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(Received: February 27/
Accepted: July 31)

Corresponding author:
Nanae Kosaka
Kao Corporation,
2606 Akabane, Ichikai-Machi,
Haga-Gun,Tochigi, 321-3497, Japan
E-mail: kosaka.nanae@kao.co.jp

ORIGINAL ARTICLE

A study on serum difference on test results in the human Cell Line Activation Test (h-CLAT): Results of 3rd Japanese inter-laboratory study

Sakiko Sono [1], Takaaki Yamada [2], Nanae Kosaka [3], Kenji Okamoto [4], Makoto Mizuno [5], Jun Sato [6], Mayumi Yoshida [7], Naoko Ota [7], Tatsuji Kodama [6], Yuko Okamoto [5], Hirofumi Kuwahara [4], Hitoshi Sakaguchi [3], Seiji Hasegawa [2], Takao Ashikaga [1] and Yasuo Ohno [8]

[1]Shiseido Co., Ltd., [2]Nippon Menard Cosmetic Co., Ltd., [3]Kao Corporation, [4]Kanebo Cosmetics Inc., [5] KOSÉ Corporation, [6] LION CORPORATION, [7] POLA CHEMICAL INDUSTORIES, INC., [8]National Institute of Health Sciences, Japan

Abstract

The human Cell Line Activation Test (h-CLAT) is an in vitro skin sensitization test based on the enhancement of CD86 and/or CD54 expression on THP-1 cells. The aim of this study was to examine the effect of differences of serum source on the results of h-CLAT. Three different lots of serum, obtained from three sources, were compared with the serum used in the previous Japanese ring study. With each serum, cellular proliferation in subculture, cytotoxicity, and CD86/CD54 expression on THP-1 cells were measured following exposure to two known allergens (dinitrochlorobenzene (DNCB) and nickel sulfate (Ni)) and one non-allergen (sodium lauryl sulfate (SLS)).

There was no clear difference of cellular proliferation in subculture, cytotoxicity, or CD86/54 expression among cultures in the four sera. Although the source of serum does not appear to influence the result of h-CLAT, the validity of the test should nevertheless be confirmed when serum from a new source is introduced.

Key words: *skin sensitization, alternative test, serum, h-CLAT*

Introduction

Because of increasing social concern about animal welfare and the use of animals in testing, many non-animal tests have been proposed as alternatives. There is particular interest in developing alternative methods for skin sensitization testing (De Silva *et al.*, 1996). Measuring phenotypic changes, such as changes of CD86 or CD54 expression on dendritic cells, induced by sensitizers was an important approach for developing alternative methods of evaluating skin sensitization potential (Aiba *et al.*, 1997; Hopper *et al.*, 1995). 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) (Ashikaga, 2006). In our previous study, we indicated 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 need to confirm the general versatility and robust-

ness of h-CLAT. In this study, we focused on the influence of the source of fetal bovine serum (FBS) in the culture medium, because inter-lot differences of biological materials, such as serum, are to be expected (Cartwight *et al.*, 1994, Technical report No.4 1990) and it is unknown how serum difference affects the result of h-CLAT. Therefore, we have examined the effect of serum source on cell growth, cytotoxicity, and expression of CD86 or CD54 of h-CLAT, with the aim of collecting background data to support the robustness of h-CLAT as an alternative, non-animal test method in two of seven Japanese laboratories participating in the h-CLAT program.

Materials and Methods

Collaborating laboratories

Laboratory C: Shiseido In Vitro Toxicology Research Laboratory, Laboratory D: Research Laboratories, Nippon Menard Cosmetic Co., Ltd.

Cells and culture

THP-1 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured in RPMI 1640 medium (Invitrogen Corp., Carlsbad, CA, USA) with 10% FBS (v/v), 0.05 mM 2-mercaptoethanol and 1% antibiotic-antimycotic mixture (Invitrogen Corp.).

Four kinds of FBS were used: one was that used in the previous ring study (MP Biomedical, Irvine, CA, USA, lot#. 2688H). This lot was used as a reference lot in this study. The others were purchased from different manufacturers or sourced from different lots (X: Hyclone Laboratories, Logan, Utah, lot#. AQA22909, Y: Invitrogen Corp., Carlsbad, CA, USA, lot#. 560557, Z: MP Biomedical, Irvine, CA, USA, lot#. 8076H).

To confirm the inter-laboratory reproducibility of h-CLAT, the tests were conducted at two laboratories with the same THP-1 cell lot and the same lots of fetal bovine serum.

The same subculture method was used in both laboratories. The cell culture concentration was kept below 1×10^7 cells/mL

Test chemicals and application doses

All chemicals have been evaluated and classified with the LLNA (Gerberick et al., 2005). Two well-known allergens (dinitrochlorobenzene (DNCB) and nickel sulfate (Ni)) and one non-allergen (sodium lauryl sulfate (SLS)) were evaluated. The vehicle was saline (for Ni and SLS) or DMSO (for DNCB).

All chemicals were examined at the CV75, which was determined by PI assay. All CV75 doses of test chemicals used in this study are shown in Table 2. Each laboratory conducted PI assay and determined the application dose separately.

Cytotoxicity assay

Cell viability was measured by propidium iodide (PI) assay as described previously (Kosaka *et al.*, 2008). The CV75 (μg/mL) (CV75: estimated concentration affording 75% cell viability) value for each test chemical was calculated.

h-CLAT procedure

THP-1 cells were seeded at between 0.1×10^6 and 0.2×10^6 cells/mL, and pre-cultured for 48 h or 72 h. After the incubation, THP-1 cells were plated at 1×10^6 cells/ml in a 24-well plate and treated for 24 h with test chemical. The expressions of CD86 and CD54 on the cells were analyzed as described previously (Kosaka *et al.*, 2008).

Data analysis

Both laboratories independently evaluated the test chemicals once a week for 3 weeks (3, 4 and 5 weeks after the start of cell culture).

The relative fluorescence intensity (RFI) was used as an indicator of CD86/ CD54 expression and was calculated as described previously (Kosaka *et al.*, 2008).

RFI values above 150 and 200 for CD86 and CD54 expression, respectively, were considered positive response following exposure to a chemical. When the cell viability was less than 50% in even one experiment, the data at that concentration was excluded from the analysis of the data. The reason is that the diffuse labeling cytoplasmic structures that occur due to cell membrane destruction will interfere with fluorescent measurements (Becker et al., 1994). Also, data were not included in the analysis when the cell viability with DNCB at CV75 was not within a range of 60-90%.

Statistical analysis

The Student's *t*-test was used to analyze data for significant differences. The value was regarded as significant at $p<0.01$.

