (RC) cells and established HeLa cells. We compared the cytotoxicities of each cell type *in vitro* and the Draize eye irritation test *in vivo*, and compared the cytotoxicities among three types of cells. There was a good correlation between the cytotoxicities of each cells *in vitro* and the Draize score *in vivo*, and a correlation among three types of cells. The same sensitivities among the three types of cells were observed. These data suggest that, using either RCN, RC or HeLa cells, the cytotoxicity test *in vitro* may be useful as a substitute for the Draize eye irritation test.

Introduction

The Draize rabbit eye irritation test has been used to assess the potential eye irritation of most chemicals, cosmetics and consumer products[1]. This method, however, has been criticized for its lack of interlaboratory reproducibility, the need for skillful judgment and the pain caused to animals as a result of severe and permanent damage to the eye[2, 3]. Therefore, an alternative method to the Draize eye irritation test is required.

Recently, a large number of cytotoxicity tests on cultured cells have been developed as alternatives to the Draize test. These tests are based on colony forming ability[1-6], absorption of neutral red dye[7, 8] and uridine uptake[9].

Several investigators have examined the cytotoxicity of various substances based on the colony forming ability of primary corneal epithelial cells[5], an established cell line of rabbit cornea (SIRC cell)[10] and also in other cell lines[5, 11]. In a previous study[4], we examined the cytotoxicities of 52 chemicals using freshly isolated rabbit corneal (RC) cells. There was a close correlation between *in vitro* and *in vivo*. Results, which suggested that the colony forming assay using cultured cells is one of the most appropriate alternatives to *in vivo* testing. However, we did not use conjunctival cells for these cytotoxicity tests. The Draize eye irritation score was estimated on the basis of the reaction by the cornea (73 % of total Draize score), conjunctiva (18 %) and iris (9 %)[1]. The corneal ratio (73 %) of the total Draize score was higher than the conjunctival ratio (18 %), but the conjunctiva and the cornea were both directly exposed to irritants. Furthermore, it was observed that the *in vivo* irritative responses differed between the conjunctiva and the cornea. Ether-banded non-ionic detergents caused severe corneal damage following anesthesia[12, 13]. In our preliminary study, a lower concentration of anionic detergents caused a reaction only in the conjunctiva.

This study, therefore, was designed to determine whether freshly isolated conjunctival (RCN) cells were appropriate as an *in vitro* replacement to the Draize rabbit eye irritation test as well as

freshly isolated corneal (RC) cells in our previous study. In addition, we examined the cytotoxicity of the universally established HeLa cell line, and compared the cytotoxicities of RCN, RC and Hela cells.

Furthermore, in a previous study[4], we examined the cytotoxicity of the ingredients of cosmetics (detergents and other chemicals). In this study, we additionally examined the cytotoxicities of shampoos and hair rinses as well as the detergents used in cosmetics.

Materials and Methods

Cell culture:

Primary RCN and RC cells and established HeLa cells were used in this experiment.

RCN cells were separated from the rabbit conjunctiva and RC cells were separated from the rabbit cornea. The conjunctivas and the corneas from a Japanese white rabbit were rinsed with phosphate-buffered saline without calcium or magnesium. Conjunctivas and corneas were cut into small pieces with a knife. About 15 pieces were put into a 6-cm diameter dish and incubated in Eagle's minimum essential medium with 10 % fetal bovine serum at 37 ℃ in a CO_2 incubator for 5-7 days. Cell outgrowth was observed around the cultured pieces of conjunctivas and corneas. Primary cultures were trypsinized briefly, subcultured for 4 days at 10^5 cells/75 cm^2 flask. Secondary cultures were trypsinized briefly, suspended in culture medium containing 10 % dimethylsulphoxidd and stocked in liquid nitrogen. Both cell types were subcultured every 4 days. The cloning efficiency of both cell types before passage 5 was about 50 %, but declined gradually thereafter. Therefore only cells between passages 3 and 5 were used in cytotoxicity tests.

