ORIGINAL ARTICLE

Assessment of the human Cell Line Activation Test (h-CLAT) for Skin Sensitization; Results of the First Japanese Inter-laboratory Study

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Abstract

The human Cell Line Activation Test (h-CLAT) is an in vitro skin sensitization test based on the enhancement by sensitizers of CD86 and/or CD54 expression on THP-1 cells. The aim of this study is to confirm the transferability and reproducibility of the h-CLAT protocol. Seven Japanese laboratories participated in this h-CLAT ring study. First, two well-known sensitizers (dinitrochlorobenzene (DNCB) and nickel sulfate (Ni)) and one non-sensitizer (sodium lauryl sulfate (SLS)) were evaluated at each laboratory with the same protocol at the same application dose. All laboratories correctly evaluated the skin sensitization potential of these three chemicals. Next, four sensitizers and one non-sensitizer were tested as a second trial. There were two false-negatives (ethylene diamine and eugenol) in some laboratories. Finally, chemicals tested in the second trial were re-evaluated with doses individually determined by each laboratory as a third trial. The results were almost the same as the results obtained when all the laboratories tested the same application doses. These results suggest that for more precise evaluation of difficult samples (e. g., unstable or water-insoluble chemicals), modifications of the protocol and prediction model are needed. However, the protocol was easily transferred to all laboratories and there were only a few false-negatives among 56 tests (8 chemicals at 7 laboratories).

Key words: *Skin sensitization, alternatives, THP-1, reproducibility, h-CLAT*

Introduction

Because of increasing social concern about animal welfare and the use of animals in testing, many alternative, non-animal tests have been proposed. There is particular interest in developing alternative methods for skin sensitization testing (De Silva et al., 1996). Measuring phenotypic changes, such as CD86 or CD54 expression on dendritic cells, induced by sensitizers is an important approach for developing alternative methods of evaluating skin sensitization potential (Aiba et al., 1997; Hopper et al., 1995). However, the effects of chemicals on the surface phenotype of dendritic cells are dependent on the source of peripheral blood used to obtain the cells; in other words, the effect varied from donor to donor (Aiba et al., 1997; Rougier et al., 2000). Furthermore, it is not easy to obtain sufficient fresh peripheral blood. In order to overcome these problems, we tested human leukemia cell lines, such as THP-1, as surrogates for dendritic cells. We have reported that THP-1 cells, which show enhanced CD86 and/or CD54 expression when treated with sensitizers, can be used in an in vitro skin sensitization test

(Ashikaga et al., 2002; Yoshida et al., 2003), and we named this test the human cell line activation test (h-CLAT). In our previous study, we optimized the test conditions (Ashikaga et al., 2006) and confirmed good predictive performance using nine chemicals (Sakaguchi et al., 2006). When the criteria for positive response of CD86 and CD54 in h-CLAT were set at 150% and 200% respectively, the correspondence between in vivo and in vitro was more than 90% (Ashikaga et al., 2007). h-CLAT could predict the sensitization potential of preservatives, which are well-known sensitizers (Sakaguchi et al., 2007). These results suggested that h-CLAT could be a useful in vitro test system for predicting sensitizing properties of chemicals. Before submission of h-CLAT to a public center for validation of alternative methods, we required further data, especially on inter-laboratory reproducibility among multiple laboratories. Therefore, this inter-laboratory study was set up to confirm the transferability and reproducibility of the h-CLAT protocol. Seven Japanese laboratories participated in this study, with the support of the Ministry of Health, Labor and Welfare.

Materials and Methods

Study management and SOP

An initial test protocol was developed based on our previous study (Ashikaga et al., 2006). In the light of subsequent experiments, a refined and detailed standard operating procedure (SOP) was defined for conducting further study.