Results

Cellular proliferation in subculture

THP-1 cells were cultured with four different FBS during two months by both labs. Each lab evaluated the proliferation of cells cultured with each

FBS once a week during the two months (at week 3 through week 9 cultures). After culturing for 72h, the cell number cultured with each FBS in each lab is shown in Figure 1. *P* values were calculated by Student's *t*-test in order to evaluate a statistical significance between the Reference FBS and the each new lot. There were no significant differences in cell number after 72h culture in both laboratories (Table 1).

Comparison of cytotoxicity with each FBS

The values of CV75 for three chemicals were calculated at the 3rd week after the start of cell culture in each laboratory (Table 2). The value of CV75 was essentially independent of the source of FBS. These results suggested that the use of FBS from different sources had little influence on viability.

There were relatively large differences between laboratories in the case of Ni, but not the other chemicals.

Expression of CD86 or CD54 in response to the three chemicals

Two well-known sensitizers (DNCB and Ni) and one non-sensitizer (SLS) were tested every week during 3 to 5 weeks after the start of cell culture with the serum samples in both laboratories. Table 3 shows the mean RFI values. DNCB 5.0 μg/mL did not induce CD54 augmentation with lot. "X" and lot. "Z" in Lab. D. However, the values obtained fell only slightly below the criterion for positivity. The RFI values of CD86 and CD54 for DNCB and Ni were above the positive criterion, and that for SLS were negative irrespective of serum lots in both laboratories, except for 2 values of CD54 RFI in Lab. D. Therefore, the test chemicals were generally evaluated correctly in both laboratories.

RFI values in all tests at 3-5 weeks

Fig. 2-a and 2-b show all the RFI values for each chemical in three tests during weeks 3 to 5 using

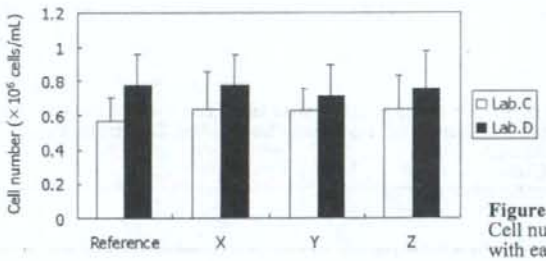

Figure 1
Cell number of each THP-1 cell cultured with each FBS after 72h culture

Table 1
Statistical analysis of cell number of each THP-1 cell lot after 72h culture

FBS lot	P value	
	Lab.C	Lab.D
Reference	-	-
X	0.51	0.98
Y	0.46	0.53
Z	0.47	0.84

P values were calculated by Student's *t*-test in order to evaluate a statistical significance between the reference lot in this study, and the each new lot.

Table 2
CV75 values (mg/mL) of DNCB, Ni, and SLS

Samples	Laboratory	Reference FBS	X	Y	Z
DNCB CV75 (μg/mL)	Lab. C	3.6	3.9	3.8	3.5
	Lab. D	4.0	3.7	3.8	4.1
Ni CV75 (μg/mL)	Lab. C	100	96	96	91
	Lab. D	130	130	160	160
SLS CV75 (μg/mL)	Lab. C	40	37	39	38
	Lab. D	42	40	41	47

The CV75 (estimated concentration affording 75% cell viability) values of the three chemicals were calculated from the results of cytotoxicity tests at the 3rd week after the start of cell culture in each laboratory.
Upper= Lab. C, Lower=Lab. D

FBS from different sources in both laboratories. The RFI values for Ni were below the criterion (150%) for CD86 in 4 out of 24 tests (Fig. 2-a). The RFI value for DNCB was below the criterion (200%) for CD54 in one out of 24 tests (Fig. 2-b). The RFI values for SLS were below the criteria for CD86 and CD54 in all tests. All lots of FBS allowed stable and correct identification of sensitivity to chemicals as positive (test agents) or negative (SLS control). These results indicate that the use of FBS from different sources had little effect on evaluation of the sensitizing potential of these chemicals.

Discussion

Several in vitro skin sensitization methods using cell lines have been reported (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.*, 2006). However, more data was needed, especially about the effects of possible variables, before the tests could be considered suitable for practical purposes. In this study, we focused on the effect of the source of FBS in the culture medium on h-CLAT results.

FBS is essential for in vitro cell culture. The role of serum in cell culture work is largely undefined (Barnes, *et al.*, 1980, Jayme, *et al.*, 1988). It has been proposed that the main role of serum in cell proliferation is as a provider of complex hormones (Bottenstein *et al.*, 1979; Phillips *et al.*, 1981) and other growth factors influencing attachment (e.g., fibronectin), propagational factors (e.g., insulin), transport factors (e.g., transferrin), and trace metals (Jayme *et al.*, 1985). Therefore, an appropriate serum for cell culture must be selected for use in vitro tests, because biological materials such as serum typically display lot-to lot variation (Cartwight *et al.*, 1994; Technical report No.4 1990).

Many factors, such as the quality, type, and concentration of the components in different FBS lots, may affect the cellular growth rate (Boone *et al.*, 1971). It was reported that three lots of FBS

Table 3
Average RFI values (%) in each laboratory

Two well-known sensitizers (DNCB and Ni) and one non-sensitizer (SLS) were tested every week during 3 to 5 weeks after the start of cell culture with FBS from different sources in both laboratories. The results are the mean of the three tests during 3~5 weeks in each case.
(a): RFI values for CD86, (b): RFI values for CD54.
Results; *: negative, **: LLAN and h-CLAT predictions differ.

(A) CD86

CD86	Lab.C				Lab.D			
	Reference	Lot.X	Lot.Y	Lot.Z	Reference	Lot.X	Lot.Y	Lot.Z
DNCB CV75	265 ± 80.3	345 ± 14.3	340 ± 106	322 ± 103	290 ± 124	257 ± 50.8	324 ± 117	279 ± 33.0
DNCB 5 μg/mL	174 ± 82.8	278 ± 42.5	237 ± 84.4	198 ± 76.1	229 ± 55.3	169 ± 51.1	155 ± 59.2	170 ± 37.1
Ni CV75	237 ± 1.8	279 ± 64.9	302 ± 57.1	254 ± 58.6	210 ± 126	202 ± 84.9	241 ± 143	222 ± 123
SLS CV75	73 ± 7.6*	93 ± 19.1*	91 ± 24.5*	68 ± 4.9*	88 ± 51.1*	100 ± 38.9*	79 ± 14.0*	70 ± 25.4*

