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Corresponding author:
Mr. Makoto Mizuno
KOSÉ Corporation,
Fundamental Research Laboratories, 1-18-4,
Azusawa, Itabashi-ku, Tokyo, 174-0051, Japan
E-mail: m-mizuno@kose.co.jp

An *In Vitro* Tier Evaluation for the Identification of Cosmetic Ingredients Which are Not Ocular Irritants

Shigenobu Hagino, Yuuko Okazaki and Hiroshi Itagaki

Shiseido Research Centre, Yokohama, Japan

Summary — A tier evaluation system was assessed as an alternative method for the identification of cosmetic ingredients which are not ocular irritants. The system employed monolayer cultures of SIRC cells, an established cell line originally derived from the rabbit cornea, and a three-dimensional living dermal model (LDM), MATREX™, which consists of human dermal fibroblasts in a contracted collagen lattice. Effects on the cell monolayer cultures were determined by using SIRC cell-Crystal Violet staining (SIRC-CVS), and effects on the LDM were assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A non-irritating ingredient was defined as a compound having a maximal average total score (MAS) of 5 or less in the Draize eye test, as this is the criterion used in the Japanese draft guidance for evaluating cosmetic ingredients. Among 34 test substances with known characteristics, 30 were classified accurately. Based on these encouraging results, the possibility of simplifying the MTT assay on the LDM for more-practical use, by selecting only three concentration levels to discriminate non-irritants from irritants, was assessed. The simplified method, involving a three-dose set (the three-dose method), was confirmed as being suitable for the identification of non-irritating ingredients, with triethanolamine used as a negative reference standard. Finally, the LDM was used to evaluate compounds at similar concentrations to those tested *in vivo*, aiming to predict the concentration at which an ingredient can be formulated into products without causing eye irritation. On the basis of previous validation data and our additional results, it was found that test samples that resulted in a cell viability of 50% or more in this model, could be classified as non-irritating ingredients. In all, these results indicate that the tier evaluation system may be suitable for the evaluation of ingredients intended to be used in cosmetics and medicated cosmetics in Japan.

Key words: *alternative method, cytotoxicity, Draize eye test, eye irritation, MATREX™, SIRC cells, three-dimensional dermal model, three-dose method, tier evaluation system.*

Address for correspondence: *S. Hagino, Shiseido Research Centre, 2-12-1, Fukuura, Kanazawa-ku, Yokohama-shi 236-8643, Japan.*
E-mail: *shigenobu.hagino@to.shiseido.co.jp*

Introduction

The determination of eye irritation potential is required for the hazard assessment of chemicals. The rabbit eye test (the Draize eye test), which was developed by Draize *et al.* (1), has become the international standard assay for acute ocular toxicity (as in OECD Test Guideline 405; 2). However, the method has been criticised for scientific and animal welfare reasons, and, as a result, great efforts to develop substitute *in vitro* eye irritation tests have been made in academia, and by individual companies, industry trade associations and public institutions, especially the European Centre for the Validation of Alternative Methods (ECVAM), the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) and the Japanese Centre for the Validation of Alternative Methods (JaCVAM). Two alternative tests, in which tissues collected from slaughterhouses are used, i.e. the bovine corneal opacity and permeability (BCOP) test and the isolated chicken eye (ICE) test, have been endorsed as alternative methods for the identification of ocular corrosives and severe irritants, by ICCVAM in 2006, and by ECVAM in 2007. These were important steps toward the elimination of the use of animals in eye irritation testing. However, the Draize eye test has also been used to identify non-irritating ingredients in safety assessment of chemicals, and especially those for use in cosmetics and medicated cosmetics, in Japan. Therefore, to further reduce the use of animals in testing, an *in vitro* test method to identify non-irritating ingredients needed to be developed, validated and endorsed as soon as possible.

In Japan, cytotoxicity tests for identifying non-irritating ingredients were presented in *Guidance on Alternative Appraisal Methods for Determining the Eye Irritation Potential of Cosmetic Raw Materials* (see 3). This was based on the results of a validation programme entitled *Study on Test Methods to Evaluate the Safety of Cosmetics Containing New Ingredients*, supported by funds from the Japanese Ministry of Health and Welfare (4). The guidance states that, if a test substance is found to be non-irritant on the basis of alternative methods alone, and will not be formulated into

products at a concentration in excess of 10%, then it may be appraised as a non-irritant, without additional animal testing. The guidance also notes that artificial dermal models can be used for the identification and classification of non-irritants and irritants. The cut-off point between non-irritants and irritants is a maximal average total score (MAS) of 5, which was selected as the maximum score consistent with the absence of corneal injury, taking into account the significant variability of the Draize eye test score.

In this study, a novel tier evaluation system, combining SIRC cell monolayer cultures and a three-dimensional, living dermal model (LDM), was designed and re-analysed within the framework of the previous Japanese validation studies. Effects on the cell monolayer cultures were determined by using SIRC cell-Crystal Violet staining (SIRC-CVS), which was developed by Itagaki *et al.* (5). This method was previously shown to have the potential to discriminate between non-irritants and irritants in the Japanese validation study. Effects on the LDM were assessed by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The LDM, based on a three-dimensional dermal model developed by Bell *et al.* (6) and Gay *et al.* (7), was included on the basis of its expected validity for the identification of non-irritating ingredients and its expected ability to evaluate water-insoluble ingredients. The data used for the preliminary verification of the tier evaluation system were taken from the reports by Ohno *et al.* (4), Tani *et al.* (8) and Ohuchi *et al.* (9). After verification of the tier system, the possibility of simplifying the MTT assay on the LDM for more-practical use, by selecting only three concentration levels to discriminate non-irritants from irritants, was assessed. It was felt that the original method used for obtaining 50% inhibitory concentration (IC50) values, required significant resources in terms of time, cost and manpower. The new, simplified three-dose method was evaluated by applying it to a set of compounds used in the previous Japanese validation studies.

In addition, we considered it necessary, for practical purposes, to be able to predict the concentration at which an ingredient can be formulated into products, without causing eye irritation. For this purpose, we used the LDM with similar concentrations of test compounds to those used in the *in vivo* tests.

Materials and Methods

Test substances

The test substances used are shown in Table 1. Nine of the 22 substances were selected from among the chemicals used in the previous Japanese validation studies (4). The other 13 substances were selected from among chemicals for which eye irritation data had been previously reported (5, 10, 11). These were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan), Sigma-Aldrich Corp. (St. Louis, MO, USA), Tokyo Chemical Industry Co. Ltd (TCI; Tokyo, Japan), or Wako Pure Chemical Industries, Ltd (Osaka, Japan). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Dojindo Laboratories (Kumamoto, Japan).

Test kit and procedures

The MATREX™ kit was purchased from Toyobo Co. Ltd (Osaka, Japan). The kit contained the LDM, which consists of human dermal fibroblasts cultured in a bovine type-I collagen lattice, to maintain a three-dimensional structure. The test was conducted according to a standard operating procedure (SOP) based on the kit supplier's procedure. The required number of LDM samples were placed in the wells of a six-well plate and 5ml of assay medium (supplied in the kit) were decanted onto the surface of each LDM, and the plate left for 30 minutes at room temperature to remove any residual conditioned medium. Then, the 5ml aliquot of assay medium was aspirated from each well, and 1.2ml of fresh assay medium were added underneath each LDM. A polyethylene ring was placed on the surface of the LDM, and silicone sealant applied around the area of exposure. An 80µl (or 80mg, in the case of a solid) aliquot of test substance was then applied to the surface of the LDM, within the polyethylene ring. The concentrations of the test samples were usually 1, 5 and 10%, for discrimination between non-irritants and irritants. To obtain the IC50 values for each substance, the test was performed with five or more concentration levels, as necessary.

The solvents used for diluting the test substances were distilled water, 50% (v/v) dimethyl sulphoxide or ethylene glycol, selected on the basis of the solubility characteristics of the substance. When none of these solvents were suitable, liquid paraffin was employed. The stepwise determination of whether a test substance dissolved, or if whether it was suspended uniformly, was based on visual observation. The LDM samples were exposed to the test substances for 24 hours at 37°C, in a 5% (v/v) CO_2 incubator. After incubation, the test substances were removed from the wells by washing thoroughly with the assay medium dispensed from a wash bottle.