Cells and culture

THP-1 cells (ATCC No. TIB-202) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA USA) with 10% FBS (v/v) (MP Biomedicals, Morgan Irvine, CA, USA, Cat. No. 29165, Lot. No. 2688H), 0.05 mM 2-mercaptoethanol and 1% Antibiotic-Antimycotic (Invitrogen Corp., Carlsbad, CA USA).

h-CLAT procedure

THP-1 cells were seeded at between $0.1x10^6$ and $0.2x10^6$ cells/mL, and pre-cultured for 48 h or 72 h. After the incubation, THP-1 cells were plated at 1 x 10^6 cells/ml in a 24-well plate and treated for 24 h with test chemical. The final concentration of DMSO, when this was used as a solvent, in culture media was less than 0.2%. Chemical-treated cells were washed twice with PBS(-) containing 0.1% BSA. Then, the cells were treated with 0.01% globulins, Cohn fraction II, III (Sigma-Aldrich) for FcR blocking, for 10 min at 4℃. Cell staining was done at 4° C for 30 min. Anti-human CD86 antibody was obtained from BD-PharMingen (Clone: Fun-1, San Diego, CA, USA). Anti-human CD54 antibody was obtained from DAKO (Clone: 6.5B5, Glostrup, Denmark). FITC labeled-mouse IgG1 was purchased from DAKO (Clone; DAK-G01, Glostrup, Denmark) and used as an isotype control. Cells were washed once with PBS(-) containing 0.1% BSA, and expression of cell surface antigens was analyzed by flow cytometry. Dead cells were gated out by staining with propidium iodide (PI, 0.625 μg/ml). In total, 10,000 living cells were analyzed. When the cell viability was less than 50%, Relative Fluorescence Intensity (RFI) was not calculated because of diffuse labeling of cytoplasmic structures due to cell membrane destruction (Becker et al., 1992). RFI was used as an in-

Table 1 Test chemicals and common dose setting (ND= No data).

Test chemicals	LLNA EC3(%)	Potency category by LLNA	Common CV75 (μg/mL)	vehicle for h-CLAT
p-Benzoquinone (BQ)	0.0099	Extreme	3.5	DMSO
1-Chloro-2,4-dinitrobenzene (DNCB)	0.05	Extreme	6.0	DMSO
Glutaraldehyde (GA)	0.1	Strong	8.0	Saline
Ethylene diamine (ED)	2.2	Moderate	250	Saline
Nickel sulfate (Ni)	4.8	Moderate	150	Saline
Eugenol (EU)	13	Weak	150	DMSO
Lactic acid (LA)	Not calculated	Non-sensitizer	2800	Saline
Sodium lauryl sulfate (SLS)	N.D.	False potitive	60	Saline

dicator of CD86 and CD54 expression and was calculated as follows:

$$\text{RFI (\%)} = \frac{\text{(MFI of chemical-treated cells} - \text{MFI of chemical-treated Isotype control cells)}}{\text{(MFI of vehicle control cells} - \text{MFI of vehicle Isotype control cells)}} \times 100$$

MFI = (Geometric) Mean fluorescence intensity

Test chemicals and application doses

Eight test chemicals are shown in Table 1. All chemicals have been evaluated and classified with the LLNA (Gerberick et al., 2005). Six sensitizers were evaluated: two extreme, one strong, two moderate, and one weak allergens, as classified by LLNA. Two non-sensitizers were also evaluated: one non-classified allergenic chemical and the other false positive by LLNA. All chemicals were purchased from Sigma-Aldrich. In first and second trials, application doses were determined from the results of cytotoxicity tests conducted at two laboratories. Cytotoxicity was evaluated by flow cytometry with propidium iodide (PI) (PI assay). From the PI assay data, eight doses based on the dose estimated to give 75% cell viability (CV75) were used [1.2 x CV75, 1 x CV75, 1/1.2xCV75 (or 0.8333xCV75), $1/1.2^2$xCV75 (or 0.6944xCV75), $1/1.2^3$xCV75 (or 0.5787xCV75), $1/1.2^4$xCV75 (or 0.4822xCV75), $1/1.2^5$xCV75 (or 0.4019xCV75) and $1/1.2^6$x CV75 (or 0.3349xCV75)]. The appropriateness of this dose setting was confirmed by the evaluation of more than 60 chemicals (Ashikaga et al., 2007). All CV75 doses of test chemicals used in this study are shown in Table 1. The vehicle was saline or DMSO (SIGMA-ALDRICH, Cat. No. 154938, purity ≧99.9%). In the third trial, each laboratory individually conducted cytotoxicity testing for determination of the application doses.

Data analysis

Tests were performed three times with each chemical. The values of cell viability and CD86/54 expression were calculated as the mean of the three tests. The average of three experiments at any dose should exceed the positive criterion ("CD86 ≧ 150 or CD54 ≧ 200") in order for the test chemical to be considered as 'positive'.