(B) CD54

CD54	Lab.C				Lab.D			
	Reference	Lot.X	Lot.Y	Lot.Z	Reference	Lot.X	Lot.Y	Lot.Z
DNCB CV75	393 ± 95.2	521 ± 153.0	433 ± 53.7	256 ± 55.6	240 ± 4.4	242 ± 14.8	288 ± 77.2	278 ± 23.8
DNCB 5 μg/mL	386 ± 170	505 ± 118	489 ± 135	242 ± 85.4	249 ± 44.5	207 ± 29.7	197 ± 70.9	197 ± 74.0
Ni CV75	975 ± 372	1005 ± 648	1027 ± 332	701 ± 234	1077 ± 62	944 ± 363	936 ± 190**	919 ± 391**
SLS CV75	91 ± 27.5*	119 ± 25.4*	91 ± 20.1*	77 ± 12.4*	89 ± 48.2*	90 ± 22.8*	81 ± 56.9*	86 ± 49.5*

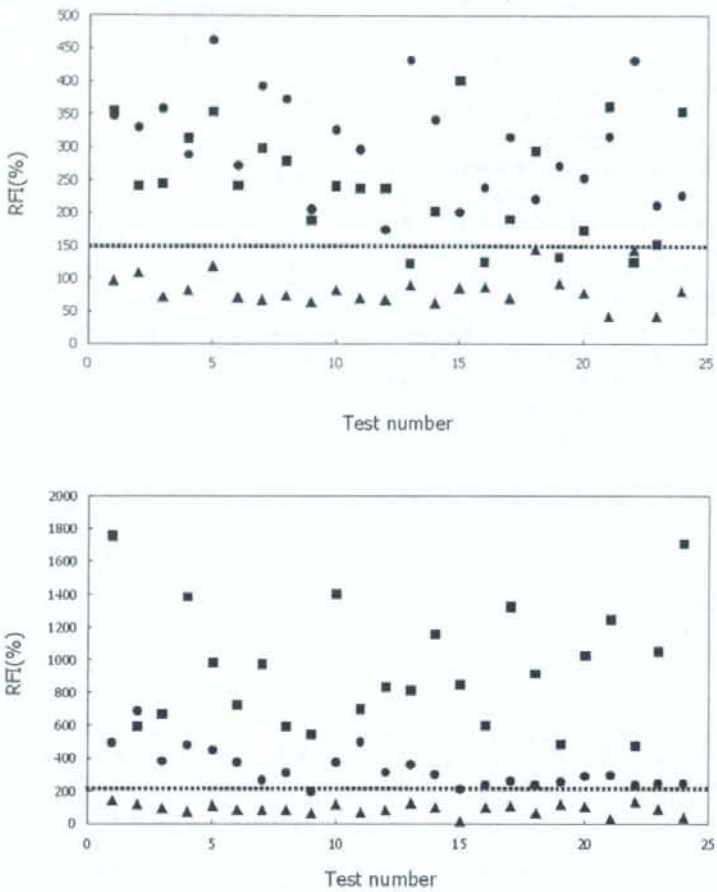

Figure 2
RFI values (%) in all tests at 3-5 weeks

RFI values of the three test chemicals during 3~5 weeks with FBS from different sources in both laboratories. Fig. 2-a = RFI values for CD86, Fig. 2-b = RFI values for CD54. ● = DNCB; ■ = Ni; ▲ = SLS. Small dotted line: positive criterion for CD86; dashed line: positive criterion for CD54.

from different manufacturers showed different abilities to support cell growth (Xiaoyang *et al.* 2006). However, in this study, the source of FBS had little effect on cellular proliferation in subculture in either of the laboratories, except for a slight decrease after week 8 in Lab. D (Data not shown). These results indicate that the source of FBS had little effect on cellular proliferation in subculture in either of the laboratories.

Since cell viability may be influenced by the source of FBS (Howard *et al.* 1980), we examined the cytotoxicity of the three chemicals at the 3rd week of cell culture using FBS from various sources. However, the value of CV75 was essentially independent of the source of FBS in each laboratory. There were relatively large differences between laboratories in the case of Ni, but not the other chemicals. The reason for this may be the steepness of the dose-response relationship for Ni.

Cell lines, which are cultured once in the presence of an endotoxin, show changes in biological characteristics or phenotypes. FBS may contain significant levels of lipopolysaccharide (LPS), and it is particularly important to avoid en-

dotoxin contamination of cell culture (Kirikae *et al.*, 1997). Moreover, LPS stimulates the production of cytokines and the expression of co-stimulatory molecules by dendritic cells through a soluble CD14-dependent pathway (Verhasselt *et al.*, 1997), and monocyte-derived dendritic cells exposed to 1 ng/mL LPS showed strong up-regulation of the surface expression of HLA-DR, the co-stimulatory molecule CD86, and the adhesion molecule CD54 (Karine *et al.*, 1999). However, in the present study, the use of FBS from different sources had little effect on changes of expression of CD86 or CD54.

In conclusion, the current findings suggest that the source of FBS is not critical for the h-CLAT. Nevertheless, it is prudent to confirm the suitability of FBS from a new source before conducting the test. We propose the following criteria for the selection of appropriate FBS, DNCB and Ni are positive with both CD86 and CD54 expression over the positive criterion. In contrast, SLS is negative.

Acknowledgements

This study was supported by a Grant-in-Aid from Ministry of Health, Labor and Welfare.

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(Received: February 27/
Accepted: July 31)

Corresponding author:
Ms. Sakiko Sono,
Shiseido Co., Ltd., Quality Assurance Center,
2-12-1, Fukuura, Kanazawa-ku,
Yokohama, 236-8643, Japan
E-mail: sakiko.sono@to.shiseido.co.jp

ORIGINAL ARTICLE

Effects of pre-culture conditions on the human Cell Line Activation Test (h-CLAT) results: Results of the 4th Japanese inter-laboratory study

Makoto Mizuno[1], Mayumi Yoshida[2], Tatsuji Kodama[3], Nanae Kosaka[4], Kenji Okamoto[5], Sakiko Sono[6], Takaaki Yamada[7], Seiji Hasegawa[7], Takao Ashikaga[6], Hirofumi Kuwahara[5], Hitoshi Sakaguchi[4], Jun Sato[3], Naoko Ota[2], Yuko Okamoto[1] and Yasuo Ohno[8]

[1]KOSÉ Corporation, [2]POLA CHEMICAL INDUSTORIES, INC., [3]LION CORPORATION, [4]Kao Corporation, [5]Kanebo Cosmetic Inc., [6]Shiseido Co., Ltd., [7]Nippon Menard Cosmetic Co., Ltd., [8]National Institute of Health Science, Japan

Abstract

The human Cell Line Activation Test (h-CLAT) is an in-vitro skin sensitization method based on the enhancement of CD86 and/or CD54 in THP-1 cells. Experimental conditions for h-CLAT were optimized in our previous study. This protocol defines that THP-1 cells are seeded between $0.1x10^6$ and $0.2x10^6$ cells/mL, and pre-cultured for 48h or 72h before treated with a test chemical. In this study we evaluated effects of pre-culture conditions on the h-CLAT results minutely. We cultivated the cells on nine pre-culture conditions before exposure to allergens (dinitrochlorobenzene (DNCB) and nickel sulfate (Ni)) or non-allergen (sodium lauryl sulfate (SLS)), and then measured CD86 and CD54 expressions on these cells after the exposure. All laboratories almost correctly evaluated the skin sensitization potential of these three chemicals on any pre-culture conditions. However only low CD86 and CD54 RFI values induced by DNCB tend to be obtained as the final cell concentration on pre-culture became higher. For maintaining the response of THP-1 cells to allergens and distinguishing allergens and non-allergens more clearly, THP-1 cells should be avoided being in over-growth conditions during pre-culture. Therefore a supplementary experimental condition about pre-culture for h-CLAT that final cell concentration in pre-culture must not exceed $1.0x10^6$ cells/mL was defined.