Each treated LDM was submerged in 1.2ml of MTT solution (0.333mg/ml in assay medium), for 3–4 hours at 37°C. After exposure to the MTT solution, the centre of the LDM tissue was excised, by using an 8mm diameter skin biopsy punch (RS-

Table 1: Details of test substances

CAS No.	Substance	Supplier	Source of *in vivo* data
100-51-6	Benzyl alcohol	Kanto	
71-35-3	Butanol	Kanto	
21245-02-3	2-Ethylhexyl *p*-dimethylaminobenzoate	TCI	
9004-81-3	Polyethyleneglycol monolaurate (10 EO)	Wako	
9002-93-1	Polyoxyethylene octylphenylether (10 EO)	Wako	Ohno *et al.* (4)
9005-64-5	Polyoxyethylene sorbitan monolaurate (20 EO)	Wako	
10124-65-9	Potassium laurate	Kanto	
54-21-7	Sodium salicylate	Wako	
102-71-6	Triethanolamine	Kanto	
78-76-2	Bromo-2-butane	Aldrich	
588-96-5	4-Bromophenetole	Aldrich	
140-72-7	Cetylpyridinium bromide	Sigma	
2370-63-0	2-Ethoxyethyl methacrylate	Aldrich	
589-34-4	3-Methylhexane	Aldrich	Bagley *et al.* (10)
108-10-1	Methyl isobutyl ketone	Aldrich	
135-98-8	1-Methylpropylbenzene	Sigma-Aldrich	
598-98-1	Methyl trimethyl acetate	Aldrich	
25322-68-3	Polyethylene glycol 600	Aldrich	
57-55-6	Propylene glycol	Aldrich	
9005-46-3	Sodium caseinate	Wako	Itagaki *et al.* (5)
8012-95-1	Liquid paraffin	Kanto	Nakamura *et al.* (11)
111-01-3	Squalane	Wako	

6330; Toyobo Co. Ltd, Osaka, Japan). As an indicator of cell viability, the MTT formazan dye was extracted with 0.3ml of isopropanol containing 0.04N HCl, over a 2-hour incubation period. The absorbance was measured at 570nm, by using a microplate reader (Benchmark Plus; Bio-Rad Laboratories, Hercules, CA, USA). The cell viability was expressed as the percentage of living cells with respect to untreated controls or solvent-treated controls handled in the same manner. The IC50 value was calculated by the interpolation of two dose–response data sets, if necessary.

Table 2: Comparison of classification by the SIRC-CVS only, and by the tier evaluation system comprising SIRC-CVS and the LDM MTT assay

	Classification by SIRC-CVS only		Classification by the tier evaluation system	
	Positive	Negative	Positive	Negative
***In vivo* irritants (MAS > 5)**	20	2	20	2
***In vivo* non-irritants (MAS ≤ 5)**	3	9	2	10
Sensitivity (%)	91		91	
Specificity (%)	75		83	
Positive predictivity (%)	87		91	
Negative predictivity (%)	82		83	
Accuracy (%)	85		88	

10% solutions of 34 substances were classified by SIRC-CVS only, or by the tier evaluation system, after initial classification on the basis of pH.

SIRC cell culture and testing

As the cytotoxicity data for the SIRC (Statens Serum Institut Rabbit Cornea) cells were obtained from the literature, the methods used were as described in Tani *et al.* (8).

Verification of the performance of the tier evaluation system

The performance of the tier evaluation system was measured in terms of its sensitivity, specificity, positive predictivity, negative predictivity and accuracy, as follows, where TP, TN, FP and FN are true positive, true negative, false positive and false negative, respectively:

Sensitivity = TP/(TP + FN)
Specificity = TN/(TN + FP)
Positive predictivity = TP/(TP + FP)
Negative predictivity = TN/(TN + FN)
Accuracy = (TP + TN)/(TP + TN + FP + FN)

Results

Design and verification of the tier evaluation system

The results of Japanese validation studies reported by Ohno *et al.* (4), Tani *et al.* (8) and Ohuchi *et al.* (9), were re-analysed by using the tier evaluation system, focusing on the reduction of false-positives and on the effective evaluation of water-insoluble ingredients. The authors' laboratory was one of the participating laboratories in these validation studies. The design of the tier system for the identification of non-irritating ingredients, combining SIRC cell monolayer cultures and the LDM, and including a pH criterion, is shown in Figure 1. The techniques employed were SIRC-CVS of the monolayer cell cultures, and an MTT assay on the LDM samples supplied in the MATREX kit.

Five of the 34 substances were classified as positive irritants, on the basis of their pH (i.e. pH $\leq$ 2 or pH $\geq$ 11.5, in a 10% solution in water). One of these five positive substances, calcium thioglycolate, was a false-positive. Eleven of the 29 substances that were not classified as positive on the basis of their pH, were deemed to be negative (non-irritants) by SIRC-CVS, with polyoxyethylene sorbitan monolaurate (20 EO; Tween-20) used as a reference substance for ocular non-irritancy, according to the Japanese draft guidance. Two water-insoluble lower alcohols, butanol and benzyl alcohol, were classified as false-negatives. One of the 18 substances classified as positive by SIRC-CVS, was classified as negative in the LDM MTT assay, with triethanolamine used as a reference substance for ocular non-irritancy. Triethanolamine was the preferred reference substance for ocular non-irritancy with the LDM, as opposed to Tween-20 in the case of the monolayer cell culture. In the evaluation employing the LDM, polyethyleneglycol monolaurate (10 EO) was classified as a false-positive. Sixteen of the 17 substances classified as positive in the LDM MTT assay, were true-positives.

Table 2 compares the results from the monolayer cell culture only and from the tier evaluation system with both the monolayer cell culture and the LDM. The combination of the two models improved the specificity from 75% to 83%, and the accuracy increased from 85% to 88%.

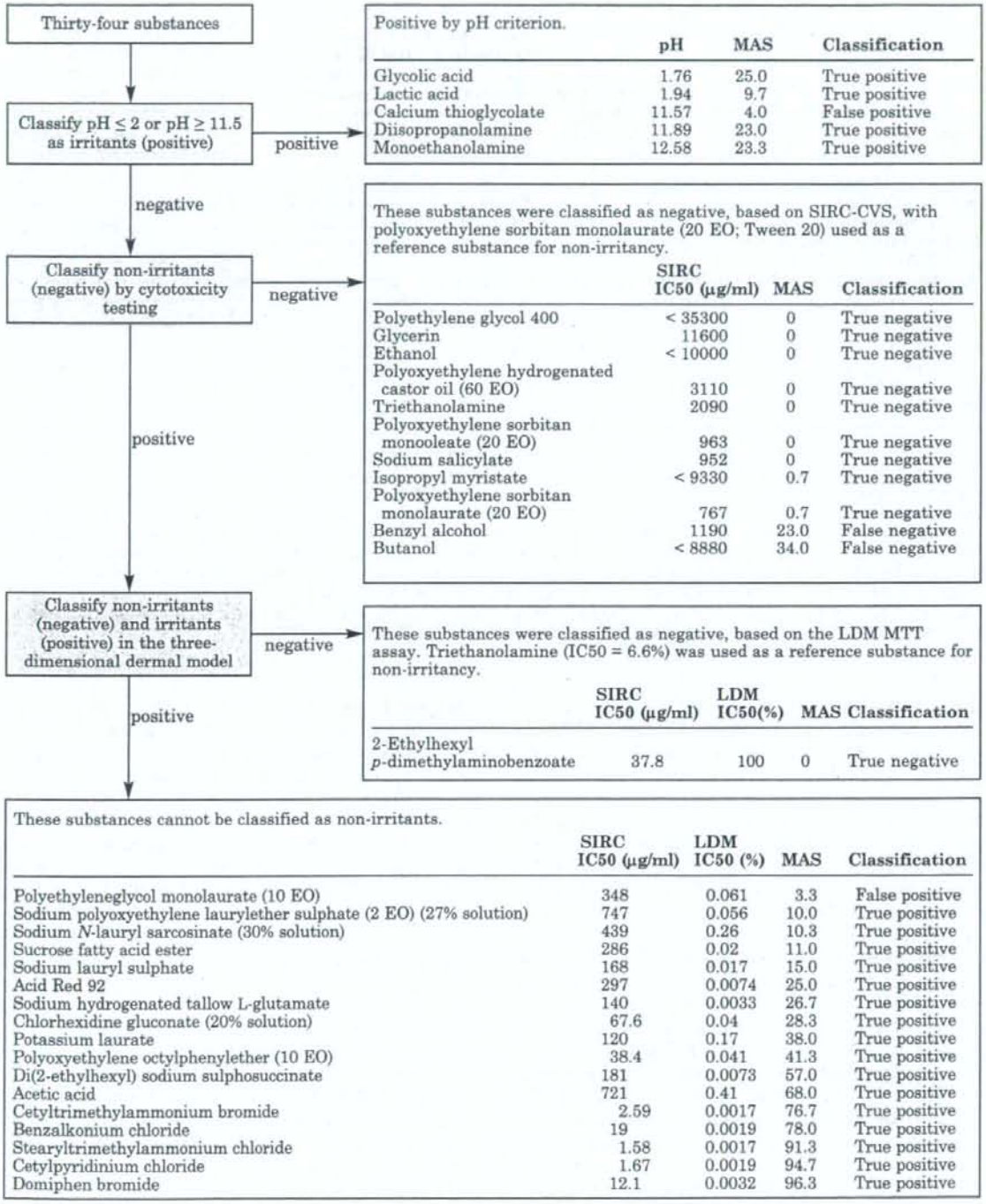
Verification of the use of the three-dose method in the MTT assay of the LDM

A simplified dose concentration set of 1, 5, and 10%, was evaluated for discriminating between non-irritants and irritants with the three-dimensional LDM (Figure 2). Table 3 compares the results from this three-dose method used alone, with those from previous Japanese validation studies (9), which obtained IC50 values for use as an index of irritancy potential by the standard multiple-dose method. The former results corresponded well with the latter, with respect to the classification of a substance as non-irritant or irritant, when the IC50 value for triethanolamine was used as the cut-off point value. The triethanolamine cut-off points were 4.6% for the three-dose LDM MTT assay and 6.6% for the standard multiple-dose method. In the case of the three-dose LDM MTT assay, an IC50 value of <1% means that the cell viability in measurements repeated three times was less than 50%, following the application of the test sample at a concentration of 1%. Similarly, an IC50 value of >10% means that the viability in measurements repeated three times was greater than 50%, following the application of the test sample at a concentration of 10%. The IC50 values of three measurements for butanol were 7.2%, 8.6% and <10%, meaning that the average could not be calculated exactly. Therefore, the IC50 of butanol was expressed as IC50 $\geq$7.2%. All substances could be classified on the basis of the IC50 data, including the data expressed as IC50 <1% or IC50 >10%.