Cell culture conditions

THP-1 cells cultured in Lab "F" showed unacceptably low viability (less than 50%) when treated with 5 μg/mL DNCB (CV75/1.2), which was used as a positive control in every experiment. For that reason, the dose-response of DNCB in Lab "F" was different from the results in other laboratories (Fig. 1-a). However, when freshly cultured THP-1 cells were introduced in Lab "F", the results were very similar to those in the other laboratories (Fig. 1-b). Both the firstly tested cell and the freshly cultured cell originated from a same lot of THP-1.

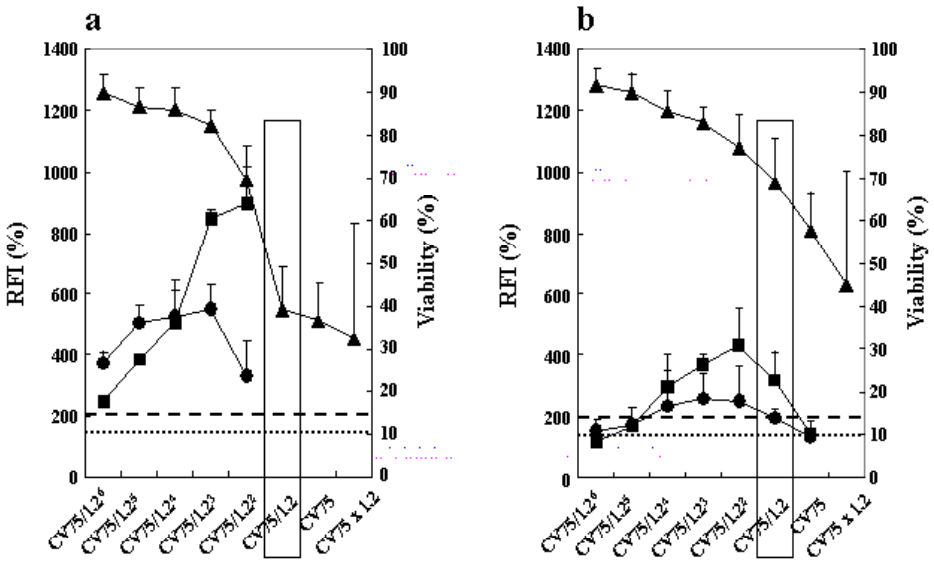

Fig. 1 Improvement of reproducibility (example 1)
At laboratory "F", the cell viability at 5 μg/mL DNCB (CV75/1.2) was improved when DNCB was re-evaluated with freshly cultured THP-1 cells. a=First experiment; b=Second experiment. ▲= Viability; ●= CD86; ■= CD54. Small dotted line: criterion for CD86 positivity; dashed line: criterion for CD200 positivity.

The viability of the freshly cultured cell treated with 5mg/mL of DNCB (CV75/1.2) was about 70%. On the other hand, that of the firstly tested cell was less than 40%, and the value meant condition of the cell was not good. Condition of the firstly tested cell could have decreased due to wrong operation such as over-growth during cell culture. Moreover, in Lab "D" the dose-response of Ni was initially different from those in the other laboratories (Fig. 2-a). Cell viability of control cells at this time was less than 90%, which was unusual. When Lab "D" re-evaluated Ni with freshly cultured THP-1 cells, the viability of control cells was over 90% and both CD86 and CD54 were enhanced by treatment of the test cells with Ni (Fig. 2-b). These results suggested that tight control of cell culture conditions is important for good reproducibility in the test.

Inter-laboratory reproducibility with three well-known chemicals

As a first trial, seven laboratories tested two well-known sensitizers (DNCB and Ni) and one non-sensitizer (SLS), after the introduction of tighter control of cell culture conditions. Fig. 3 shows the inter-laboratory reproducibility for