Key words : *h-CLAT, skin sensitization, alternatives, THP-1, culture*

Introduction

The guinea pig maximization test (Magnusson and Klingman, 1969) and the local lymph node assay (LLNA) (Kimber *et al.*, 1986) have been universally used to assess the sensitizing potential of chemicals. On the other hand, many researchers have made efforts to develop alternative tests because of animal welfare, time reduction and cost (De Silva *et al.*, 1996). One of the most interested approaches for developing in-vitro sensitization methods is measuring phenotypic changes of dendritic cells such as increase of CD86 or CD54 expression (Aiba *et al.*, 1997), and internalization of MHC class II molecules (Becker *et al.*, 1997). However, their attempts have been restricted due to difficulties in using dendritic cells. It was reported that the response to allergens on the surface phenotype of dendritic cells varies from do-

nor-to-donor (Aiba *et al.*, 1997; Rougier *et al.*, 2000). Moreover, abundant dendritic cells are needed for routinely testing the sensitizing potential of chemicals.

In order to resolve these problems, Ashikaga *et al.* (2002) and Yoshida *et al.* (2003) tried to use THP-1 cells, human acute monocytic leukemia cell line, as a substitute for dendritic cells. They illustrated that surface molecules such as CD86 or CD54 on THP-1 cells were up-regulated when treated with allergens and also showed the possibility that measuring changes in CD86 and/or CD54 expression on THP-1 cells could be used as an in-vitro skin sensitization test method. These two laboratories cooperatively optimized the protocol for this assay and named this method the human cell line activation test (h-CLAT) (Ashikaga *et al.*, 2006). Then an inter-laboratory validation with nine chemicals using this protocol was carried out and both laboratories gave a good prediction of the sensitizing potential (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). It was also reported that h-CLAT could predict sensitization potential of preservatives, which were well-known sensitizers (Sakaguchi *et al.*, 2007). Recently the inter-laboratory study by seven Japanese laboratories was carried out and suggested that the transferability of the protocol and inter-laboratory reproducibility of the test were basically good (Ashikaga *et al.*, 2008).

For a successful submission of h-CLAT to a public center for validation of alternative methods, we need to examine the test conditions in details and improve the test protocol more suitable. The effects of differences of cell lot (Kosaka et al., 2008) and serum source (Sono *et al.*, 2008) on the results of h-CLAT were investigated. In this paper we examined effects of incubation time and initial cell concentration during pre-culture on the result of h-CLAT with three laboratories among seven Japanese laboratories.

Materials and Methods

Collaborating laboratories

Laboratory E: KOSÉ Corporation Fundamental Research Laboratories, F: POLA CHEMICAL INDUSTORIES, INC. Research Laboratories, G: LION CORPORATION Research Laboratories.

Cells and culture

THP-1 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured as described previously (Kosaka *et al.*, 2008). All studies have finished within two months from starting the culture.

Cytotoxicity test

Cell viability was measured by propidium iodide (PI) assay as described previously (Kosaka *et al.*, 2008). The CV75 (μg/mL) (CV75: estimated concentration affording 75% cell viability) value for each test chemical was calculated.

Chemicals and applying doses

Two well-known allergens (dinitrochlorobenzene (DNCB) and nickel sulfate (Ni)) and one non-allergen (sodium lauryl sulfate (SLS)) were evaluated. All chemicals were purchased from SIGMA-ALDRICH (St. Louis, MO, USA) and were dissolved as described previously (Kosaka *et al.*, 2008). In order to determine the applying doses, each laboratory conducted PI-assay and decided the appropriate dose for each chemical individually. In addition, as a positive control in h-CLAT, 5 μg/mL of DNCB was evaluated.

Pre-culture conditions

We evaluated a total of nine pre-culture conditions in this study. THP-1 cells were seeded at 0.1×10^6, 0.2×10^6 or 0.3×10^6 cells/mL, and were cultivated for 24 h, 48 h or 72 h in cell flasks. We started these nine pre-cultures from a cell population simultaneously.

h-CLAT procedure

After pre-culture, THP-1 cells were plated at 1.0×10^6 cells/mL in a 24-well plate and treated for 24 h with test chemicals. The expressions of CD86 and CD54 on the cells were analyzed as described previously (Kosaka *et al.*, 2008). Lab. E and F performed the test with each pre-culture condition three times, and Lab. G performed twice.

Data analysis

Relative Fluorescence Intensity (RFI) was used as an indicator of CD86 and CD54 expression and was calculated as described previously (Kosaka *et al.*, 2008).

The positive criteria for CD86 and CD54 expression were RFI value of 150 and 200, respectively. The RFI value should exceed the positive criteria (CD86 $\geq$ 150 or CD54 $\geq$ 200) in order to be

considered a 'positive' outcome.

When the cell viability was less than 50%, RFI was not calculated. The reason is that the diffuse labeling cytoplasmic structures that occur due to cell membrane destruction will interfere with fluorescent measurements (Becker *et al.*, 1992).

Statistical analysis

Tukey's multiple comparison method was used to analyze data for significant differences. The value was regarded as significant at $p < 0.05$.

Results

Application dose of chemicals

Cytotoxicity tests were conducted at each laboratory at third week from starting cultivation and the CV75 values for each test chemical were calculated. We used these CV75 values for application doses for h-CLAT through this study as a basic rule. However some CV75 values obtained in this cytotoxicity test were thought to be inappropriate for the study referring to the test conditions in our previous study (Ashikaga *et al.*, 2008). Because 200 μg/mL of Ni and 90 μg/mL of SLS for Lab. F were over the doses we had used and enough cytotoxicity had been shown at the concentrations below these values in our previous study, these concentrations were supposed to be too much cytotoxic. It was also supposed that 2.0 μg/mL of DNCB for Lab. F and 49 μg/mL of SLS for Lab. G were lower for the study because these doses had not shown any cytotoxicity in each laboratories at that study. In such cases, the laboratory decided appropriate application doses for h-CLAT taking the CV75 values from the present cytotoxicity test result and our previous study data into consideration. The CV75 values from these cytotoxicity tests and the values that we used as application doses for h-CLAT in this study are shown in Table 1.

