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Which Cytotoxicity Tests are Useful for Prediction of Skin Irritation by Surfactants?

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Abstract

With the aim to predict human skin irritation, cytotoxicity tests have recently been introduced on a worldwide basis. Many papers have appeared reporting data with reconstituted human dermal models, skin explants and cultured monolayer cells as alternatives to *in vivo* skin irritation tests. To answer the question which cytotoxicity tests are the most useful for prediction of human skin irritancy, we compared results of MTT assays using rabbit skin explants (*3D - Histoculture* : organ model), a reconstituted human dermal model (Gunze™ cultured 3-Dimensional Skin Model : 3-D culture model), cultured monolayers of human dermal fibroblasts (NB1RGB : monolayer cells), a Draize primary skin irritation test with rabbits (Draize rabbit test) and human patch testing with 12 surfactants.

Comparison of *in vivo* human data with the *in vitro* test and Draize rabbit test findings did not demonstrate a good correlation. In particular, monolayer cells showed many false positives. The organ model was the most accurate in term of consistence with human patch results, similar in this respect to the Draize rabbit test.

We therefore consider that the organ model is useful to evaluate the potential for causation of human skin irritation by new surfactants.

Keywords: *skin irritation, reconstituted human dermal model, skin explants, in vitro, fibroblasts, and surfactant*

Introduction

There is great interest in establishing alternatives to animals for test purposes, and many investigators have proposed various approaches for predicting the potential of chemicals to cause skin corrosion or irritation. These include cytotoxicity tests using cultured monolayer cells such as normal human keratinocytes (Dickson et al., 1994, Muller-Decker et al., 1994, Laurence et al., 1996, Shibata et al., 1997), an immortalized human keratinocyte

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cell line (HaCaT: Wilhelm et al., 1994, Brosin, 1997), normal human fibroblasts (Shin et al., 1996), a human epithelial cell line (Konishi et al., 1977a, 1977b), and other cells (Jain et al., 1992, Kotani et al., 1994, Clare and Cormak, 1996, DeLeo et al., 1996). The data have in general shown good correlations with *in vivo* findings, at least with surfactants. In addition, a reconstituted human dermal model and skin explants have recently been employed to investigate the mechanisms of skin irritation or corrosion (Oliver et al., 1988, Li et al, 1991, Gay et al., 1992, Slivka and Zeigler, 1993, Monterio-Riviere et al., 1997). Good correlations with *in vivo* results have been reported using various substances (Botham et al., 1992, Laska et al., 1992, Osborne and Perkins, 1994, Roguet et al., 1994, 1998, Pierard 1995, Kotani et al., 1996, Kojima et al., 1996,1998, Monterio-Riviere et al., 1997, Morota et al., 1997, 1999, Augustin, 1998, Genno et al., 1998).

For the purpose to answer the question which cytotoxicity tests are the most useful for prediction of skin irritancy. We have compared a number of cytotoxicity tests with human skin and rabbit primary skin data.

Methods we selected were MTT (3 - [4,5-dimethyl - thiazol - 2 - yl] - 2,5 - diphenyl tetrazolium bromide) reduction assay using rabbit skin explants (*3D-Histoculture* : organ model), a reconstituted human dermal model (Gunze^{TM} cultured 3-Dimensional Skin Model : 3-D culture model) and cultured monolayer human dermal fibroblasts (NB1RGB : monolayer cells), and we used 12 surfactants as test chemicals. The results were then compared with data from Draize primary skin irritation with rabbits (Draize rabbit test), and human patch tests for the same lot of the compounds.

Materials and Methods

Test substances

The twelve surfactants employed are listed in Table 1 along with their suppliers and abbreviations. Those with a purity >99% were obtained from Sigma, New York, USA, and with a purity > 95% were supplied by Tokyo Kasei Kogyo, Tokyo, Japan.

Table 1 Summary of details for the test substances employed.