HeLa cells were subcultured in a manner similar to the method used for RCN and RC cells. The cloning efficiency of HeLa cells was almost 100 %.

Test samples :

We chose 15 test samples consisting of 7 detergents, 5 shampoos and 3 hair rinses. (Table 1) Detergents were of technical grade, and represented those usually used as ingredients of cosmetics. Shampoos and rinses were commercial products marketed in Japan.

Table 1. TEST SAMPLES TESTED IN CYTOTOXICITY TESTS WITH RCN, RC AND HELA CELLS *IN VITRO* AND RABBIT EYE IRRITANCY TEST *IN VIVO*

Test samples	Abbreviation	solvent[a]
Non-ionic detergents		
1. Polyxyethylene Glycol Monolaulate	POE-GML	A
2. Polyoxyethylene Oleyl Ether	POE-OE	C
Anionic detergents		
3. Sodium Lauryl Sulfate	SLS	A
4. Sodium N-Lauroyl-L-Gulutamate	SLGL	A
Cationic detergents		
5. Stearyl Trimethyl Ammonium Chloride	STAC	A
6. Distearyl dimethyl Ammonium Chloride	DMAC	C
Amphoteric detergent		
7. 2-Alkyl-N-Carboxymethyl-N-Hydroxyethyl Imidazolinium Betaine	ACIB	A
Shampoos		
8. Shampoo 1 (Anionic, amphoteric and non-ionic detergents ; 17 %)	S1	B
9. Shampoo 2 (Anionic and amphoteric detergents ; 23 %)	S2	B
10. Shampoo 3 (Anionic and non-ionic detergents ; 27 %)	S3	B
11. Shampoo 4 (Anionic, amphoteric and non-ionic detergents ; 16 %)	S4	B
12. Shampoo 5 (Anionic, amphoteric and non-ionic detergents ; 14 %)	S5	B
Hair rinses		
13. Rinse 1 (Cationic and non-ionic detergents ; 3.5 %)	R1	B
14. Rinse 2 (Cationic and anionic detergents ; 4.0 %)	R2	B
15. Rinse 3 (Cationic and non-ionic detergents ; 3.5 %)	R3	B

a) Solvents ; A = Phosphate buffered saline, B = Ethanol, C = 50 % ethanol in phosphate buffered saline.

Shampoos were mainly formulated as anionic and amphoteric detergents. Hair rinses were mainly formulated as cationic detergents.

Cytotoxicity test :

RCN, RC and HeLa cell exponential cultures were trypsinized briefly, suspended in culturl medium. RCN and RC cells were seeded at 150 cells/60 mm diameter dish of 3 dishes/dose. HeLa cells were seeded at 75 cells/60 mm diameter dish. After 6-12 hr., these cells were treated *in situ* with test samples for 20 min. at 37 ℃, then washed twice with 5 ml phosphate-buffered saline, refed with complete medium and allowed to form colonies. Test samples listed in Table 1 were dissolved in phosphate-buffered saline, ethanol or 50 % ethanol in phosphate-buffered saline on a weight/weight basis, sterilized through a filter and put into the culture medium directly with micropipettes. The final concentrations of solvents were less than 2 %, which had no effect on cell survival. After 10-12 days, colonies were fixed with ethanol, stained with 5 % Giemsa solution, and the colonies containing more than 50 cells were counted. The surviving fractin was expressed as the ratio of the number of colonies raised in the treated dish to the number of colonies raised in the untreated dish. For the *in vitro* data, the survival fraction, calculated from the individual scores of 3-6 independent experiments, was plotted against each test sample concentration tested to obtain a does-response plot. The concentration of test sample allowing 50 % survival (LD50) was selected as a representative score during evaluatiom of the majority of the test samples.