Proliferation rate of THP-1 cells during pre-culture

We measured the cell number in culture medium all the time at the end of pre-culture. The average number of each pre-culture condition in each laboratory was calculated and summarized in Table 2. Statistically significant difference of proliferation rate of the cells in some pre-culture conditions between Lab. E and the others was observed.

When cells were seeded at 0.3×10^6 cells/mL, and were cultivated for 72 h, average cell number of each laboratory was 1.67×10^6 cells/mL for Lab. E, 0.82×10^6 cells/mL for Lab. F and 0.82×10^6 cells/mL for Lab. G. All of these average numbers were over 0.8×10^6 cells/mL. As a matter of course the cell concentration at the end of pre-culture tended to increase as seeded cell number increased and culture time became longer. Lab. E, having the largest cell proliferation rate among these laboratories, showed marked this tendency.

It is considered that the initial cell concentration and cultivation time are unrelated the prolif-

Table 1
The values of CV75 (μg/mL) of DNCB, Ni, SLS and the test dose for these chemicals
The values of CV75 of DNCB, Ni and SLS were measured by cytotoxicity test at third week from starting cultivation in each laboratory. Lab. E used CV75 values as test doses. Lab. F and Lab. G decided an appropriate dose of each chemical for h-CLAT taking these CV75 values and the previous study data into consideration individually. The test dose was shown in parentheses at the right side of CV75 only when the dose was different from CV75.

Lab.	Chemicals		
	DNCB	Ni	SLS
	CV75 (μg/mL)		
E	3.8	157	57
F	2.0 (2.5)	200 (100)	90 (60)
G	5.0 (4.6)	153	49 (63)

Table 2
The cell concentration ($x10^6$ cells/mL) at the end of pre-culture
The cell concentration at the end of pre-culture was measured and the average concentration of each pre-culture condition in each laboratory is shown. These average concentrations were calculated from three individual experiments in Lab. E and F, from two experiments in Lab. G. Data are expressed as mean ± SD.
*Indicates a significant difference from the other laboratories with $p < 0.05$ as calculated by Tukey's multiple comparison method.

Lab.	Initial cell concentration ($x10^6$ cells/mL)	Pre-culture time (h) 24h	48h	72h
		Final cell concentration ($x10^6$ cells/mL)		
E	0.1	0.17 ± 0.03	0.32 ± 0.09	0.71 ± 0.14*
	0.2	0.30 ± 0.02	0.61 ± 0.20	1.22 ± 0.17*
	0.3	0.50 ± 0.04	0.91 ± 0.08*	1.67 ± 0.10*
F	0.1	0.15 ± 0.04	0.20 ± 0.03	0.44 ± 0.03
	0.2	0.31 ± 0.10	0.41 ± 0.08	0.54 ± 0.16
	0.3	0.52 ± 0.08	0.55 ± 0.16	0.82 ± 0.30
G	0.1	0.09 ± 0.03	0.19 ± 0.01	0.29 ± 0.08
	0.2	0.26 ± 0.02	0.38 ± 0.01	0.63 ± 0.02
	0.3	0.36 ± 0.04	0.54 ± 0.01	0.82 ± 0.09

eration rate for the cells during exponential phase. However the proliferation rate of THP-1 cells for the first 24 h was slow and the rate increased after 24h. Especially in Lab. G, the proliferation rate during the first 24 h was very slow. This indicated that the cells in the first 24 h period of pre-culture were in lag phase. And also the proliferation rate became smaller as the initial cell concentration became larger at 72h in Lab. E and F. This indicated that the cells in these conditions were in the stationary phase.

The predictivity of sensitization potential for each chemical in each laboratory

We measured RFI values for CD86 and CD54 expression following each pre-culture condition and exposure to chemical, then judged the result as positive if either RFI of CD86 or CD54 was equal to or over the criterion and as negative if both RFI of CD86 and CD54 were below the criterion. The percentage that we predicted the sensitization potential correctly of each chemical in each laboratory was calculated. The percentage of predictivity and the number of experiments are summarized in Table 3. The number of experiment was different from each chemical because the data were excluded from the calculation when cell viability was below 50%.

Lab E and F showed more than 80% of high predictivity of any chemical except 5 μg/mL of DNCB. The predictivity of 5 μg/mL DNCB, which we conducted as a positive control, was 79% for Lab. E and only 64% for Lab. F.

Lab. G showed the perfect predictivities about all chemicals in this study.

Average RFI values and cell viabilities in each Laboratory

We calculated average RFI values for CD86 and CD54, and cell viabilities for each chemical in each laboratory and summarized in Table 4.

About CD86 RFI average values, the value for

Table 3
The predictivity of sensitizing potential for each chemical in each laboratory

Lab.	Chemicals			
	DNCB (CV75)	Ni (CV75)	SLS (CV75)	DNCB (5 μg/mL)
	Predictivity (%)			
E	93% (25/27)	100% (27/27)	100% (23/23)	79% (19/24)
F	92% (23/25)	81% (21/26)	100% (17/17)	67% (14/21)
G	100% (18/18)	100% (18/18)	100% (18/18)	100% (18/18)

RFI values for CD86 and CD54 expression on THP-1 were measured following each pre-culture condition and exposure to chemical. We judged the result as positive if either RFI of CD86 was equal to or more than 150% or RFI of CD54 was equal to or more than 200%, as negative if both RFIs of CD86 and CD54 were below the criterion. The percentage that we predicted the sensitization potential of each chemical correctly was calculated. The numbers of experiment judging correctly and total number of experiment are also shown as a fraction number. In the table, CV75 indicates the test doses using for h-CLAT, does not indicate the concentration obtained the cytotoxicity assay in Table 1.

Table 4
Average RFI values (%) and cell viability (%) of each chemical in each laboratory
Average CD86 and CD54 RFI values and cell viability of each chemical in each laboratory were calculated. Data are expressed as mean ± SD. † Indicates a false negative result. * Indicates a significant difference from the other laboratories, ** indicates a significant difference between these laboratories with $p < 0.05$ as calculated by Tukey's multiple comparison method. In the table, CV75 indicates the test doses using for h-CLAT, does not indicate the concentration obtained the cytotoxicity assay in Table 1.