Test substance(Supplier)	Abbreviation
Cationic Surfactants	
Benzethonium Chloride (Katayama Chemical Industry)	BC
Cetylpyridinium Chloride (Sigma)	CC
Distearyl Dimethyl Ammonium Chloride (Tokyo Kasei Kogyo)	DDAC
Stearyl Trimethyl Ammonium Chloride (Tokyo Kasei Kogyo)	STAC
Anionic Surfactants	
Potassium Laurate (Kanto Chemical)	PL
Sodium Lauryl Sulphate (Sigma)	SLS
Nonionic Surfactants	
Polyoxyethylene 23 Lauryl Ether	POE-LE
Polyoxyethylene 10 Oleyl Ether	POE-OE
Polyoxyethylene Sorbitan Monolaurate (20E.O. Sigma)	Tween 20
Polyoxyethylene Sorbitan Monoorate (20E.O. Sigma)	Tween 80
Sucrose Fatty Acid Ester (Tokyo Kasei Kogyo)	SFAE
t-Octylphenoxypolyethoxyethanol (Sigma)	Triton X-100

Test methods

1. Cytotoxicity tests

1-1 Cytotoxicity in *3D-Histoculture* (Rabbit skin explant : organ model)

The rabbit skin explants for the organ model are produced and supplied by Kyokuto Pharmaceutical Indust. Co. Ltd., Tokyo, Japan. The explant are 5.0 mm in diameter and consist of living rabbit dermis and epidermis.

Test substances were dispersed at a variety of different concentrations in white petroleum ;white vaseline (Sun White P-200, Nikko SC Corp., Tokyo, Japan) or applied as 100 % when liquids, and 20 mg aliquots were applied to explants on collagen gel in 24 well-plates (Falcon, Becton Dickinson Labware, Tokyo, Japan). One ml of medium (Dulbecco's modified Eagle's Minimum Essential Medium: MEM supplemented with 5 % fetal bovine serum) was added to each well and the explants were cultured for 24 hr at 37℃ in a 5 % CO_2 environment (Tabai Espec, Osaka, Japan). The explants were washed with phosphated buffer saline (pH 6.8), than placed in culture medium containing MTT (Sigma, St Louis, MO,USA) at 0.5 mg/ml. Two hours later the MTT formazan crystals formed were extraced with 200 μl of isopropanol and the absorbance was measured at 540 nm with a microplate reader (Emax, Molecular Devices, Menlo Park, CA, USA). The ratio of the absorbance for treated vs. non-treated cases was calculated and the MTT_{50} value was estimated from duplicate dose-response curves (MTT assay, Mosmann, 1983).

1-2 Cytotoxicity in the Gunze™ cultured 3-Dimensional Skin Model (3-D culture model)

The 3-D culture model, which simulates human skin, is produced and supplied by Gunze Co. Ltd., Kyoto, Japan. This model maintains barrier functions, and consists of living human dermis and epidermis. Fibroblasts derived from neonatal human foreskins are seeded in collagen. After four weeks in culture, epidermal keratinocytes are seeded onto this collagen sponge in polypropyrene ring (inner diameter 6.3mm), which grow into a functional multilayered epidermis overlaid with stratum corneum (Morota et al., 1998).

Fifty mg aliquots of test substances applied as described in the organ model system. With each preparation on 24 well-plates, 1 ml of medium (Eagle's MEM supplemented with 10 % fetal bovine serum) was added. After 24 hr culture, the cytotoxicity was evaluated by the MTT assay as described above.

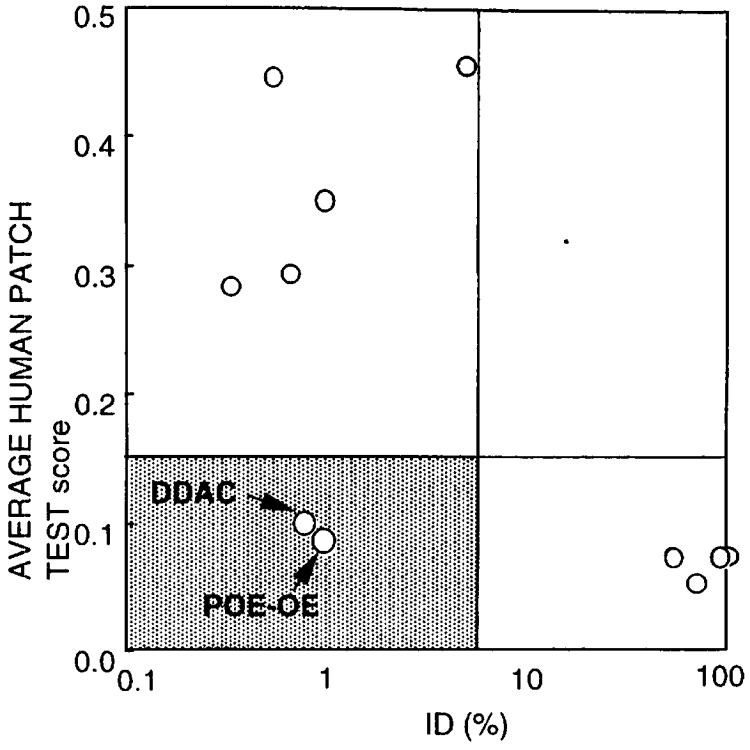
1-3 Cytotoxicity in human dermal fibroblasts (NB1RGB : monolayer cells)