Animal test :

Japanese white rabbits of both sexes were used. Test samples were prepared by same method as described above and put into the right eye directly by a micropipette. The left eye served as the control. At least 4 different concentrations of test sample were used im in vivo testing. Corneal, iris and conjunctival responses were scored[13, 14, 15] at 1, 3, 6, 24, 96 and 168 hr. Fluorescein stain was used to aid the determination of the extent of corneal damage. For the *in vivo* data, the arithmetical mean Draize score, calculated from individual scores of 3-6 rabbits at 6 different exposure times, was plotted against each test sample concentration tested to obtain a standard measure (DS20) for calculating comparative potency. This value was within the range of the score actually observed during evaluation of the majority of the test samples.

Characterization of intermediate filaments of RNC and RC cells :

RCN and RC cells were grown on cover-slips, washed with phosphate-buffered saline and fixed for 5 min. at −20℃ with methanol. Cells were incubated either with a monoclonal antibody prepared against cytokeratins Nos. 1 to 19 (Boehringer Mannheim, Penzberg, FRG) or with a monoclonal antibody against vimentin (Amersham, Tokyo, Japan) for 60 min. Cells were then washed with phosphate-buffered saline, stained with fluorescein-isothiocyanate conjugated anti-mouse IgG (Amersham, Tokyo, Japan) and mounted with Eukitt (O. Kindler, FRG) for viewing under an Olympus photomicroscope equipped with epiflturescent illumination.

Results

Characterization of intermediate filaments of RCN and RC cells :

Characterization of intermediate filaments of RCN and RC cells was performed at passage 3 by staining with a monoclonal antibody prepared against either cytokeratin or vimentin type intermediate filaments. RCN cells were stained with monoclonal antibody raised against vimentin, but not with one against cytokeratin. RC cells, too, were stained with a monoclonal antibody raised against vimentin, but not with one against cytokeratin.

Cytotoxicity and eye irritation tests of test samples :

We studied the cytotoxic effect of 15 test samples which included detergents and cosmetic products on RCN, RC and HeLa cells, and studied the eye irritation caused by 7 detergents, 2 shampoos and 2 hair rinses. The dose-response plots for 6 typical test samples,

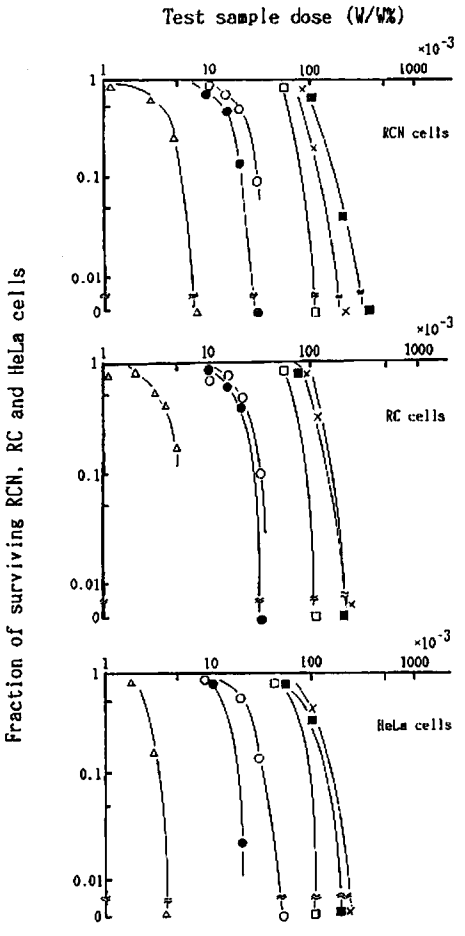

Fig. 1. Survival of RCN, RC and HeLa cells treated with test samples (6 out of 15 test samples tested; ○, POE-GML; ●, SLS; △, STAC; ×, ACIB; ■, S2; □, R2). See Table 1 for the full name of test samples.