Prediction of the concentration at which an ingredient can be formulated into products without causing eye irritation

IC50 values previously obtained by using the standard multiple-dose method in the LDM (MATREX) MTT assay (9) were compared with the concentra-

Figure 1: Verification of the tier evaluation system, comprising the SIRC-CVS and LDM MTT assay, for the identification of non-irritating cosmetic ingredients



Positive by pH criterion.

	pH	MAS	Classification
Glycolic acid	1.76	25.0	True positive
Lactic acid	1.94	9.7	True positive
Calcium thioglycolate	11.57	4.0	False positive
Diisopropanolamine	11.89	23.0	True positive
Monoethanolamine	12.58	23.3	True positive

These substances were classified as negative, based on SIRC-CVS, with polyoxyethylene sorbitan monolaurate (20 EO; Tween 20) used as a reference substance for non-irritancy.

	SIRC IC50 (μg/ml)	MAS	Classification
Polyethylene glycol 400	< 35300	0	True negative
Glycerin	11600	0	True negative
Ethanol	< 10000	0	True negative
Polyoxyethylene hydrogenated castor oil (60 EO)	3110	0	True negative
Triethanolamine	2090	0	True negative
Polyoxyethylene sorbitan monooleate (20 EO)	963	0	True negative
Sodium salicylate	952	0	True negative
Isopropyl myristate	< 9330	0.7	True negative
Polyoxyethylene sorbitan monolaurate (20 EO)	767	0.7	True negative
Benzyl alcohol	1190	23.0	False negative
Butanol	< 8880	34.0	False negative

These substances were classified as negative, based on the LDM MTT assay. Triethanolamine (IC50 = 6.6%) was used as a reference substance for non-irritancy.

	SIRC IC50 (μg/ml)	LDM IC50(%)	MAS	Classification
2-Ethylhexyl *p*-dimethylaminobenzoate	37.8	100	0	True negative

These substances cannot be classified as non-irritants.

	SIRC IC50 (μg/ml)	LDM IC50 (%)	MAS	Classification
Polyethyleneglycol monolaurate (10 EO)	348	0.061	3.3	False positive
Sodium polyoxyethylene laurylether sulphate (2 EO) (27% solution)	747	0.056	10.0	True positive
Sodium *N*-lauryl sarcosinate (30% solution)	439	0.26	10.3	True positive
Sucrose fatty acid ester	286	0.02	11.0	True positive
Sodium lauryl sulphate	168	0.017	15.0	True positive
Acid Red 92	297	0.0074	25.0	True positive
Sodium hydrogenated tallow L-glutamate	140	0.0033	26.7	True positive
Chlorhexidine gluconate (20% solution)	67.6	0.04	28.3	True positive
Potassium laurate	120	0.17	38.0	True positive
Polyoxyethylene octylphenylether (10 EO)	38.4	0.041	41.3	True positive
Di(2-ethylhexyl) sodium sulphosuccinate	181	0.0073	57.0	True positive
Acetic acid	721	0.41	68.0	True positive
Cetyltrimethylammonium bromide	2.59	0.0017	76.7	True positive
Benzalkonium chloride	19	0.0019	78.0	True positive
Stearyltrimethylammonium chloride	1.58	0.0017	91.3	True positive
Cetylpyridinium chloride	1.67	0.0019	94.7	True positive
Domiphen bromide	12.1	0.0032	96.3	True positive

The data were taken from Ohno et al. *(4), Tani* et al. *(8) and Ohuchi* et al. *(9). Non-irritants (negative) were defined as those having a MAS of 5 or less in the Draize eye test. The eye irritancies (MAS) of 10% solutions of the substances were predicted, based on the IC50 in the two models, after classification according to pH.*

Table 3: A comparison of results from the simplified three-dose LDM MTT assay method for the evaluation of non-irritating ingredients, and from the standard LDM MTT assay reported in Japanese validation studies

Substance	Draize eye test MAS at 10% concn[a]	Results of the simplified three-dose LDM MTT assay method for evaluation of non-irritating ingredients[b]		Results from the standard LDM MTT assay used in the Japanese validation studies[b,c]	
		IC50 (%)	Classification	IC50 (%)	Classification
2-Ethylhexyl *p*-dimethylaminobenzoate	0	> 10	True negative	100	True negative
Sodium salicylate	0	8.9 ± 0.6	True negative	8.9 ± 2.4	True negative
Triethanolamine	0	4.6 ± 3.4	True negative	6.6 ± 1.9	True negative
Polyoxyethylene sorbitan monolaurate (20 EO)	0.7	< 1	True positive	0.063 ± 0.008	True positive
Polyethyleneglycol monolaurate (10 EO)	3.3	< 1	True positive	0.061 ± 0.003	True positive
Benzyl alcohol	23.0	6.0 ± 0.4	False negative	7.3 ± 0.9	False negative
Butanol	34.0	≥ 7.2	False negative	9.1 ± 2.6	False negative
Potassium laurate	38.0	< 1	True positive	0.17 ± 0.05	True positive
Polyoxyethylene octylphenylether (10 EO)	41.3	< 1	True positive	0.041 ± 0.017	True positive

Comparison of results of the simplified three-dose LDM MTT assay method with previous results from the standard LDM MTT assay used in the Japanese validation studies. [a]*The* in vivo *classification of 10% solutions of the substances were based on the criterion of a MAS of 5 or less in the Draize eye test;* [b]*The* in vitro *data included triethanolamine as a reference substance for non-irritancy. The values are presented as the mean ± SD, n = 3. An IC50 value of <1 means that the cell viability in measurements repeated three times, was less than 50%, following the application of the test sample at 1% concentration; an IC50 value of >10 means that the viability in measurements repeated three times, was greater than 50%, following the application of the test sample of 10% concentration. The IC50 values from three measurements of butanol were 7.2%, 8.6% and <10%, thus the average could not be calculated, therefore the IC50 of butanol was expressed as IC50 ≥7.2;* [c]*The results obtained with the standard LDM MTT assay in the Japanese validation studies are those reported by Ohuchi* et al. *(9).*

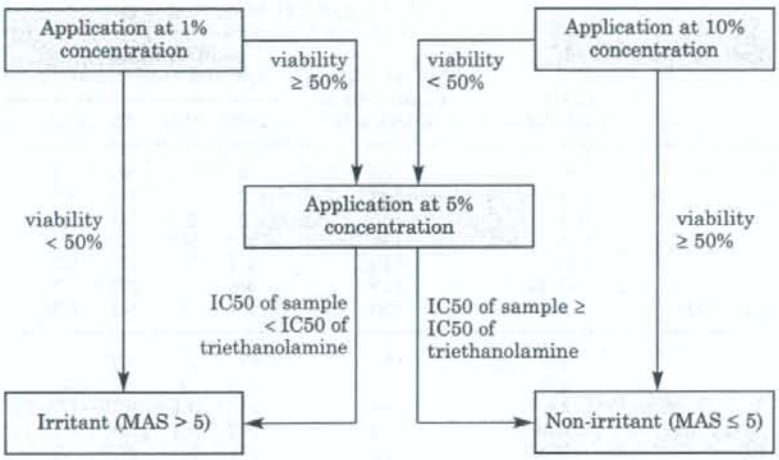
tions classified as non-irritant *in vivo* in the Japanese validation studies (4; Table 4). The *in vivo* concentrations evaluated as non-irritant were obtained from Draize eye test data, though the available concentration intervals were wide. The IC50 values previously obtained with the LDM, were less than or equal to the concentrations classified as non-irritant in the Draize eye test. No conclusions could be reached for ethanol, polyoxyethylene hydrogenated caster oil (60 EO) or benzyl alcohol, because of the wide concentration intervals used in the existing data for the Draize eye test. It was concluded that, when the viability in the LDM was greater than or equal to 50%, the test substance lacked ocular irritancy at the applied concentration. This finding is important for the identification of non-irritating ingredients. On the other hand, the difference between the IC50 value and the concentration evaluated as non-irritant in the Draize eye test, is expected to result in false-positives. For example, the LDM predicts that cetylpyridinum chloride can be formulated into products without causing eye irritation, at a concentration of 0.0019% or less. As 0.1% cetylpyridinum chloride did not cause eye irritation in the Draize eye test, the concentration range between these two concentrations is expected to show up as false-positive with our model.

When we evaluated the results for a number of other substances reported by Bagley *et al.* (10), Itagaki *et al.* (5) and Nakamura *et al.* (11), some showed a marked difference between their IC50 values obtained by using the standard multiple-dose LDM MTT assay method, and the concentrations deemed to be non-irritant in the Draize eye test, as shown in Table 5. Nevertheless, test substances at concentrations below the IC50 value might be non-irritant.