Cytotoxicity to normal human skin fibroblasts (NB1RGB, Riken Cell Bank, Tukuba, Ibaraki, Japan) was evaluated by the MTT assay. The cell doubling time was 24 hr and the culture medium was Dulbecco's Modified MEM (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (JRH Bioscience, Lenexs, KS, USA). A 100 μl suspension of 4×10^3 cells/ml was added to each well of 96 well-plates (Falcon) and incubated for 3 days. Test substances were dissolved or suspended in distilled water at different concentrations and added to five or six wells per concentration. After 24 hr, the cytotoxicity was evaluated by the MTT assay.

2. In vivo

2-1 Draize primary skin irritation test with rabbits (Draize rabbit test)

Three Japanese white female rabbits with a weight of 2.9 to 3.5 kg were used for each test substance. The rabbits were shaved on the back and lateral areas. One hundred-fifty mg of test substances at different concentrations (% : w/w), dispersed in white petroleum, were applied to intact skin under 1.7 cm diameter gauze patches. After 24 hr the patches were removed and the skin response was scored for erythema and oedema for each rabbit at 1 and 24 hr after the removal (Draize et al, 1944, Food and Drug Administration, 1959). The average score was calculated at each time for



]Fig. 1. Comparison of Draize primary skin scores with rabbits and average human patch test socres for surfactants.
ID : Irritation Dose of more than 5.0 % is positive and under 5.0 % is negative. Average human patch test score : more than 0.15 is positive and under 0.15 is negative. Gray zone shows false positives, DDAC and POE-OE.

each substance and the maximum value was used as the Draize score. Furthermore, we obtained an Irritation Dose : ID(%) inducing clear erythema and oedema for each chemical substance .

2-2 Human patch test

Forty healthy volunteers of both sexes, aged 20-50 years, cooperated for this patch testing. We applied 15 mg preparations of test materials at 5 % in white petrolatum using Finn Chambers® (Norgesplaster A/S, Norway) and Scanpor® tape (Epitest Ltd Oy, Tuusula, Finland) to the upper arm. After 24 hr the patches were removed and the skin response was scored for erythema and oedema for each volunteer at 1 and 24 hr after the removal (Japanese standard criteria : Kawamura et al., 1970). The average score was calculated at each time for each substance and the maximum value was used as the human patch score.

Results

Our aim was to predict human skin irritancy for new chemical substances. We first assessed the accuracy of the Draize rabbit test that is widely used at present. As shown in Fig. 1, comparison of results with ID and human patch test finding did not reveal a good correlation (coefficient − 0.644 as shown in Table 2). However, with defined criteria for each test (a human patch test score of more than 0.15 is positive and an ID of more than 5.0 % is positive) a concordance of prediction was 83.3% (10/12, Table 3). The two surfactants, distearyl dimethyl ammonium chloride (DDAC) and polyoxyethylene 10 oleyl ether

Table 2. Correlation of various skin irritation assay vs. human skin irritancy results.

Test assay	Marker	Human patch test	Draize rabbit test	Monolayer cells	3-D Culture model	Organ model
Human patch test	Average score	*	- 0.644	- 0.378	-0.362	-0.686
Draize rabbit test	ID		*	0.622	0.587	0.366
Monolayer cells	MTT_{50}			*	0.464	0.369
3-D Culture model	MTT_{50}				*	0.825
Organ model	MTT_{50}					*

*Transformed logarithmic values used (except human patch test).