Table 2. LD50 VALUES FROM *IN VITRO* CYTOTOXICITY TESTS AND *IN VIVO* DRAIZE SCORES

Tdst samples[a)]	*In vitro* testing (LD 50[b)]) RCN (w/w % ×10²)	RC (w/w % ×10²)	HeLa (w/w % ×10²)	*In vivo* testing DS 20[c)] (w/w %)	Draize rank[d)]
Non-ionic detergents					
1. POE-GML	20	20	23	85	Non
2. POE-OE	4.2	4.7	6.2	5.5	Severe
Anionic detergents					
3. SLS	19	15	12	4.5	Moderate
4. SLGL	160	150	230	13.5	Mild
Cationic detergents					
5. STAC	3.8	4.2	2.5	2.2	Severe
6. DMAC	7.5	5.8	7.2	2.4	Moderate
Amphoteric detergent					
7. ACIB	90	90	96	30	Non
Shampoos					
8. S1	120	130	140	39	Slight
9. S2	70	70	70	43	Slight
10. S3	70	70	70		
11. S4	140	140	140		
12. S5	140	130	140		
Hair rinses					
13. R1	240	130	90	>100(105)	Slight
14. R2	130	180	140	22	Mild
15. R3	160	270	110		

a) See Table 1 for description of chemicals.
b) LD 50; Concentration of the test sample extrapolated from the dose-survival curve giving 50 % cell survival.
c) LS 20; Concentration of the test sample extrapolated from the dose-response curve giving a Draize test score of 20.
d) Draize rank; Ocular irritancy classification based on Draize test scores according to the scale of Kay and Calandra (1962).

obtained from *in vitro* testing, are shown in Fig. 1. The LD 50 values from the dose-response plots represent the 50 % survival ratio of the concentration of the test samples. Table 2 shows the LD 50 values for all samples tested *in vitro* using RCN, RC and HeLa cells and the concentration of each chemical predicted to cause a Draize score of 20 in the rabbit eye *in vivo*. The last column in Table 2 gives the eye irritancy classification for each of the test samples based on the Draize test scores according to the scale of Kay and Calandra[15]. Cationic detergents, such as stearyl trimethyl ammonium chloride and distearyl dimethyl ammonium chloride, and ether-banded non-ionic detergents, such as polyoxyethylene oleyl ether (7E.O.), showed the highest toxicity both *in vitro* and *in vivo*. Anionic, amphoteric and ester banded non-ionic detergents, such as polyoxyethylene glycol monolaulate (10E.O.), showed moderate or weak toxicity both *in vitro* and *in vivo*. Furthermore, Shampoor and hair rinses showed

weak toxicity both *in vitro* and *in vivo*.

Comparison between in vitro and in vivo data :

Comparison between *in vitro* data using the concentration of LD 50 values and *in vivo* using the concentration of the Draize score of 20 are shown in Fig. 2. Good correlations were obtained betweem *in vitro* data and *in vivo* data. The correlation coefficients calculated from these data were RCN vs. DS 20 = 0.77, RC vs. DS 20 = 0.75 and HeLa vs. DS 20 = 0.72.

Comparison among in vitro data :

LD 50 values determined for test samples were compared among RCN, RC and HeLa cells. There was a close correlation among three types of cells. The correlation coefficients were RCN vs. RC = 0.99, RCN vs. HeLa = 0.94 and RC vs. HeLa = 0.97. (Fig. 3)

Discussion

In this study, we examined the cytotoxicity of various detergents and cosmetics using freshly isolated rabbit conjunctival (RCN),corneal (RC) cells and established HeLa cells. RCN and RC cells were used between passage 3 and 5 before a decrease in cloning efficiency. Both cell types were stained with the monoclonal antibody raised against vimentin type intermediate filament, but not with one against cytokeratin. These results showed that RCN and RC cells used in this study were derived from the embryonic mesoderm. RCN cells are thought to be fibroblast cells of the rabbit conjunctival mucosa and RC cells are thought to be endothelial and stromal cells of the cornea.