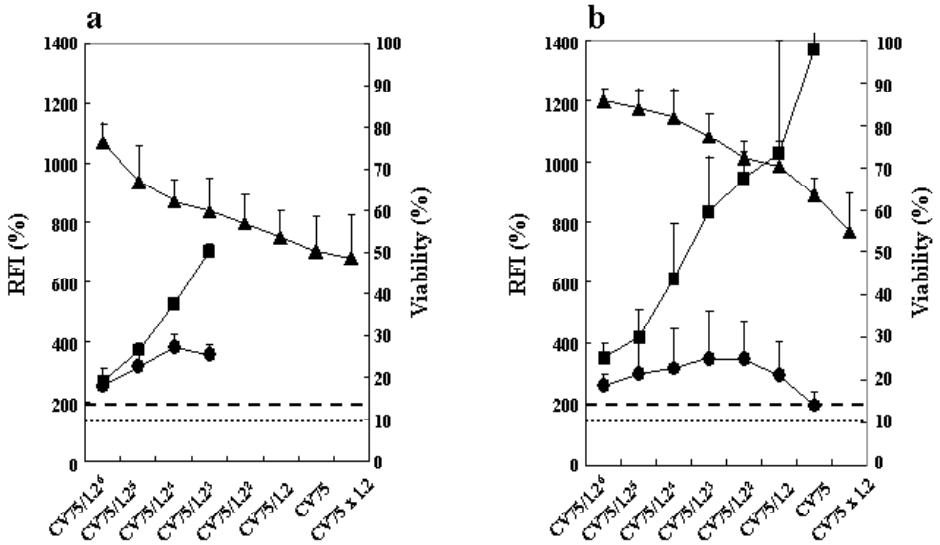

Fig. 2 Improvement of reproducibility (example 2)
At laboratory "D", the cell viability of control cells was improved when freshly cultured THP-1 cells were used, and the dose-response curve of Ni was similar to those in the other laboratories. a=First experiment; b=Second experiment. ▲= Viability; ●= CD86; ■= CD54. Small dotted line: criterion for CD86 positivity; dashed line: criterion for CD200 positivity.

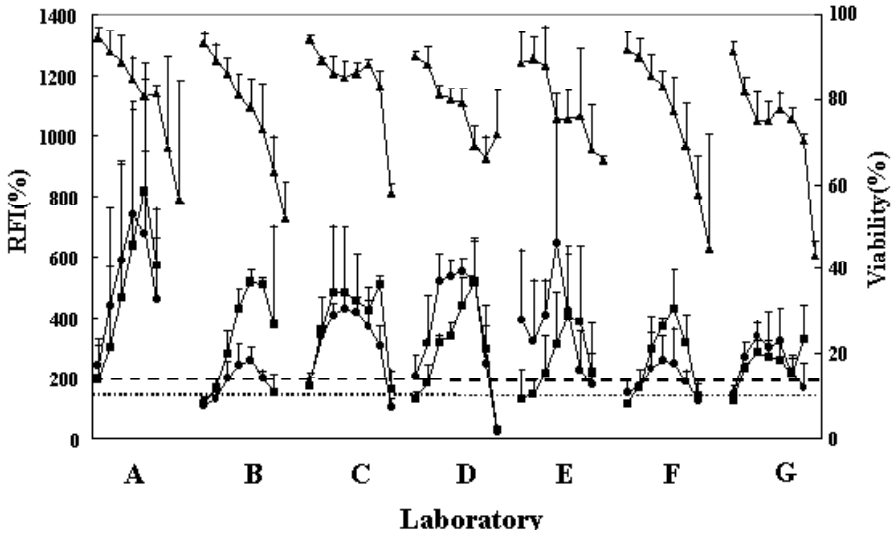

Fig. 3 Inter-laboratory reproducibility of prediction for DNCB
▲= Viability; ●= CD86; ■= CD54. Small dotted line: criterion for CD86 positivity; dashed line: criterion for CD200 positivity.

DNCB. In all laboratories, DNCB clearly enhanced both CD86 and CD54 at several doses and the dose-response relationships were basically similar. Both CD86 and CD54 were augmented dose-dependently at lower doses and their expression was suppressed due to cytotoxicity at higher doses. Ni also enhanced both CD86 and CD54 of THP-1 cells in all laboratories (Fig. 4). In particular, CD54 expression was remarkably induced by the Ni treatment in a dose-dependent manner. On the other hand, SLS, a non-sensitizer, did not affect CD86 or CD54 expression at any dose, including higher "subtoxic doses", in all laboratories (Fig. 5). All seven laboratories correctly evaluated the sensitizing potential of these three chemicals. The reproducibility of dose-response relationships among laboratories was excellent.