	Lab.	Chemicals			
		DNCB (CV75)	Ni (CV75)	SLS (CV75)	DNCB (5 μg/mL)
CD86 RFI (%)	E	338 ± 193	223 ± 107	81.6 ± 25.4	233 ± 117
	F	332 ± 144	197 ± 67	69.8 ± 12.4	227 ± 182
	G	347 ± 82	211 ± 43	68.1 ± 10.9	343 ± 83*
CD54 RFI (%)	E	264 ± 140	437 ± 165	99.1 ± 24.9	159 ± 91†
	F	357 ± 180	323 ± 154	101.7 ± 36.7	260 ± 211
	G	537 ± 200*	1754 ± 303*	92.6 ± 22.6	547 ± 215*
viability (%)	E	74.6 ± 7.6	74.5 ± 9.2	75.4 ± 12.1**	69.2 ± 7.3
	F	81.5 ± 8.6*	72.9 ± 8.5	80.7 ± 16.6	69.2 ± 7.0
	G	75.0 ± 7.3	69.0 ± 7.9	87.6 ± 2.9**	75.2 ± 6.5*

5 μg/mL of DNCB in Lab. G were statistically significantly larger than those of the other laboratories. On CV75 of DNCB, Ni and SLS, the values were almost same and no statistically significant difference was observed among three laboratories.

About CD54, all the values by sensitizers in Lab. G were much larger than those of the other laboratories.

About cell viability, the viability for DNCB in Lab. F, for 5 μg/mL of DNCB in Lab. G were statistic

ally significantly larger than those of the other laboratories, and the statistical significant difference of the viability for SLS was observed between Lab. E and G.

CD86 Average RFI value in each pre-culture condition

CD86 average RFI value in each pre-culture condition in each chemical is summarized in Table 5(a).

The RFI average values for CV75 of DNCB

Table 5(a)

Average CD86 RFI values (%) of each pre-culture condition in each laboratory in each chemical

Average RFI values of each pre-culture condition in each laboratory are summarized. Table 5(a) is for CD86. Data are expressed as mean ± SD. The numbers of experiment are shown as a number in parentheses. † Indicates a false negative result. * Indicates a significant difference from the other laboratories, ** indicates a significant difference between these laboratories with $p < 0.05$ as calculated by Tukey's multiple comparison method. In the tables, CV75 indicates the test doses using for h-CLAT, does not indicate the concentration obtained the cytotoxicity assay in Table 1.

(a)

Chemicals	Lab.	Initial cell concentration ($x10^5$ cells/mL)	Pre-culture time (h) 24h		48h		72h	
			CD86 RFI (%)					
DNCB (CV75)	E	0.1	519 ± 185	(3)	532 ± 280	(3)	194 ± 54.7	(3)
		0.2	540 ± 212	(3)	257 ± 45.0	(3)	206 ± 15.9	(3)
		0.3	366 ± 151	(3)	250 ± 109	(3)	175 ± 31.0	(3)
	F	0.1	276 ± 109	(3)	382 ± 134	(3)	544 ± 95.1*	(3)
		0.2	199 ± 125	(3)	269 ± 61.0	(2)	249 ± 119	(3)
		0.3	369 ± 175	(3)	375 ± 101	(2)	318 ± 149	(3)
	G	0.1	425 ± 137	(2)	355 ± 219	(2)	315 ± 10.9	(2)
		0.2	407 ± 60.7	(2)	380 ± 25.4	(2)	307 ± 3.6	(2)
		0.3	339 ± 44.1	(2)	319 ± 29.2	(2)	276 ± 34.0	(2)
Ni (CV75)	E	0.1	181 ± 35.8	(3)	368 ± 197	(3)	200 ± 56.0	(3)
		0.2	153 ± 128	(3)	212 ± 91.3	(3)	187 ± 58.9	(3)
		0.3	298 ± 77.1	(3)	152 ± 14.4**	(3)	251 ± 93.2	(3)
	F	0.1	186 ± 57.9	(3)	233 ± 19.2	(3)	271 ± 17.0	(3)
		0.2	153 ± 50.2	(3)	117 ± 46.0†	(3)	132 ± 55.6†	(3)
		0.3	227 ± 85.8	(3)	264 ± 43.8**	(2)	210 ± 36.1	(3)
	G	0.1	258 ± 17.1	(2)	227 ± 42.0	(2)	178 ± 12.7	(2)
		0.2	248 ± 43.2	(2)	182 ± 2.6	(2)	192 ± 7.9	(2)
		0.3	255 ± 43.9	(2)	208 ± 47.3	(2)	153 ± 16.5	(2)
SLS (CV75)	E	0.1	116 ± 0.9	(2)	63.9 ± 23.2	(2)	91.7 ± 31.4	(3)
		0.2	86.8 ± 13.9	(2)	63.8 ± 30.2	(3)	86.0 ± 18.8	(3)
		0.3	64.8 ± 19.9	(2)	79.5 ± 25.9	(3)	81.4 ± 19.7	(3)
	F	0.1	61.4 ± 5.6	(2)	67.8 ± 6.4	(2)	82.2 ± 28.8	(2)
		0.2	61.1 ± 8.0	(2)	65.0 ± 12.1	(2)	70.3 ± 22.9	(2)
		0.3	72.4 ± 0.8	(2)	67.6	(1)	78.9 ± 1.9	(2)
	G	0.1	79.4 ± 13.4	(2)	71.8 ± 12.8	(2)	64.7 ± 3.4	(2)
		0.2	76.9 ± 5.9	(2)	66.5 ± 2.0	(2)	61.0 ± 7.6	(2)
		0.3	78.9 ± 9.0	(2)	59.8 ± 0.1	(2)	53.4 ± 10.2	(2)
DNCB (5 μg/mL)	E	0.1	354 ± 126	(3)	278 ± 29.1	(3)	143 ± 48.5†	(3)
		0.2	386 ± 149	(3)	167 ± 64.0	(3)	153 ± 3.9	(2)
		0.3	234 ± 107	(3)	150 ± 23.3	(2)	145 ± 84.2†	(2)
	F	0.1	188 ± 40	(2)	265 ± 225	(3)	225 ± 356	(2)
		0.2	160 ± 96	(3)	91 ± 21.3†	(2)	266 ± 218	(3)
		0.3	138†	(1)	147 ± 62.0†	(2)	341 ± 292	(3)
	G	0.1	427 ± 103	(2)	381 ± 125	(2)	263 ± 96.4	(2)
		0.2	421 ± 43.9	(2)	362 ± 20.7*	(2)	285 ± 4.4	(2)
		0.3	381 ± 24.9	(2)	287 ± 100	(2)	284 ± 45.6	(2)

in Lab. E and G tended to be lower as the initial cell concentration became higher and culture time became longer. In Lab. F, the values for CV75 of DNCB were lower than the values of other initial cell concentrations when cells were seeded at $0.2x10^6$ cells/mL.