We examined the cytotoxicity of 7 detergents, 5 shampoos and 3 hair rinses based on the colony forming abilities of RCN, RC and HeLa cells. Cationic detergents and ether - banded non - ionic detergent, such as polyoxyethylene oleyl ether (7E.O.), produced a higher cytotoxicity on RCN, RC and HeLa cells than anionic, amphoteric and ester - banded non - ionic detergents, such as polyoxyethylene glycol monolaulate (10E.O.). The results of tests using cationic detergents were in agreement with the ranking reported by others[4, 5, 9] Cationic detergents commonly used in hair rinses and

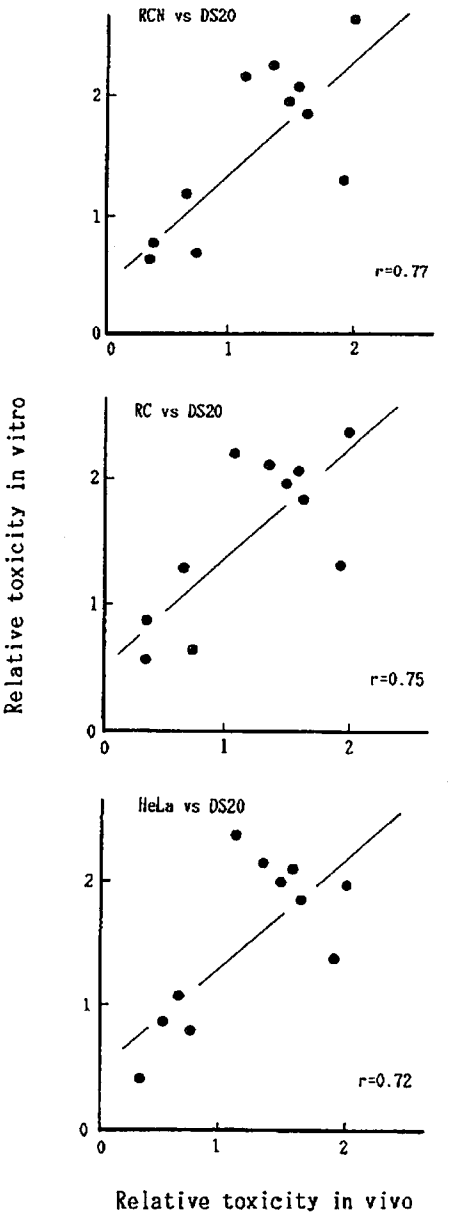

Fig. 2. Rank correlation of the cytotoxicities of RCN, RC and HeLa cells *in vitro*, and eye irritation in the Draize test for the 15 test samples. Logarithmic valuse of the concentration (W/W %) of each test sample predicted to cause a Draize score of 20 *in vivo* were plotted as the relative toxicity *in vivo*, and logarithmic values of the concentration multiplied by 10^3 (W/W %) of each test sample allowing 50 % survival was used as the relative toxicity *in vitro*. Correlation coefficients are RCN vs. DS 20 = 0.77, RC vs.DS 20 = 0.75 and HeLa vs. DS 20 = 0.72.