Inter-laboratory reproducibility of five additional chemicals

Next, four sensitizers, covering diverse sensitizing potentials, and one non-sensitizer were tested as a

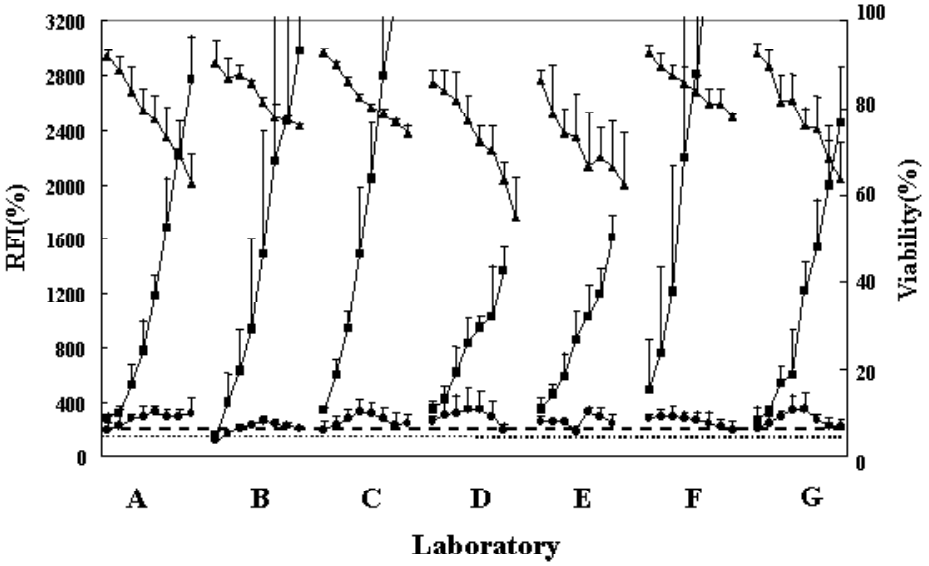

Fig. 4 Inter-laboratory reproducibility of prediction for Ni
▲= Viability; ●= CD86; ■= CD54. Small dotted line= criterion for CD86 positivity; dashed line: criterion for CD200 positivity.

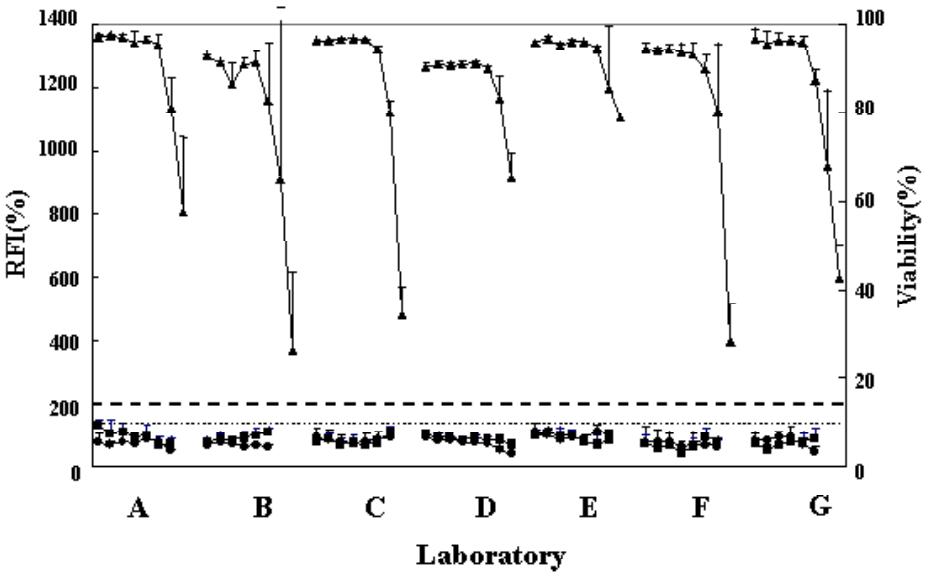

Fig. 5 Inter laboratory reproducibility of SLS
▲= Viability; ●= CD86; ■= CD54. Small dotted line= criterion for CD86 positivity; dashed line: criterion for CD200 positivity.

second trial. In the second trial, all five chemicals were tested based on common CV75 values. The results for the five chemicals in seven laboratories are summarized in Table 2. Thirty-three out of 35 tests corresponded with LLNA. There were two false-negatives (ethylene diamine and eugenol), but no false-positives. The overall accuracy of the 1st and 2nd trials was about 96%. In summary, the reproducibility of h-CLAT was basically good. Either CD86 or CD54 was slightly enhanced in the two false-negative cases (data not shown), but the increases did not meet the criteria for positivity.