About Ni, there was not any characteristic trend in Lab. E. In Lab. F, the values for Ni were lower than those of other initial cell concentrations when cells were seeded at $0.2x10^6$ cells/mL. The values for Ni in Lab. G tended to be lower as the initial cell concentration became higher and culture

Table 5(b)
Average CD86 RFI values (%) of each pre-culture condition in each laboratory in each chemical
Average RFI values of each pre-culture condition in each laboratory are summarized. Table 5(b) is for CD54. Data are expressed as mean ± SD. The numbers of experiment are shown as a number in parentheses. † Indicates a false negative result. * Indicates a significant difference from the other laboratories, ** indicates a significant difference between these laboratories with $p < 0.05$ as calculated by Tukey's multiple comparison method. In the tables, CV75 indicates the test doses using for h-CLAT, does not indicate the concentration obtained the cytotoxicity assay in Table 1.

(b)

Chemicals	Lab.	Initial cell concentration ($x10^6$ cells/mL)	Pre-culture time (h) 24h		Pre-culture time (h) 48h		Pre-culture time (h) 72h	
			CD54 RFI (%)					
DNCB (CV75)	E	0.1	415 ± 79.1	(3)	394 ± 161	(3)	132 ± 84.0†	(3)
		0.2	393 ± 26.3	(3)	250 ± 106	(3)	132 ± 22.7†	(3)
		0.3	275 ± 176	(3)	264 ± 17.2	(3)	117 ± 22.2**†	(3)
	F	0.1	299 ± 152**	(3)	457 ± 327	(3)	489 ± 200	(3)
		0.2	322 ± 206	(3)	407 ± 128	(2)	320 ± 252	(3)
		0.3	342 ± 95.2	(3)	361 ± 160	(2)	238 ± 100	(3)
	G	0.1	830 ± 264**	(2)	579 ± 69.9	(2)	444 ± 214	(2)
		0.2	662 ± 55.2	(2)	612 ± 179	(2)	269 ± 55.6	(2)
		0.3	591 ± 211	(2)	427 ± 96.5	(2)	419 ± 168**	(2)
Ni (CV75)	E	0.1	365 ± 93.1	(3)	591 ± 263	(3)	556 ± 148	(3)
		0.2	378 ± 139	(3)	495 ± 240	(3)	386 ± 98.6	(3)
		0.3	427 ± 222	(3)	388 ± 144	(3)	348 ± 50.5	(3)
	F	0.1	274 ± 103	(3)	389 ± 198	(3)	416 ± 227	(3)
		0.2	262 ± 97.8	(3)	310 ± 201	(3)	206 ± 130	(3)
		0.3	288 ± 115	(3)	411 ± 192	(2)	383 ± 177	(3)
	G	0.1	1964 ± 118*	(2)	2026 ± 373*	(2)	1589 ± 4.4*	(2)
		0.2	1615 ± 322*	(2)	1950 ± 83.3*	(2)	1549 ± 216*	(2)
		0.3	1841 ± 183*	(2)	1826 ± 637*	(2)	1424 ± 273*	(2)
SLS (CV75)	E	0.1	91.7 ± 13.8	(2)	126 ± 34.8	(2)	117 ± 27.1	(3)
		0.2	86.0 ± 16.6	(2)	112 ± 13.0	(3)	113 ± 11.9	(3)
		0.3	81.4 ± 6.1	(2)	76.7 ± 30.9	(3)	84.7 ± 24.8	(3)
	F	0.1	94.6 ± 37.8	(2)	110 ± 41.4	(2)	142 ± 44.5	(2)
		0.2	94.9 ± 30.6	(2)	107 ± 50.6	(2)	66.3 ± 29.2	(2)
		0.3	97.5 ± 61.2	(2)	139	(1)	82.9 ± 5.8	(2)
	G	0.1	118 ± 59.5	(2)	107 ± 7.9	(2)	94.1 ± 16.5	(2)
		0.2	85.7 ± 27.7	(2)	99.4 ± 14.4	(2)	76.6 ± 7.7	(2)
		0.3	90.2 ± 1.9	(2)	93.5 ± 13.7	(2)	68.9 ± 1.4	(2)
DNCB (5 μg/mL)	E	0.1	256 ± 103	(3)	234 ± 125	(3)	104 ± 26.9†	(3)
		0.2	190 ± 16.2†	(3)	123 ± 78.1†	(3)	71 ± 33.1†	(2)
		0.3	175 ± 106**†	(3)	149 ± 58.7†	(2)	65 ± 5.6†	(2)
	F	0.1	324 ± 15.3	(2)	377 ± 505	(3)	150 ± 190	(2)
		0.2	222 ± 151	(3)	103 ± 67.0†	(2)	269 ± 212	(3)
		0.3	395	(1)	111 ± 82.2†	(2)	310 ± 108	(3)
	G	0.1	782 ± 53.5*	(2)	647 ± 163	(2)	435 ± 294	(2)
		0.2	692 ± 82.7*	(2)	689 ± 283**	(2)	281 ± 46.9	(2)
		0.3	650 ± 150.6**	(2)	344 ± 19.0	(2)	400 ± 167	(2)

time became longer.

There was not any characteristic trend in Lab. E and F about SLS. The values for SLS in Lab. G tended to be lower as the initial cell concentration became higher and culture time became longer.

For 5 μg/mL of DNCB, the RFI average values in Lab. E and G tended to be lower as the initial cell concentration became higher and culture time became longer. When pre-cultivated for 72 h, two average CD86 values of nine conditions didn't reach the criterion of 150% in Lab. E. There was not any characteristic trend in Lab. F.

Some statistically significant differences between laboratories in some pre-culture conditions were observed, but no statistically significant difference between pre-culture conditions in any laboratories was observed in these results.

CD54 Average RFI value in each pre-culture condition

CD54 average RFI value in each pre-culture condition in each chemical is summarized in Table 5(b).