Discussion

Monolayer cell cultures and three-dimensional dermal models have previously been examined separately as alternative methods for eye irritancy testing in several validation studies (4, 12–16). Since we considered that a combination of these models might be more reliable, we designed a tier evaluation system for identifying ingredients characterised as non-irritating in the Japanese validation studies reported by Ohno *et al.* (4), Tani *et al.* (8) and Ohuchi *et al.* (9). The inclusion of the three-

Figure 2: The evaluation scheme for discriminating non-irritating ingredients with a MAS of 5 or less in the Draize eye test



The eye irritancies of solutions of the substances were evaluated by using the LDM MTT assay, with three application concentrations of 1, 5 and 10% (maximally). The IC50 values for the substances were calculated from the viability data at two concentrations of 5% and 1 or 10%. Triethanolamine was used as a reference substance for non-irritancy.

dimensional LDM in the tier evaluation system gives the advantage that it is applicable for use with all types of substances and formulations, regardless of their solubility or form (liquid or powder). As both of the combined models detect cytotoxicity as an endpoint, the classification of non-irritancy should be essentially consistent between them both. However, 2-ethylhexyl *p*-dimethylaminobenzoate, which was classified as a false-positive in the monolayer cell culture staining assay, was correctly classified as a non-irritant in the standard LDM MTT assay, suggesting that the tier evaluation system might indeed be advantageous. Differences in the results from the two assays may arise, not only from differences in the test conditions, but also from the variations in cellular environments, including any effects resulting from their respective culture media.

The fact that two water-insoluble lower alcohols were among the four false-negative substances, might indicate that the application of a 10% suspension in water in the previous *in vivo* test was inappropriate, because the suspension would have readily separated, thus allowing the neat alcohols to contact the ocular tissue. Calcium thioglycolate, evaluated as false-positive by pH measurement, should be re-evaluated after neutralisation, in order to better-reflect the physiological situation. From the viewpoint of product development in general, all substances evaluated as positive from the pH criterion, should be further evaluated following neutralisation.

The sensitivity, specificity and accuracy of the tier evaluation system were 91%, 83% and 88%, respectively. Therefore it might be reasonable to use the tier evaluation system in the same manner as the evaluation procedure outlined in the Japanese draft guidance. That is, if a test substance is found to be non-irritant on the basis of this tier evaluation system, and if it will not be formulated in products at a concentration in excess of 10%, it may be appraised as a non-irritant, without the need for animal testing.

It has been reported that a range of cytotoxicity tests performed on monolayer cell cultures supplemented with calf serum gave almost the same results, regardless of the type of cell line (SIRC, HeLa or CHL) or the endpoint of cytotoxicity (i.e. Crystal Violet staining, Neutral Red uptake or MTT reduction; 8, 17, 18). This implies that various combinations of cell lines and endpoints are potentially applicable as part of a tier evaluation system.

To overcome the disadvantage that evaluation of IC50 by the standard multiple-dose method, requires significant resources in terms of time, cost and manpower, we examined a simplified method for the LDM MTT assay with a three-dose set, to see whether it might be more practical. The results were consistent with the standard IC50 data reported by Ohuchi *et al.* (9), suggesting that this three-dose method may be sufficiently useful for

Table 4: The relationship between IC50 values obtained with the standard LDM MTT assay and concentrations evaluated as non-irritant in the Draize eye test

Test substance	LDM IC50 (%)	Draize eye irritation test results: Concentration evaluated as (MAS ≤ 5)	MAS at each applied concentration: 100%	10%	1%	0.1%
Isotonic sodium chloride solution	100	100	**0**	NT	NT	NT
2-Ethylhexyl *p*-dimethylaminobenzoate	100	100	**0**	0	NT	NT
Isopropyl myristate	100	100	**0**	0.7	NT	NT
Silicic anhydride	100	100	**2.7**	NT	NT	NT
Glycerin	100	100	**4.7**	0	NT	NT
Polyethylene glycol 400	67–100	100	**4.0**	0	NT	NT
Polyoxyethylene sorbitan monooleate (20 EO)	2.4	100	**4.7**	0	NT	NT
Sodium salicylate	8.9	10	83.7	**0**	NT	NT
Triethanolamine	6.6	10	8.0	**0**	NT	NT
Calcium thioglycolate	4.6	10	79.7	**4.0**	NT	NT
Polyoxyethylene sorbitan monolaurate (20 EO)	0.063	10	NT	**0.7**	NT	NT
Polyethyleneglycol monolaurate (10 EO)	0.061	10	NT	**3.3**	NT	NT
Acid Red 92	0.0074	1	71.0	25.0	**0.7**	NT
Cetylpyridinium chloride	0.0019	0.1	NT	94.7	34.7	**2.7**
Ethanol	43	10	32.7	0	NT	NT
Polyoxyethylene hydrogenated castor oil (60 EO)	28.0	10	NT	0	NT	NT
Benzyl alcohol	7.3	1	31.0	23.0	0	NT

The data were taken from Ohno et al. *(4) and Ohuchi* et al. *(9). The stated value for each LDM IC50, is the mean of data obtained from 3–7 laboratories in the previous Japanese validation studies, one of which was the authors' laboratory. The IC50 for polyethylene glycol 400 was 100% in 3 laboratories and 67, 78, 82, 85% in the other 4 laboratories. "Not tested" is shown as NT. No conclusion could be reached for ethanol, polyoxyethylene hydrogenated castor oil (60 EO) or benzyl alcohol, because of the large concentration intervals in the available Draize eye test data. The bold figures indicate the concentrations at which the MAS in the* in vivo *test was ≤ 5.*

the discrimination of non-irritants and irritants. In addition, the potential omission of one or two doses on the basis of previously-available data or known structure–activity relationships, etc., might be permissible.

In the three-dose method, the IC50 (%) of triethanolamine was used as a cut-off value between non-irritants and irritants. Though Tween-20 was recommended as a reference substance for non-irritancy in the cytotoxicity test with the monolayer cell cultures, as outlined in the Japanese draft guidance for evaluating cosmetic ingredients, this surfactant seemed to be excessively cytotoxic to the three-dimensional LDM, and was thus unsuitable as a negative reference substance. Therefore, triethanolamine was selected as a negative reference substance for use with the LDM, for the following reasons: the MAS in the Draize eye test was zero for a 10% solution of triethanolamine (4); the IC50 of triethanolamine was about 5% with the LDM, and this is deemed to be a suitable cut-off for discriminating between irritating and non-irritating ingredients; and triethanolamine is readily water-soluble and is stable under our usage conditions.

When the IC50 values obtained by using the standard LDM MTT assay in this study and in the previous validation studies, were compared with the concentrations classified as non-irritant in the Draize eye test, for the purpose of predicting the concentrations at which ingredients can be formulated into products without causing eye irritation, it was noted that the IC50 values were less than, or equal to, the concentrations classified as non-irritant in the Draize eye test. The relationship between the IC50 values obtained in the standard LDM MTT assay and the concentrations classified as non-irritant in the Draize eye test, suggests that the IC50 values might be taken as the maximum concentrations that could be formulated into products without causing eye irritation.

Itagaki *et al.* (19) reported that a tier evaluation system consisting of SIRC-CVS and an assay using collagen gel constructed from collagen and human

dermal fibroblasts, was applicable to cosmetic ingredients and products. The LDM in the MATREX kit and the collagen gel were both made on the basis of the method reported by Bell *et al.* (6) and Gay *et al.* (7). Although there are differences in test conditions between the LDM MTT assay and the collagen gel assay — especially with respect to application time (24 hours *versus* 1 hour), and in the predictive criterion used in the Draize eye test (MAS 5 *versus* MAS 15) — both tier systems are useful for the prediction of irritancy of cosmetic ingredients. The LDM MTT assay is more conservative than the collagen gel assay, and thus may offer a greater margin of safety.

Other *in vitro* tests that employ similar concentrations of test substances to those used in the *in vivo* test, such as the chorioallantoic membrane (HET-CAM) or chorioallantoic membrane-trypan blue staining (CAM-TB) tests (20–23), may also be helpful in predicting the concentration at which an ingredient can be formulated into products, without causing eye irritation.

Although the IC50 values obtained with the LDM in this study and in the previous Japanese validation studies, were shown to be useful as an index for predicting eye irritancy, false-positives were, and still remain, an issue. Although relatively few non-irritants were evaluated, the appearance of some false-positives in the data in Tables 4 and 5, is fully consistent with extensive in-house data that we have accumulated (not shown). It is possible that non-irritating ingredients should be finally identified by comparing the IC50 values with those of standard substances selected by taking into account existing experience in the market, the type of usage, physicochemical properties, etc. The results from the standard LDM MTT assay and the *in vivo* data showed a high Spearman's rank correlation coefficient of 0.832 (3). As results accumulate, it may be feasible to identify standard IC50 ranges, within which ingredients could be formulated into products without causing eye irritation, for particular product groups.