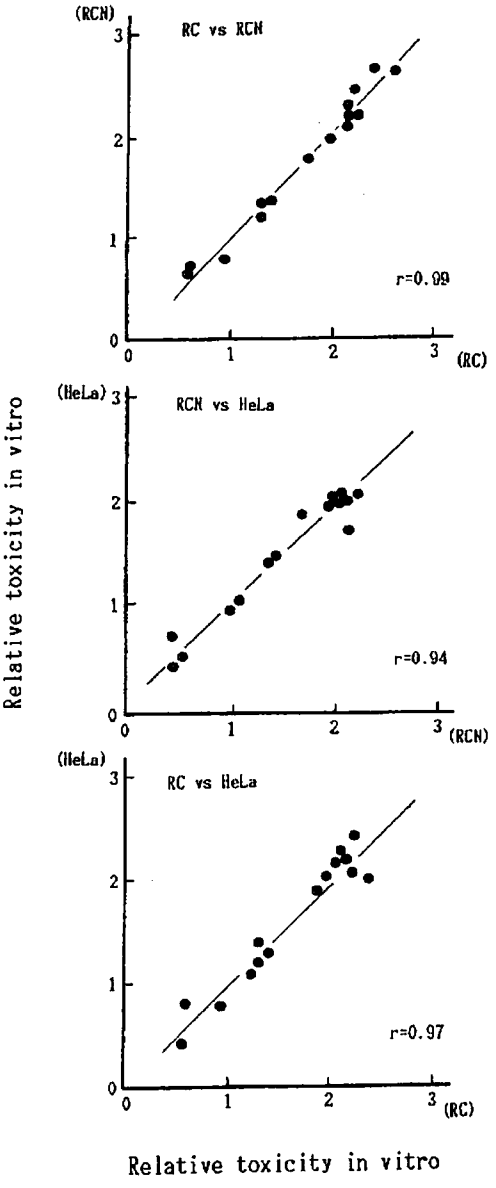

Fig. 3. Rank correlation among cytotoxicities of RCN, RC and HeLa cells *in vitro* for 15 test samples. Logarithmic values of the concentration multiplied by 10^3 (w/w %) of each test sample allowing 50 % survival was used as the relative toxicity *in vitro*. Correlation coefficients are RCN vs. RC = 0.99, RCN vs. HeLa = 0.94 and RC vs. HeLa = 0.97.

cosmetics, are irritating *in vivo*. The results of ether - banded non - ionic detergents were also in agreement with a previous study[4]. Ether - banded non - ionic detergents are known to cause severe corneal damage following anesthesia in the rabbit eyes[12, 13]. Since an ether - banded non - ionic detergent was put into the eye, it was dangerous that we did not notice corneal damage possibly as a result of anesthesia. Accordingly, ether - banded non - ionic detergents must be screened using the cytotoxicity test. Shampoos and hair rinses had weak cytotoxic effects on RCN, RC and HeLa cells. The results for shampoos were similar to the result of cytotoxicity using SIRC cells[10]. The cytotoxicity of shampoos, mainly formulated with anionic and amphoteric detergents, and hair rinses, mainly formulated with cationic detergents, produced a lower cytotoxicity than the detergents themselves. The reason for this is thought to be that shampoos and hair rinses contain a low percentage of detergents : 14 - 27 % and 3.5 - 4.0 % respectively.

There were good correlations between the relative toxicity *in vitro* and the relative toxicity *in vivo* and there were strong correlations among cytotoxicity of RCN, RC and HeLa cells. This observation was in agreement with that of another group[7] who compared among 5 cell types. Their data indicated that primary rabbit corneal cells were more sensitive to a given concentration of test sample than the established cell lines. However, according to our data primary RCN and RC cells and established HeLa cells all showed the same sensitivities. On the other hand, different *in vivo* responses between the conjunctiva and the cornea were observed when the eye irritation response of detergents was tested. Ether - banded non - ionic detergents caused severe corneal damage following anesthesia[12, 13]. In our preliminary study, anionic detergents caused the corneal and the conjunctival damage, but a lower concentration of anionic detergents caused a reaction only in the conjunctiva. It is thought that the different structure of the detergents tested caused the difference in response between the

conjunctiva and the cornea *in vivo*. However, our *in vitro* observation showed that the sensitivities to cytotoxicity between the primary conjunctival (RCN) and corneal (RC) cells were the same. The different cytotoxic responses between the conjunctiva and the cornea *in vivo*, was not observed between the conjunctival (RCN) cells and the corneal (RC) cells *in vitro*.

These data therefore indicate that, similar to a previous study using RC cells[4], the cytotoxicity based on the colony forming ability using freshly isolated RCN and RC cells and established HeLa cells can be successfully used for preliminary screening and for determining the range of the toxic concentration of potentially irritative compounds. These *in vitro* assays were economical, easy to execute, objective and could potentially reduce the large number of animals required for *in vivo* tessing.

The cytotoxicity test using RCN and RC cells required a rabbit at the first step to obtain and separate conjunctival and corneal cells. However the cytotoxicity test using HeLa cells did not require any animals, and HeLa cells could be cultured more easy than RCN and RC cells. Therefore, we believe that the cytotoxicity test based on colony forming ability using HeLa cells is easier and more efficient since it dose not require animals.

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