Inter-laboratory reproducibility, including dose finding

In order to further assure of the performance of the assay, chemicals tested in the second trial were evaluated again in a third trial. In this trial, each laboratory individually performed cytotoxicity assay, and determined the application doses based on their own results. Table 3 shows CV75 values (estimated dose affording 75% cell viability) at each

Table 2 Summary of the first and second trials
The results of evaluation of eight chemicals in the seven laboratories are summarized.
Results; += positive; -= negative. Battery (CD86/CD54). *; Laboratory "D" judged BQ positive as a result of eight experiments. Shaded cell= LLAN and h-CLAT predictions differ.

	Laboratory						
Test chemical	**A**	**B**	**C**	**D**	**E**	**F**	**G**
p-Benzoquinone (BQ)	+ (+/+)	+ (+/+)	+ (+/+)	+ (+*/-)	+ (+/-)	+ (+/+)	+ (+/-)
Glutaraldehyde (GA)	+ (+/+)	+ (+/+)	+ (+/+)	+ (+/-)	+ (+/-)	+ (+/+)	+ (-/+)
Ethylene diamine (ED)	+ (+/-)	+ (+/-)	- (-/-)	+ (+/-)	+ (+/-)	+ (+/-)	+ (+/-)
Eugenol (EU)	+ (+/+)	+ (-/+)	+ (+/+)	+ (+/-)	+ (+/+)	+ (+/+)	- (-/-)
Lactic acid (LA)	- (-/-)	- (-/-)	- (-/-)	- (-/-)	- (-/-)	- (-/-)	- (-/-)

Table 3 Values of CV75 at each laboratory in the third trial
Grand mean= mean value of all seven laboratories; SD= standard deviation; CV= coefficient of variation.

Test chemical	Common CV75 in the second trial	CV75s (μg/mL) in the third trial									
		A	**B**	**C**	**D**	**E**	**F**	**G**	Grand mean	SD	CV
DNCB	6.0	4.3	4.2	5.0	4.6	3.6	4.6	6.4	4.7	0.9	0.19
pBQ	3.5	2.6	5.5	3.2	7.3	5.4	2.8	3.7	4.3	1.7	0.40
GA	8.0	9.7	12	12	20	7.5	8.2	9.2	11	4.3	0.38
ED	250	267	256	274	278	248	370	277	281	41	0.14
EU	150	153	161	155	202	177	190	120	165	27	0.17
LA	2800	2730	2754	3045	3055	2997	3300	2920	2972	195	0.07

Table 4 Summary of the third trial
The results of re-evaluation of five chemicals in the seven laboratories are summarized.
Results; += positive; -= negative. Battery (CD86/CD54). Hatched cell= LLAN and h-CLAT predictions differ.

	Laboratory						
Test chemical	**A**	**B**	**C**	**D**	**E**	**F**	**G**
BQ	+ (+/+)	+ (+/+)	+ (+/+)	+ (+/-)	+ (+/-)	+ (+/+)	+ (+/-)
GA	+ (+/+)	+ (+/+)	+ (+/+)	+ (+/-)	+ (+/+)	+ (+/+)	+ (+/+)
ED	+ (+/-)	+ (+/-)	- (-/-)	- (-/-)	+ (+/-)	+ (+/-)	+ (+/+)
EU	+ (+/+)	- (-/-)	+ (+/-)	+ (+/-)	+ (+/-)	+ (+/+)	+ (+/+)
LA	- (-/-)	- (-/-)	- (-/-)	- (-/-)	- (-/-)	- (-/-)	- (-/-)

laboratory. There was some variation of CV75 among laboratories. This might have been caused by differences of culture conditions between laboratories, because the same lots of serum and the same cell line were used at all laboratories. However, the CV75s at the individual laboratory were very close to the common CV75s used in the second trial. The coefficient of variation for each test chemical was between 0.07 and 0.4, and the range of CV value was good compared with that in another inter-laboratory study on cytotoxicity assay (Tani et al., 1999). The results of the third trial are summarized in Table 4. Among the five test chemicals, p-benzoquinone, glutaraldehyde and lactic acid were correctly evaluated at all laboratories. On the other hand, two laboratories missed the sensitizing potential of ethylene diamine or eugenol. These results are almost the same as those in the second trial.