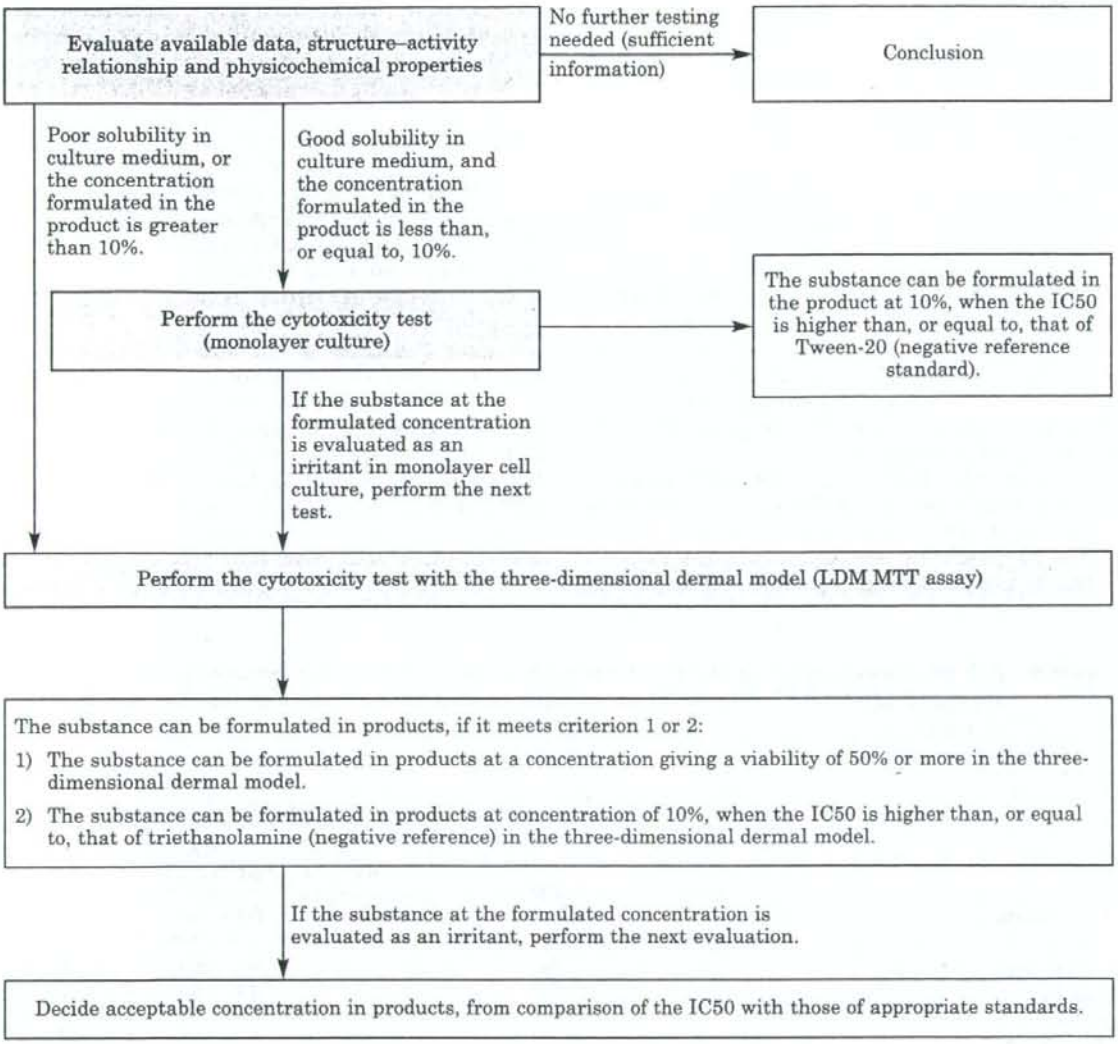
Figure 3 illustrates the tier evaluation plan utilising SIRC cell monolayer cultures and the LDM for the identification of non-irritating ingredients. Our results indicate that the tier evaluation system should reduce the number of false-positive, as well as false-negative, results, and so should be helpful in developing ingredients for cosmetics or medicated cosmetics. Basically, ingredients can be formulated in products at a concentration giving a viability of 50% or more in

Table 5: A further evaluation of the relationship between IC50 values obtained with the standard LDM MTT assay and concentrations evaluated as non-irritant in the Draize eye test

			Draize eye irritation test results				
				MAS at each applied concentration			
Test substance	**Solvent**	**LDM IC50 (%)**	**Concentration evaluated as (MAS ≤ 5)**	**100%**	**10%**	**1%**	**0.1%**
Polyethylene glycol 600	–	100	100	**2.0**	NT	NT	NT
Sodium caseinate solution (10% v/v)	–	100	100	**2.0**	NT	NT	NT
Propylene glycol	–	100	100	**1.3**	NT	NT	NT
Liquid paraffin	–	100	100	**0.9**	NT	NT	NT
Squalane	–	100	100	**0.7**	NT	NT	NT
3-Methylhexane	Liquid paraffin	95.3 ± 3.2 (v/v%)	100	**0.7**	NT	NT	NT
Methyl trimethyl acetate	Liquid paraffin	30.8 ± 2.3	100	**2.7**	NT	NT	NT
Methyl isobutyl ketone	Liquid paraffin	18.1 ± 2.7	100	**4.8**	NT	NT	NT
4-Bromophenetole	Liquid paraffin	16.0 ± 3.0	100	**1.3**	NT	NT	NT
1-Methylpropylbenzene	Liquid paraffin	8.1 ± 0.9	100	**2.0**	NT	NT	NT
2-Ethoxyethyl methacrylate	Ethylene glycol	3.5 ± 1.3	100	**0**	NT	NT	NT
Bromo-2-butane	Ethylene glycol	0.63 ± 0.05	100	**0**	NT	NT	NT
Cetylpyridinium bromide	Distilled water	0.0020 ± 0.0001	0.1	NT	89.7	36.0	**2.7**

The values obtained by using the standard LDM MTT assay in the current study, are presented as the mean ± SD, n = 3. The in vivo *data were taken from Bagley* et al. *(10), Itagaki* et al. *(5) and Nakamura* et al. *(11). "Not tested" is shown as NT. The bold figures indicate the concentrations at which the MAS in the* in vivo *test was ≤ 5.*

Figure 3: A schematic illustration of the tier evaluation for the identification of non-irritating ingredients



the LDM MTT assay. The three-dose assay method would be practically convenient, and, if a substance is to be formulated in products at a concentration as high as 10%, the three-dose method shows high predictive accuracy.

Much accumulated data and experience have shown that products can be used safely for eyes when all the ingredients of the product are formulated at concentrations that have been confirmed as non-irritating for the individual ingredients alone. Therefore, it is generally considered that the testing of ingredients is useful for the safety evaluation of products, although the additional testing of combinations of ingredients and final formulations might possibly be desirable in some cases.

Conclusions

The tier evaluation system, comprising SIRC cell monolayer cultures and a three-dimensional dermal model (LDM; MATREX), for the identification of ingredients which are not ocular irritants (defined as those having an MAS of 5 or less), was shown to be useful as an alternative method, based on a re-analysis of previous validation data and some addi-

tional results. The tier evaluation system may be suitable for the evaluation of ingredients intended to be used in cosmetics and medicated cosmetics in Japan.

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Original Article

Modification of cell-surface thiols elicits activation of human monocytic cell line THP-1: Possible involvement in effect of haptens 2,4-dinitrochlorobenzene and nickel sulfate

Morihiko Hirota[1], Mie Suzuki[1], Shigenobu Hagino[1], Saori Kagatani[2], Yoshinori Sasaki[2], Setsuya Aiba[2] and Hiroshi Itagaki[1]

[1]Quality Assessment Center, Shiseido Co., Ltd., 2-12-1 Fukuura, Kanazawa-ku, Yokohama-shi, Kanagawa 236-8643, Japan
[2]Department of Dermatology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574, Japan

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ABSTRACT — Human monocytic cell line THP-1 cells are used as an indicator for *in vitro* skin sensitization testing. Although p38 mitogen-activated protein kinases (MAPKs) and intracellular redox imbalance play crucial roles in the activation of THP-1 by skin sensitizers, the trigger of cell activation has not been identified. Therefore, we examined whether haptens induce THP-1 maturation directly or indirectly. 2,4-Dinitrochlorobenzene (DNCB), but not dinitrophenol (DNP)-conjugated bovine serum albumin or DNP-conjugated fetal bovine serum, induced CD86 expression. DNCB and nickel sulfate ($NiSO_4$) also induced related changes of cell-surface thiols and phosphorylation of p38 MAPK. However, DNCB is membrane-permeable, and so its direct effect may not be confined to cell membrane proteins. Next, we found that CD86 expression and macrophage inflammatory protein-1β (MIP-1β) production were augmented by the membrane-impermeable thiol blocker 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), and these changes were suppressed by an inhibitor of the p38 MAPK pathway, SB203580. Finally, we confirmed that endocytotic activity for bovine serum albumin (BSA) Alexa Fluor 488 conjugate did not affect cell-surface thiols on THP-1 cells. Thus, our data indicate that the changes of cell-surface thiols are one of the triggers of maturation, and play a key role in activation of THP-1 cells by haptens.