Table 3. Evaluation of the performance of various skin irritation assays in predicting irritancy/non-irritancy compared to human skin finding.

Test assay	Markers	Accuracy[1] % Number	Sensitivity[2] % Number	Specificity[3] % Number	False positive % Number	False negative % Number
Draize rabbit test	ID	83.3 (10/12)	41.7 (5/12)	41.7 (5/12)	16.7 (2/12)	0.0 (0/12)
Monolayer cells	MTT_{50}	50.0 (6/12)	41.7 (5/12)	8.3 (1/12)	50.0 (6/12)	0.0 (0/12)
3-D Culture model	MTT_{50}	66.7 (8/12)	41.7 (5/12)	25.0 (3/12)	33.3 (4/12)	0.0 (0/12)
Organ model	MTT_{50}	83.3 (10/12)	33.3 (4/12)	50.0 (6/12)	8.3 (1/12)	8.3 (1/12)

1 Accuracy (concordance) is defined as the proportion of correct outcomes with a method (NIEHS,1997).

2 Sensitivity is defined as the proportion of all positive surfactants that are correctly classified as positive in a test (NIEHS,1997).

3 Specificity is defined as the proportion of all negative surfactants that are correctly classified as negative in a test (NIEHS,1997).

(POE-OE), were false positives (rate : 16.7%) (NIEHS, 1997).

To evaluate *in vitro* cytotoxicity tests, we similarly investigated correlation and concordance with human skin irritancy. In terms of correlation, scatter points with the organ model were better than the other *in vitro* data as shown in Figs. 2 to 4. The correlation coefficient with logarithmic transformed values was highest with the organ model at -0.686, vwhile that for the 3-D culture model was -0.511 and for the monolayer cells was as low as -0.362, as shown in Table 2. From these results, the organ model alone had better predictivity than the Draize rabbit test ($r = -0.644$).

For the present study, we defined MTT_{50} values greater than 30% to be negative for the organ and 3-D culture models. The maximum dose employable with the cationic surfactants like DDAC and stearyl trimethyl ammonium chloride (STAC) was at this 30 % level. Therefore, values of 30 % were arbitrarily assigned. In monolayer cells, maximal feasible concentrations are reported to be 5000 μg/ml or 10mM of chemical (Sofuni, 1993). Therefore, we defined an MTT_{50} above one of these doses as negative. The organ model concordance was best at 83.3% (10/12), compared with 66.7 % (8/12) with the 3-D culture model

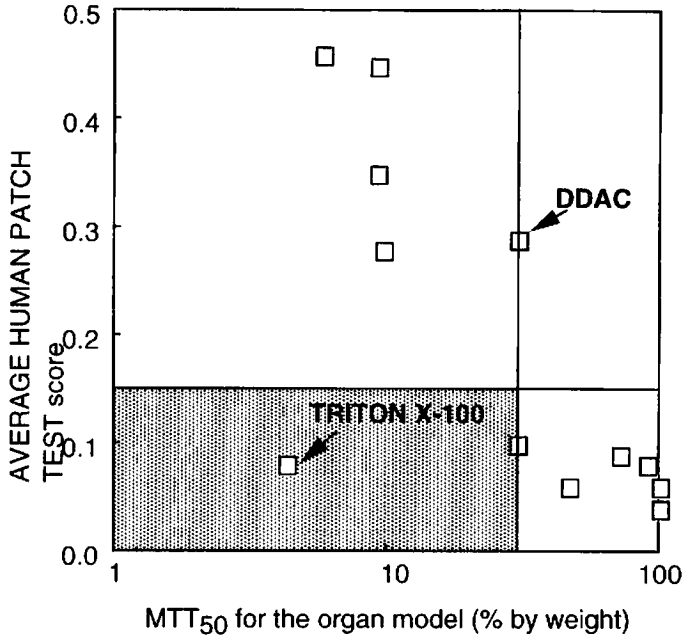


Fig. 2. Comparison of MTT_{50} values obtained with the organ model and average human patch test score for surfactants.
MTT_{50} : The ratio of the MTT readings for treated vs. non-treated was determined and the MTT_{50} value was calculated based on duplicate dose-response curves. Gray zone shows a false positive, Triton X-100, which DDAC is a false negative.