Discussion

Several in vitro skin sensitization methods using cell lines have been reported in response to current trends in animal welfare and regulatory opinion (Casati et al., 2005), but final validation and regulatory acceptance have not yet been achieved. We have reported that h-CLAT using THP-1 cells was useful for predicting skin sensitization in vitro (Ashikaga et al., 2007; Sakaguchi et al., 2007). However, more data were needed, especially about the transferability of the protocol, and the inter-laboratory reproducibility of the test, in order to support formal validation activities (Hartung et al., 2004). Therefore, we organized a multi-laboratory study involving seven laboratories. As a first step, two well-known sensitizers (DNCB and Ni) and one non-sensitizer (SLS) were evaluated. The purpose of the first trial was to establish technology transfer of the h-CLAT protocol. Because all laboratories correctly evaluated the sensitization potentials of these three chemicals, transferability of the assay was judged to be basically good. However, some differences in dose-response relationship were observed. The reproducibility improved when re-evaluation was conducted with freshly cultured THP-1 cells. These results suggest that tight control of cell culture conditions is important, especially for good reproducibility of cell-based assay in which protein expression is used as an indicator.

Based on these results, we refined the standard operating procedure (SOP). We introduced the requirements that the viability of control cells should be more than 90 %, and that the viability in the positive control should be more than 60%. After the introduction of tighter control of cell culture conditions, the reproducibility of the dose-response relationship was improved. From the result of the first trial, we concluded that the h-CLAT protocol is easy to transfer, and to further confirm the reproducibility with various kinds of chemicals, we tested four sensitizers and one non-sensitizer in a second trial. In the total of 35 tests (seven laboratories, five chemicals), there were two false-negatives (ethylene diamine and eugenol). Therefore, inter-laboratory reproducibility of the assay was basically good. Ethylene diamine is known to be very reactive with organic compounds (Agius et al., 1991), and it evaporates at room temperature. Further, eugenol showed poor water solubility at the application doses, because oil drops were observed in the cell culture medium. It would be difficult for h-CLAT to evaluate the sensitization potential of such chemicals, so the false negative results may simply reflect the particular characteristics of these two chemicals. It will be necessary to clarify the extent of applicability of h-CLAT, particularly in relation to the physicochemical properties of target molecules. Some differences in CD86/CD54 expression pattern were also observed among laboratories. This confirms the importance of predicting sensitizing potential not just with one marker, but with two or more markers. Python et al. (2007) reported that a combination of at least two markers was needed to establish a reliable evaluation of dendritic cell activation potential. We also should mention problems of h-CLAT. Test chemicals are treated with THP-1 cells in cell culture medium. Therefore, if test chemical disperse non-equally in cell culture medium (e.g., sticky, water-proof particle, oil spill, etc.), h-CLAT may not evaluate these potential correctly. In addition, THP-1 is thought to almost not have metabolic enzymes such as P-450 (Prof. Yoshida, Showa Univ., personal communication). Therefore, h-CLAT might not be able to evaluate a potential of chemical that can be changed by metabolism. Study on the applicability domain of h-CLAT remains to be done.

Finally, chemicals tested in the second trial were re-evaluated with doses determined at each individual laboratory as a third trial, to see whether more appropriate application doses could be selected, depending on the precise test conditions. However, differences of the values of CV75 between laboratories were not large. Furthermore, the results (positive/negative judgment) were almost

the same as in the second trial with common application doses. In conclusion, further study is necessary, especially to clarify the limitations of the assay. Finally, all laboratories correctly judged the sensitization potential of six test chemicals among eight chemicals. These results suggest that the h-CLAT protocol is easy to transfer, and that inter-laboratory reproducibility is basically good. We consider that h-CLAT will be ready for formal pre-validation study after further minor improvements of the method.

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References

Agius, R. M., Nee, J., McGovern, B., Robertson, A., (1991) Structure activity hypotheses in occupational asthma caused by low molecular weight substances, Ann. Occup. Hyg., 35(2), 129-37.

Aiba, S., Terunuma, A., Manome, H., and Tagami, H., (1997) Dendritic cells differently respond to haptens and irritants by their production of cytokines and expression of co-stimulatory molecules, European Journal of Immunology, 27, 3031-3038.

Ashikaga, T., Hoya, M., Itagaki, H., Katumura, Y., and Aiba, S., (2002) Evaluation of CD86 expression and MHC class II molecule internalization in THP-1 human monocyte cells as predictive endpoints for contact sensitizers, Toxicology in Vitro, 16, 711-716.