Key words: Cell-surface thiols, Cell activation, p38 MAPK, CD86, MIP-1β, THP-1

INTRODUCTION

Several groups have recently developed *in vitro* assays to predict sensitizing potential of chemicals. These approaches were based on measuring expressions of an appropriate biomarker(s) of an indicator cell line. Dendritic cells, including Langerhans cells, are considered as good candidates for indicator cells, because they are potent antigen-presenting cells and play an important role in induction of skin sensitization by simple chemicals (Aiba and Tagami, 1998). It was demonstrated *in vitro* that human monocyte-derived dendritic cells respond to haptens, such as nickel chloride ($NiCl_2$) and 2,4-dinitrochlorobenzene (DNCB), but not to irritants, such as sodium dodecyl sulfate (SDS), by significantly augmenting their expression of CD86, CD54 and HLA-DR (Aiba *et al.*, 1997; Coutant *et al.*, 1999). Furthermore, *in vitro* sensitization methods using cell lines such as THP-1 (monocytic leukemia cell line) (Ashikaga *et al.*, 2006; Yoshida *et al.*, 2003; Sakaguchi *et al.*, 2006), KG-1 (acute myelogenous leukemia cell line) (Hulette *et al.*, 2001; Yoshida *et al.*, 2003), U-937 (histiocytic lymphoma cell line) (Sakaguchi *et al.*, 2006) and MUTZ-3 (Azam *et al.*, 2006) have also been reported. In these methods, expression of surface biomarkers, such as HLA-DR, CD54 and CD86, is measured by flow cytometry after treatment of cells with haptens, such as DNCB and *p*PD. The THP-1 cell line was reported to be a good candidate indicator for *in*

Correspondence: Morihiko Hirota (E-mail: morihiko.hirota@to.shiseido.co.jp)

vitro sensitization methods from the viewpoint of inter-laboratory reproducibility (Sakaguchi *et al.*, 2006).

p38 mitogen-activated protein kinase (MAPK) plays a crucial rule in the augmentation of CD86 expression on dendritic cells, and intracellular redox imbalance is a very important upstream signal for p38 MAPK (Aiba *et al.*, 2003; Mizuashi *et al.*, 2005). Furthermore, it was reported that intracellular redox imbalance and redox-related expression of genes such as heme oxygenase-1 (HO-1) gene upon treatment with haptens were observed in THP-1 cells (Mizuashi *et al.*, 2005; Hirota and Moro, 2006). Filomeni *et al.* (2003) reported that oxidation of exofacial membrane thiol groups by exogenous membrane-impermeable oxidative-form glutathione (GSSG) triggered a decrease of intracellular glutathione (GSH) content, phosphorylation of p38 MAPK and apoptosis in U-937 cells. Furthermore, Becker *et al.* (2003) reported that coupling of sensitizers to thiol groups is a key event for activation of monocytes and monocyte-derived dendritic cells. These observations might indicate that haptens activated p38 MAPK through redox signals arising from oxidation of cell-membrane thiols. However, skin sensitizers generally have protein- and peptide-binding activity, or acquire activity by metabolic activation, so that hapten-protein or hapten-peptide complexes incorporated into antigen-presenting cells are presented to T cells in the induction phase of skin sensitization. Becker *et al.* (1992) and Girolomoni *et al.* (1990) reported that receptor-mediated endocytosis by Langerhans cells is an early event in the induction phase of skin sensitization. Thus, it remains to establish whether antigen-presenting cells and cell lines are activated by hapten-conjugated protein or by haptens directly. In this paper, we focus on a trigger of THP-1 maturation, and our results indicate that direct change of cell-surface thiols by hapten is more important in activation of THP-1 cells.

MATERIALS AND METHODS

Chemicals

DNCB, nickel sulfate hexahydrate ($NiSO_4$) and SDS were purchased from Sigma-Aldrich Corporation (St. Louis, MI, USA). 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) was purchased from Dojindo Molecular Technologies (Gaithersburg, MD, USA). Bovine serum albumin (BSA) was purchased from Wako Pure Chemicals (Osaka, Japan). Dimethyl sulfoxide (DMSO) was purchased from Kanto Chemical (Tokyo, Japan). Nonpermeable thiol reactive compound Alexa Fluor 488 C_5 maleimide, Alexa Fluor 568 C_5 maleimide, nonpermeable amine reactive compound Alexa Fluor 488 carboxylic acid succinimidyl ester, BSA Alexa Fluor 488 conjugate were purchased from Invitrogen life technologies (Carlsbad, CA, USA). FITC-labeled anti-CD86 antibody was purchased from BD-PharMingen (Clone: Fun-1, San Diego, CA, USA). FITC-labeled anti-mouse IgG1 was purchased from DAKO (Glostrup, Denmark). SB203580, an inhibitor of the p38 MAPK pathway, was purchased from Calbiochem (La Jolla, CA, USA).

Cells and culture

THP-1 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). These cells were maintained in RPMI 1640 medium (Invitrogen life technologies) with 1 % (v/v) antibiotic-antimycotic (Invitrogen life technologies), 10 % fetal bovine serum (v/v) (FBS, JRH Biosciences, Lenexa, KS, USA) and 0.05 mM 2-mercaptoethanol (2-ME) (Invitrogen life technologies) at 37°C in a 5 % CO_2 incubator. Cells were passaged by addition of fresh medium twice a week and cell density was maintained between 0.1 and 0.5 x 10^6 cells/ml.

Preparation of dinitrophenol (DNP)-conjugated BSA and DNP-conjugated FBS

100 mg/ml DNCB was dissolved in DMSO and added to 10 mg/ml BSA solution or RPMI 1640 medium containing 10 % FBS. The proteins in RPMI 1640 medium containing 10 % FBS were considered to be derived from FBS because RPMI 1640 medium itself contains little protein according to the manufacturer's information. After reaction for 24 hr at 37°C, the mixture (0.5 ml) was centrifuged in a Microcon YM-3 (Millipore, Beverley, MA, USA). Concentrated DNP-conjugated BSA and DNP-conjugated FBS were washed three times, and the concentration of non-reacted DNCB in the flow-through fraction was measured by reversed-phase high-performance liquid chromatography (RP-HPLC) on a Shimadzu Class-VP system (Shimadzu Corporation, Kyoto, Japan) using a Capcell Pak C18 UG120 S-5 (4.6 mm I.D. x 150 mm) (Shiseido, Tokyo, Japan). DNP-conjugated BSA and DNP-conjugated FBS were also assayed by Western blotting using anti-dinitrophenol (anti-DNP) antibody (Cosmo Bio, Tokyo, Japan).

The concentration of DNCB conjugated to BSA was calculated by use of the following formula: conjugated DNCB (mg) = (DNCB content before incubation with BSA or RPMI 1640 medium with 10 % FBS) – (total content of unreacted DNCB in flow-through fraction).

Flow-cytometric analysis

Flow-cytometric analyses were performed with an EPICS XL-MCL System II (Beckman Coulter, Fullerton,

CA, USA). Dead cells were gated out by staining with propidium iodide (PI, 0.625 mg/ml). A total of 10,000 living cells was analyzed. When cell viability was less than 50%, relative fluorescence intensity (RFI) was not calculated from mean fluorescence intensity (MFI) because of diffuse labeling of cytoplasmic structures due to cell membrane destruction (Becker *et al.*, 1994).

Measurement of CD86 expression by flow cytometry

THP-1 cells were seeded at 1 x 10^6 cells/ml. DNCB and DTNB were each dissolved in DMSO and added to the culture medium. The final concentration of the vehicle in the medium was under 0.2 % (v/v), which did not have any effect on cell growth (Ashikaga *et al.*, 2002). DNP-conjugated BSA or DNP-conjugated FBS was added directly to the culture medium. After 24 hr treatment with test chemicals, cells were washed with FACS buffer (phosphate-buffered saline (PBS) with 0.1 % BSA), then incubated with 50 ml of FITC-conjugated anti-human CD86 antibody (7 mg/ml) for 30 min on ice. After having been washed again with FACS buffer, cells were analyzed by flow cytometry. RFI was used as an indicator of CD86 expression and calculated as follows: RFI (%) = (MFI of sample-treated cells/ MFI of vehicle (DMSO) control cells or non-treated cells) x 100.

Analysis of cell-surface thiols and amines by flow cytometry

We used a nonpermeable thiol-reactive compound, Alexa Fluor 488 C_5 maleimide and a nonpermeable amine-reactive compound, Alexa Fluor 488 carboxylic acid succinimidyl ester, for detection of cell-surface thiols and amines. THP-1 cells were seeded at 1 x 10^6 cells/ml. DNCB and DTNB were each dissolved in DMSO and added to the culture medium. $NiSO_4$ and SDS were dissolved directly in the culture medium. After 2 hr treatment with test chemicals, THP-1 cells were recovered and washed with PBS twice, then incubated with 100 ml of Alexa Fluor 488 C_5 maleimide (10 mM) PBS solution or Alexa Fluor 488 carboxylic acid succinimidyl ester (10 mM) PBS solution for 30 min at 37°C. After having been washed again with PBS, the cells were analyzed by flow cytometry. RFI was calculated by use of the following formula: RFI (% of control) = (MFI of chemical-treated cells/ MFI of vehicle control cells or non-treated cells) x 100.