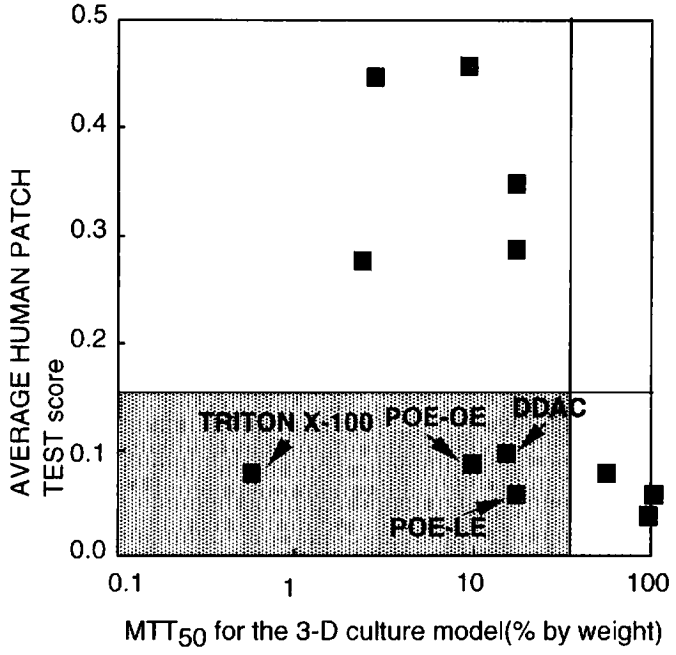


Fig. 3. Comparison of MTT_{50} values obtained with the 3-D culture model and average human patch test score for surfactants.
MTT_{50} : The ratio of the MTT readings for treated vs. non-treated was determined and the MTT_{50} value was calculated based on duplicate dose-response curves. Gray zone shows false positive, DDAC, POE-LE, POE-OE and Triton X-100.

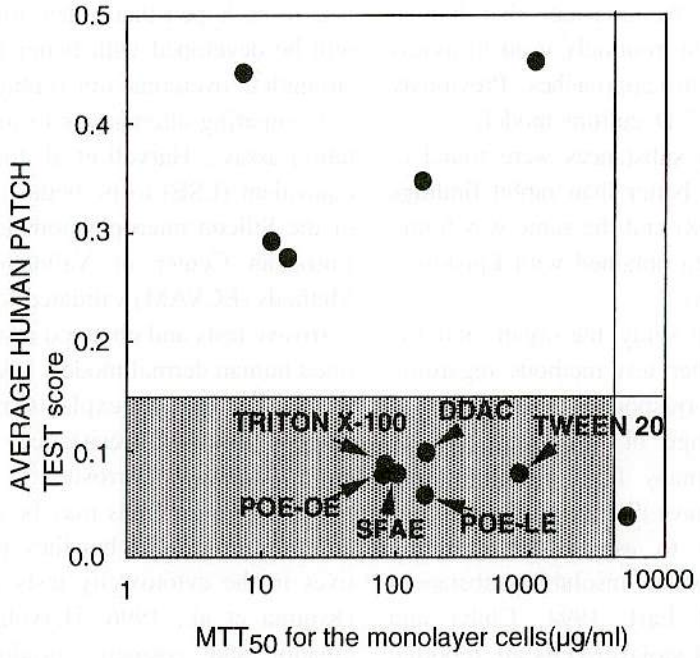


Fig. 4. Comparison of MTT_{50} values obtained with monolayer cells and average human patch test score for surfactants.
MTT_{50} : The ratio of the MTT readings for treated vs. non-treated was determined and the MTT_{50} value was calculated based on duplicate dose-response curves. Gray zone shows false positive, DDAC, POE-LE, POE-OE, SFAE, Triton X-100 and Tween 20.

and 50 % (6/12) with the monolayer cells, as shown in Table 3. The organ model gave inaccurate conclusions for t-octylphenoxypolyethoxyethanol (Triton X-100) and DDAC (Fig.2). In the 3-D culture model, four surfactants, Triton X-100, DDAC, POE-OE and polyoxyethelene 23 lauryl ether (POE-LE) were false positives (rate : 33.3%, Table 2 and Fig.3). In the monolayer cells, six surfactants, the 4 in the 3-D culture model and also SFAE, Tween 20 gave false positive results, as shown in Fig.4. The organ model demonstrated good correspondence with the human patch test.