Ashikaga, T., Yoshida, Y., Hirota, M., Yoneyama , K., Itagaki, H., Sakaguchi, H., Miyazawa, M., Ito, Y., Suzuki, H., and Toyoda, H., (2006) Development of an in vitro skin sensitization test using human cell lines; human Cell Line Activation Test (h-CLAT). I. Optimization of the h-CLAT protocol, Toxicology in Vitro, 20, 767-773.

Ashikaga, T., Kosaka, N., Sono, S., Sakaguchi, H., Suzuki H., and Itagaki H., (2007) Comparative evaluation of the in vitro skin sensitization test, human Cell Line Activation Test (h-CLAT) with LLNA and human data, The Toxicologist, 96 (1), 237.

Becker, D., Kolde, G., Reske, K. and Knop, J., (1994) An in vitro endocytotic activation of murine epidermal langerhans cells under the influence of contact allergens, Journal of Immunological Methods, 169, 195-204.

Casati, S., Aeby, P., Basketter, D. A., Cavani, A., Gennari, A., Gerberick, G. F., Griem, P., Hartung, T., Kimber, I., Lepoittevin, J. P., Meade, B.J., Pallardy, M., Rougier, N., Rousset F., Rubinstenn, G., Sallusto, F., Verheyen, G. R., and Zuang, V., (2005) Dendritic cells as a tool for the predictive identification of skin sensitisation hazard.; The Report and Recommendations of ECVAM Workshop 51, Altern. Lab. Anim., 33(1), 47-62.

De Silva, O., Basketter, D. A., and Barrat M. D., (1996) Alternative methods for skin sensitization testing, Alternative Laboratory Animals, 24, 683-705.

Gerberick, G. F, Ryan, C. A., Kern, P. S., Schlatter. H., Dearman, R. J., Kimber. I., Patlewicz, G. Y., and Basketter, D. A., (2005) Compilation of historical local lymph node data for evaluation of skin sensitization alternative methods, Dermatitis, 16(4), 157-202.

Hartung, T., Bremer, S., Casati, S., Coecke, S., Corvi, R., Fortaner, S., Gribaldo, L., Halder, M., Hoffmann, S., Roi, A. J., Prieto, P., Sabbioni, E., Scott, L., Worth, A., and Zuang, V., (2004) A modular approach to the ECVAM principles on test validity, Altern. Lab Anim., 32(5), 467-72.

Hopper, U., Degwerat, J., and Steckel, F., (1995) Use of CD1a- dendritic cells and keratinocytes to characterize cellular reaction involved in allergic contact dermatitis, Journal of Cellular Biochemistry, 21, Supple A, 11-18.

Python, F., Goebel, C., Aeby, P., (2007) Assessment of the U937 cell line for the detection of contact allergens, Toxicol. Appl. Pharmacol,. 220, 113-24.

Tani, N., (1999) Interlaboratory Validation of the In Vitro Eye irritation Tests for cosmetic Ingredients. (8) Evaluation of Cytotoxicity Tests on SIRC cells, Toxicology in Vitro, 13, 175-187.

Rougier, N., Redziniak, G., Mougin, D., Schmitt, D., and Vincent, C., (2000). In vitro evaluation of the sensitizaiton potential of weak contact allergens using Langerhans-like dendritic cells and autologous T cells, Toxicology, 145, 73-82.

Sakaguchi, H., Ashikaga, T., Miyazawa, M., Yoshida, Y., Ito, Y., Yoneyama , K., Hirota, M., Itagaki, H., Toyoda, H., and Suzuki, H., (2006) Development

of an in vitro skin sensitization test using human cell lines; human Cell Line Activation Test (h-CLAT). II. An inter-laboratory study of the h-CLAT, Toxicology in Vitro, 20, 774-784.

Sakaguchi, H., Miyazawa, M., Yoshida, Y., Ito, Y., and Suzuki, H., (2007) Prediction of preservative sensitization potential using surface marker CD86 and/or CD54 expression on human cell line, THP-1, Arch. Dermatol. Res., 298, 427-37.

Yoshida, Y., Sakaguchi, H., Ito, Y., Okuda, M., and Suzuki, H., (2003) Evaluation of the skin sensitization potential of chemicals using expression of co-stimulatory molecules, CD54 and CD86, on the naïve THP-1 cell line, Toxicology in Vitro, 17, 221-228.

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