SDS-PAGE and Western blot analysis

Protein samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, USA). The membranes were incubated with Block Ace or Immuno Block (Dainippon Sumitomo Pharma, Osaka, Japan) for 60 min at room temperature (RT), and then with rabbit polyclonal antibodies to anti-phosphorylated p38 MAPK, anti-p38 MAPK and anti-DNP antibody for 60 min at RT. They were washed three times with Wash buffer (20 mM Tris-HCl (pH 7.6), 137 mM NaCl, 0.1 % Tween-20), then incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 60 min at RT, and washed again three times with Wash buffer. Immunoreactive bands were detected by chemiluminescence measurement.

Analysis of phospho-p38 MAPK by Western blotting

After treatment with chemicals for 2 hr, phosphorylation of p38 MAPK was determined by Western blot analysis. Immunoblotting of phosphorylated p38 MAPK was performed using a p38 MAPK immunoblotting kit (Cell Signaling Technology, Beverly, MA, USA). Cells (1 x 10^6 cells/ml) were washed twice in cold PBS and resuspended in 50 ml of lysis buffer (1 % Nonidet P-40, 20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10 % glycerol, 2 mM ethylenediaminetetraacetic acid, 1 % protease inhibitor cocktail (Sigma-Aldrich), and 1 mM sodium orthovanadate). Nuclei and insoluble cell debris were removed by centrifugation at 14,000 x g for 10 min at 4°C. Postnuclear extracts were collected and used as total cell lysates. Total cell lysates were suspended in 2 x SDS sample buffer (313 mM Tris-HCl (pH 6.8), 10 % SDS, 2-ME, 50 % glycerol, and 0.01 % bromphenol blue) and heated at 95°C for 3 min. Protein samples were subjected to SDS-PAGE, and phosphorylated p38 MAPK and anti-p38 MAPK were detected by Western blotting. Rabbit polyclonal anti-phosphorylated p38 MAPK and anti-p38 MAPK antibodies were used as the primary antibodies.

Separation of cell-membrane proteins and cytoplasmic proteins from DNCB-treated THP-1, and Western blot analysis

Separation of cell-membrane proteins and cytoplasmic proteins from THP-1 cells treated with DNCB for 2 hr was performed according to the protocol of the Cytosol/Particulate Rapid Separation kit (BioVision, Mountain View, CA, USA). The separated cell-membrane proteins and cytoplasmic proteins were suspended in 2 x SDS sample buffer, and heated at 95°C for 3 min. Proteins (30 mg) were subjected to SDS-PAGE, and DNP-conjugated protein was detected by Western blotting. Rabbit polyclonal anti-DNP protein antibody was used as the primary antibody.

Measurement of MIP-1β production

Macrophage inflammatory protein-1β (MIP-1β) productions from THP-1 cells were measured as described previously (Hirota and Moro, 2006). Cultured supernatants of THP-1 cells treated with chemicals for 2 and 48 hr were recovered. The production of MIP-1β was assessed using human-specific cytokine ELISA kits (Quantikine, R&D systems, Minneapolis, MN, USA) according to the instructions of the manufacturer. The fold increase over the control was calculated by use of the following formula: MIP-1β production rate (% of control) = (MIP-1β production of chemical-treated cells/ MIP-1β production of vehicle control cells) x 100.

Analysis of endocytotic activity and cell-surface thiols by flow cytometry

We used a nonpermeable thiol-reactive compound, Alexa Fluor 568 C_5 maleimide, to label the cells. THP-1 cells were seeded at 1 x 10^6 cells/ml in culture medium. After 15 min treatment at 37°C with 150 mg/ml BSA Alexa Fluor 488 conjugate, THP-1 cells were recovered, washed with PBS three times, then incubated with 100 ml of Alexa Fluor 568 C_5 maleimide (10 mM) PBS solution for 30 min at 37°C. The cells were washed again with PBS, and analyzed by flow cytometry.

Statistical analysis

The statistical significance of differences in the RFI of cell surface thiols and amines, expressions of CD86 and MIP-1β production rate between non-treated THP-1 and chemical-treated THP-1 were analyzed using paired Student's *t* test.

RESULTS

CD86 expression on THP-1 cells treated with DNCB, DNP-conjugated BSA and DNP-conjugated FBS

In general, skin sensitizers have protein- and peptide-binding activity, or acquire their activity by metabolic activation. At first, we investigated whether hapten-treated THP-1 cells are activated by incorporation of hapten-conjugated protein or by hapten itself. We prepared DNP-conjugated BSA and DNP-conjugated FBS. Table 1 shows the amount of DNCB bound with BSA (A) or FBS (B), the amount of non-reacted DNCB, binding rate, amounts of BSA conjugated in the 50 mM and 15 mM DNP groups and amounts of 15 mM DNP-conjugated FBS in each experiment. THP-1 cells were treated with DNCB, DNP-conjugated BSA or DNP-conjugated FBS for 24 hr. When RFI of 150 % (control; 100 %) was

Table 1. CD86 expression on THP-1 treated with DNP-conjugated BSA

(A)

	DNCB conc.(μM)			Binding Rate (%)	DNP-conjugated BSA conc. (mg/ml)		BSA conc. (mg/ml)
	Before reaction (a)	non-reacted- (b)	conjugated- (a-b)		DNP (50 μM)-	DNP (15 μM)-	DNP (0 μM)-
Experiment 1.	494	195	299	61	1.9	0.83	1.9
Experiment 2.		334	160	32	3.9	1.3	3.9
Experiment 3.		322	172	35	3.3	1.1	3.3

(B)

	DNCB conc.(μM)			Binding Rate (%)	DNP-conjugated BSA conc. (mg/ml)	
	Before reaction (a)	non-reacted- (b)	conjugated- (a-b)		DNP (50 μM)-	DNP (15 μM)-
Experiment 4.	494	251	243	49	0.4	0.4
Experiment 5.		351	142	29	1.4	1.4

(A) Preparation of DNP-conjugated BSA. (B) Preparation of DNP-conjugated FBS. DNCB (494 mM (a)) DMSO solution was mixed with 10 mg/ml BSA or RPMI 1640 medium containing 10 % FBS. After reaction for 24 hr at 37 °C, non-reacted DNCB was separated from the reaction mixture and measured by HPLC (b). The DNP content in DNP-conjugated BSA or DNP-conjugated FBS was calculated by subtraction (a-b). Binding Rate (%) is the percent ratio of reacted DNCB (a-b) to total DNCB (a). The term 'conc.' represents concentrations of chemicals.

applied as the criterion as described in a previous report (Sakaguchi *et al.*, 2006), CD86 expression was induced only by direct treatment with 15 mM DNCB (Fig. 1).

Phosphorylation of p38 MAPK and changes of cell-surface thiols and amines on THP-1 cells treated with DNCB, NiSO4 and SDS

If THP-1 cells are activated by direct action of hapten as shown in Fig. 1, it is possible that the cell membrane, which is the first point of contact with haptens, triggers activation. Cell-membrane proteins, such as receptors, transport proteins and ion channels, are known to be sensors of environmental changes around cells. Further more, most proteins contain cysteine and lysine, which have nucleophilic side chains. We therefore compared changes in phosphorylation of intracellular p38 MAPK, an intracellular signaling molecule, with changes of cell membrane thiols and amines on THP-1 treated with sensitizers (DNCB and $NiSO_4$) and a non-sensitizer (SDS). DNCB and $NiSO_4$, but not SDS, induced a decrease of cell-surface thiols (Fig. 2A), and p38 MAPK were more highly phosphorylated after treatment with DNCB or $NiSO_4$ than after SDS (Fig. 2C). The change of cell-surface thiols appeared to be related to phosphorylation of p38 MAPK. On the other hand, cell-surface amines were decreased by DNCB or SDS, but not $NiSO_4$ (Fig. 2B).

Distribution of DNP-conjugated protein in DNCB-treated THP-1 cells

It was reported that exogenous non-permeable GSSG triggered a decrease of an intracellular glutathione (GSH) content and phosphorylation of p38 MAPK in U-937 cells (Filomeni *et al.*, 2003). GSSG is reported to be unable to permeate through the cell membrane (Filomeni *et al.*, 2003). On the other hand, it is not well established whether DNCB is membrane-permeable or not. Thus, we fractionated the cell-membrane proteins and cytoplasmic proteins in DNCB-treated THP-1, and detected DNCB-binding proteins by western blotting with anti-DNP protein antibody. As shown in Fig. 3, there were many DNCB-binding proteins among the cell membrane and cytoplasmic proteins. Though we expected that DNCB would affect only cell-membrane thiols, these data suggest that DNCB can activate THP-1 through modification of both cell-membrane and cytoplasmic proteins.