The correlation coefficients between *in vitro* tests were 0.825 for the organ model vs. the 3-D culture model, 0.369 for the organ model vs. the monolayer cells, and 0.366 for the 3-D culture model vs. the monolayer cells, as shown in Table 2.

Discussion

For comparison of *in vivo* data with *in vitro* skin irritation results, we have used human patch test findings with a 5% dose (solvent: white petrolatum), rather than those obtained for *in vivo* Draize rabbit test. The reason is that effects on animals may not accurately reflect impact on human health. It is reported that rabbit skin is more sensitive to irritants than those of guinea pig, rat or man, so that the existence of false positives with its use may be expected in rabbit skin test (Motoyoshi et al., 1978). Therefore, assessment of the safety of new chemical substances requires new alternatives to such animal testing. Though many papers have appeared comparing *in vitro* with animal data for prediction of skin irritancy, there has been no standarization with human

patch test results. We consider that human patch data should be routinely used to assess the accuracy of *in vitro* approaches. Previously Skin^{2TM} ZK1200 (3-D culture model) results for water insoluble substances were found to match human data better than rabbit findings (Kojima et al., 1998) and the same was found for cytotoxicity data obtained with Episkin^{TM} (Roguet et al., 1998).

From the present study, the organ model is better than the other test methods regarding correlation and correspondence with human findings for a range of surfactants. With monolayer cells, many false positives were encountered, and have also been shown to be clearly inadequate to assess skin irritation or corrosion of water insoluble substances (Chamberlian and Earl, 1994, Chiba and Toyama, 1989). Monolayer cells are reported to be useful for prediction of skin irritancy of a limited range of chemicals like water soluble substances (Konishi et al., 1977a, 1997b, Fujii et al., 1979, Osborne and Perkins, 1991, Shin et al., 1996), and have therefore been proposed for general use, because of their ease of application, cheapness, high repeatability and popularity. However, the present data would suggest that they are also not appropriate for assessing of human skin irritation by water soluble substances like surfactants.

Histological samples of 3-D cultured model show great similarity to human skin tissue. However, the barrier function is impaired and be required to improve this model. Comparing epidermis reconstructed on a de-epidermized dermis and on a fibroblast-populated collagen matrix, the sensitivity differs more than 10 fold (Ponec et al., 1995). Furthermore comparison of Skin^{2TM} ZK1200 (Ocular model : thin layer type epidermis with poor barrier function) and ZK1300 (Skin model : thick layer epidermis with good barrier function) again revealed ten fold variation (data not shown). These results show that the barrier function of skin is important due to variation in dermal absorption of a chemical. In future, it is to be hoped that a new improved version will be developed with better barrier function strength to overcome this problem.

Comparing alternatives to *in vitro* skin irritation assays, Harvell et al. found living skin equivalent (LSE) to be better than Skintex^{TM}, or the Silicon microphysiometer (1994). The European Center of Validation Acceptance Methods (ECVAM) validated four *in vitro* skin corrosive tests and obtained results of reconstituted human dermal models (Skin^{2TM} ZK1350, Episkin^{TM}) and skin explants (rat skin transcutaneous electrical resistance : TER) to be better than with Corrositex^{TM} (Fentem et al., 1998). These models may be useful to evaluate skin toxicology, but they gave false positives in the cytotoxicity tests of TritonX-100 (Kojima et al., 1996, Harvell et al., 1994), ethanol and cosmetic products containing ethanol at high concentration, and cationic surfactants (Kojima et al.,1995, 1996, Genno et al.,1998). Fentem et al. also reported the existence of many false substances in validated assays (1998).

Many problems remain to be solved, not least of which is the cost effectiveness of different approaches. In the case of kits, the price is expected the same as with animal tests. Their quality control is also problematic with possible variation in thickness and differ in other biological parameters. At third problem, with general approval from volunteers for human patch testing, *in vitro* results alone are acceptable or should the availability of animal test data be a condition? We must settle these issues to most effectively develop a new model for data accumulation and validation studies.

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