Activation of THP-1 cells treated with a membrane-impermeable thiol blocker, DTNB

As shown in Fig. 2, change of cell-surface thiols

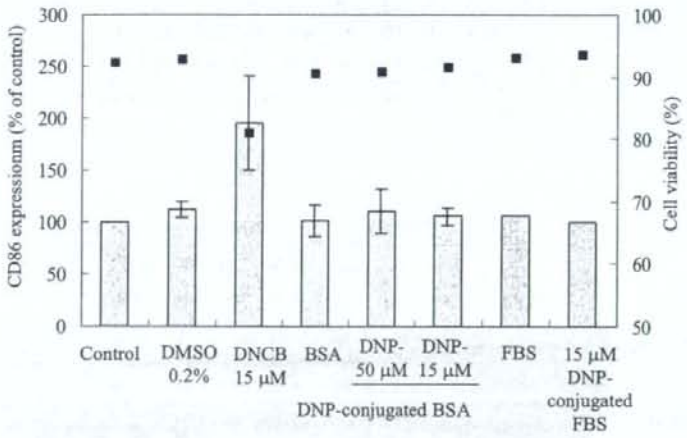

Fig. 1. THP-1 cells were treated with 15 mM DNCB, BSA, 50 mM DNP-conjugated BSA, 15 mM DNP-conjugated BSA, FBS and 15 mM DNP-conjugated FBS for 24 hr. The concentrations of BSA, 50 mM DNP-conjugated BSA, 15 mM DNP-conjugated BSA, FBS and 15 mM DNP-conjugated FBS are shown in Table 1(A) and (B). The concentration of BSA or FBS was the same with that of 50 mM DNP-conjugated BSA or 15 mM DNP-conjugated FBS, respectively. RFI values of CD86 expression were calculated as described in Materials and Methods. CD86 expression on THP-1 cells treated with DNCB (15 mM) for 24 hr was examined on the same day in each experiment. Each value of RFIs is the mean of ± S.D. of at three independent experiments. In the case of FBS and 15 mM DNP-conjugated FBS, results represented the mean of two independent experiments. Square dots indicated cell viability.

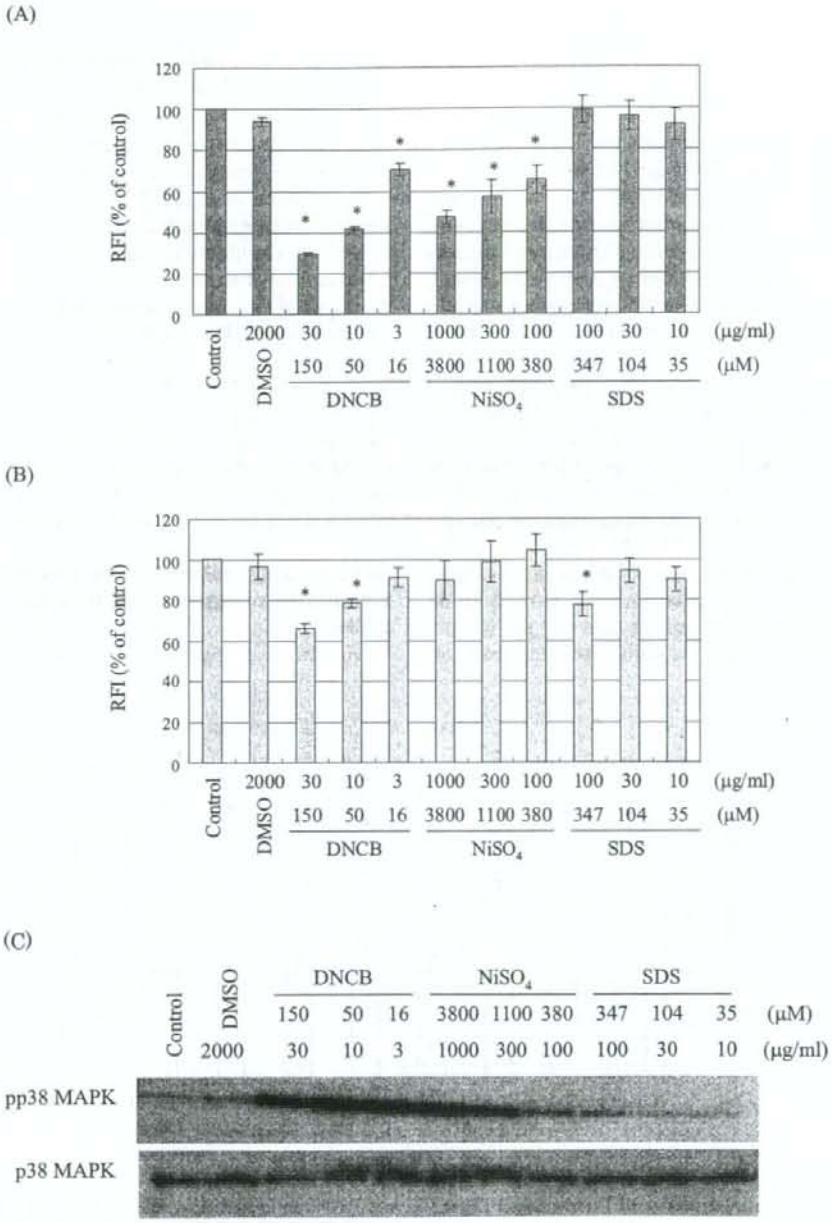

Fig. 2. Effect of chemicals on cell-surface thiols (A), amines (B) and phosphorylation of intracellular p38 MAPK (C) in THP-1 cells. THP-1 cells were exposed to test chemicals for 2 hr and cell-surface thiols and amines were measured by flow cytometry. Western blot analysis of phosphorylated p38 MAPK was performed using a p38 MAPK immunoblotting kit. Representative results of three independent sets are shown. Each value of RFIs is the mean of ± S.D. of at three independent experiments. *; $p < 0.05$.

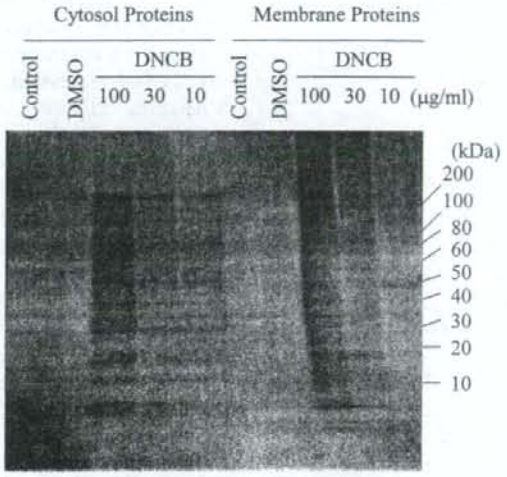

Fig. 3. Distribution of DNP-conjugated protein in DNCB-treated THP-1. THP-1 cells were exposed to DNCB for 2 hr. Separation of cell membrane proteins and cytoplasmic proteins of THP-1 cells was performed as described in Materials and Methods. The separated cell membrane proteins and cytoplasmic proteins (30 mg) were loaded on SDS-PAGE and DNP-conjugated protein was detected by Western blotting.

appeared to be related to phosphorylation of p38 MAPK, but haptens do not necessarily affect only cell-membrane proteins, if they are membrane-permeable, such as DNCB (Fig. 3). It is necessary to confirm cell activation by a membrane-impermeable thiol-modifying compound in order to prove the importance of cell-membrane thiols for cell activation. DTNB (5,5'-dithiobis (2-nitrobenzoic acid), Ellman's reagent) has been used for determining the thiols contents of cell-surface proteins because of its thiol-specific and membrane-impermeant properties (Reglinski *et al.*, 1988; Lawrence *et al.*, 1996; Laragione *et al.*, 2003). Therefore, we examined whether THP-1 cells are activated by modification of cell-surface thiols with DTNB by measuring cell-surface thiols, phosphorylation of p38 MAPK, CD86 expression and MIP-1β production, all of which have been reported to be candidate markers for *in vitro* sensitization methods (Ashikaga *et al.*, 2002; Hirota and Moro, 2006). DTNB treatment decreased cell-surface thiols and increased p38 MAPK phosphorylation (Fig. 4). CD86 expression was also augmented by 48 hr treatment with DTNB, and effect of DTNB suppressed by SB203580 (Fig. 5). Similarly, MIP-1β production was increased by treatment with DTNB for 2 and 48 hr (Fig. 6), and SB203580 suppressed increase of MIP-1β production in THP-1 cells treated with DTNB for 48 hr (Fig. 6B).

Endocytotic activity and cell-surface thiols of THP-1 cells

Receptor-mediated endocytosis has been reported as an early event of activation of LCs (Becker *et al.*, 1992; Girolomoni *et al.*, 1990). We thus investigated changes of cell-surface thiols by treatment with BSA Alexa Fluor 488 conjugate. As shown in Fig. 7, THP-1 displayed endocytic activity for BSA Alexa Fluor 488 conjugate. However, cell-surface thiols on THP-1 cells were unaffected.

DISCUSSION

According to the review by Ryan *et al.* (2005), changes in Langerhans cells as a result of exposure to chemical allergens include internalization of surface major histocompatibility complex (MHC) class II molecules via endocytosis (Becker *et al.*, 1992; Girolomoni *et al.*, 1990), induction of tyrosine phosphorylation (Kühn *et al.*, 1998), modulation of cell-surface markers (Aiba and Katz, 1990; Verrier *et al.*, 1999) and changes of cytokine expression (Enk and Katz, 1992).

We were interested in whether THP-1 cells are activated by hapten-conjugated exogenous protein, or by direct action of the hapten on the cells. In this *in vitro* study, we found that CD86 expression on THP-1 cells is augment-