The RFI average values for CV75 of DNCB in Lab. E and G tended to be lower as the initial cell concentration became higher and culture time

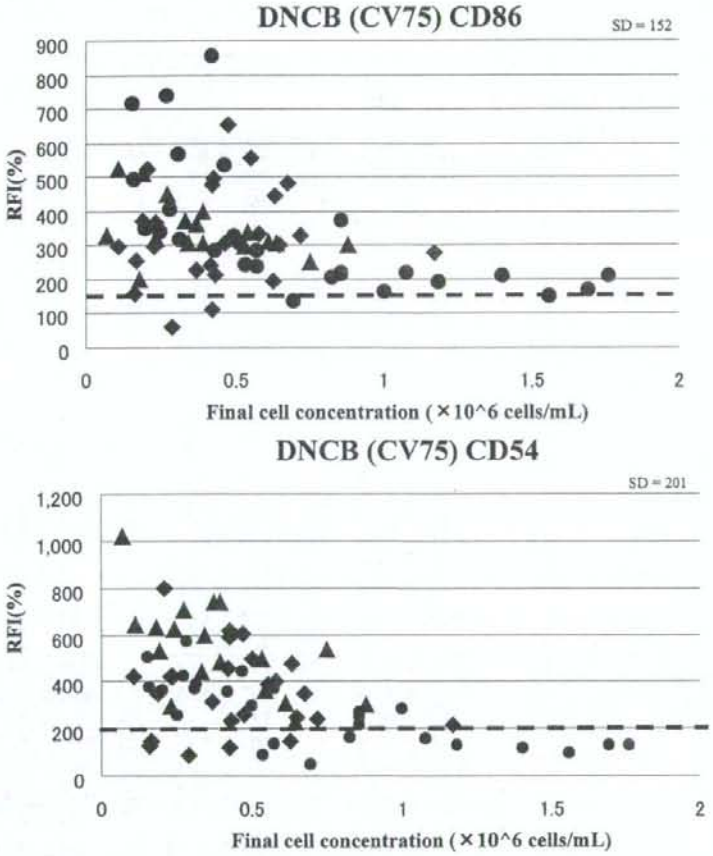

Fig. 1(a, b)
The relation between final cell concentration (x10^5 cells/mL) in pre-culture and RFI values (%)
Final cell concentration in pre-culture and RFI values of three laboratories for each chemical are shown. Fig. 1(a) is for DNCB CD86, 1(b) is for DNCB CD54. The total number of experiments is 70. Standard deviation of RFI is shown above each figure. Small dotted line shows positive criteria of CD86 or CD54. In the figures, CV75 indicates the test doses using for h-CLAT, does not indicate the concentration obtained the cytotoxicity assay in Table 1.
●: the data of Lab. E; ◆: the data of Lab. F; ▲: the data of Lab. G

became longer. When pre-cultivated for 72 h, CD54 average RFI values in Lab. E always did not reach the criterion of 200%, not even 150%. In Lab. F, CD54 RFI values for CV75 of DNCB tended to be lower when shorter pre-incubation with lower initial cell concentration or longer pre-incubation with higher initial cell concentration.

About Ni, the values in Lab. E tended to be lower when shorter pre-incubation with lower initial cell concentration or longer pre-incubation with higher initial cell concentration. In Lab. F, the values were lower than those of other initial cell concentrations when cells were seeded at $0.2x10^6$ cells/mL. The values for Ni in Lab. G tended to be lower as the initial cell concentration became higher and culture time became longer.

The values for CV75 of SLS in Lab. E tended to be lower as the initial cell concentration became lower and culture time became longer. In Lab. F, there was not any characteristic trend in CD54 values for SLS. The values in Lab. G tended to be lower as the initial cell concentration became higher and culture time became longer.

The values for 5 μg/mL of DNCB in Lab. E and G tended to be lower as the initial cell concen-

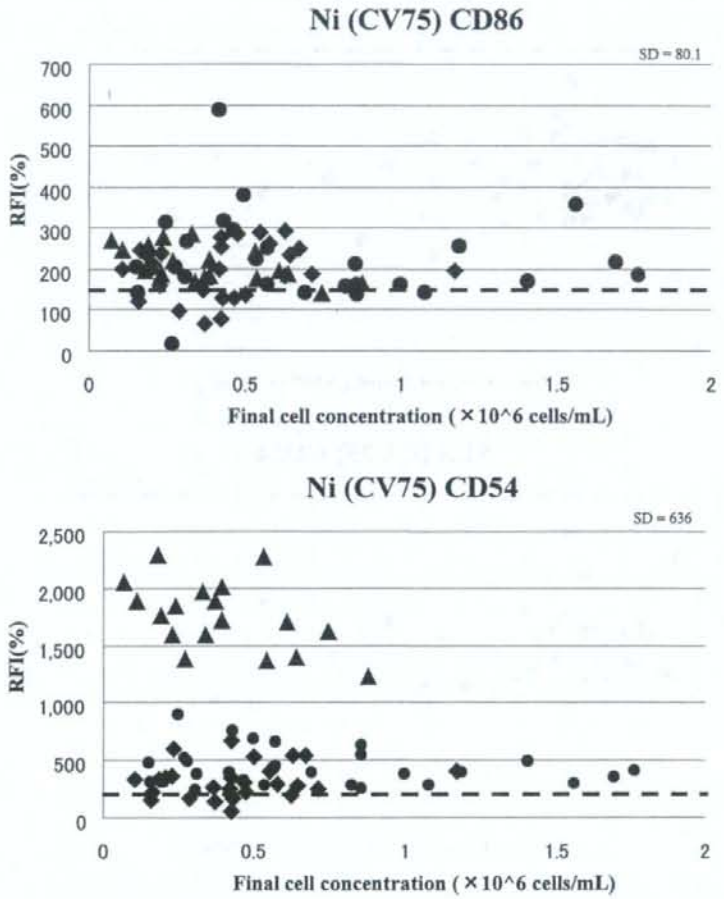

Fig. 1(c, d)
The relation between final cell concentration ($x10^6$ cells/mL) in pre-culture and RFI values (%)
Final cell concentration in pre-culture and RFI values of three laboratories for each chemical are shown. Fig. 1(c) is for Ni CD86, 1(d) is for NI CD54. The total number of experiments is 71. Standard deviation of RFI is shown above each figure. Small dotted line shows positive criteria of CD86 or CD54. In the figures, CV75 indicates the test doses using for h-CLAT, does not indicate the concentration obtained the cytotoxicity assay in Table 1.

●: the data of Lab. E; ◆: the data of Lab. F; ▲: the data of Lab. G

tration became higher and culture time became longer. In Lab. E, only two CD54 RFI average values of nine pre-culture conditions reached the criterion. There was not any characteristic trend in Lab. F.

Some statistically significant differences between laboratories in some pre-culture conditions were observed, but no statistically significant difference between pre-culture conditions in any laboratories was observed in these results.

The relation between final cell concentration in pre-culture and RFI values

We took the data of nine pre-culture conditions in three laboratories together and evaluated the relation between final cell concentration in pre-culture and RFI values for each chemical. The data were shown in Fig. 1(a) for DNCB CD86, 1(b) for DNCB CD54, 1(c) for Ni CD86, 1(d) for Ni CD54, 1(e) for SLS CD86, 1(f) for SLS CD54, 1(g) for 5 μg/mL of DNCB CD86 and 1(h) for 5 μg/mL of DNCB CD54.

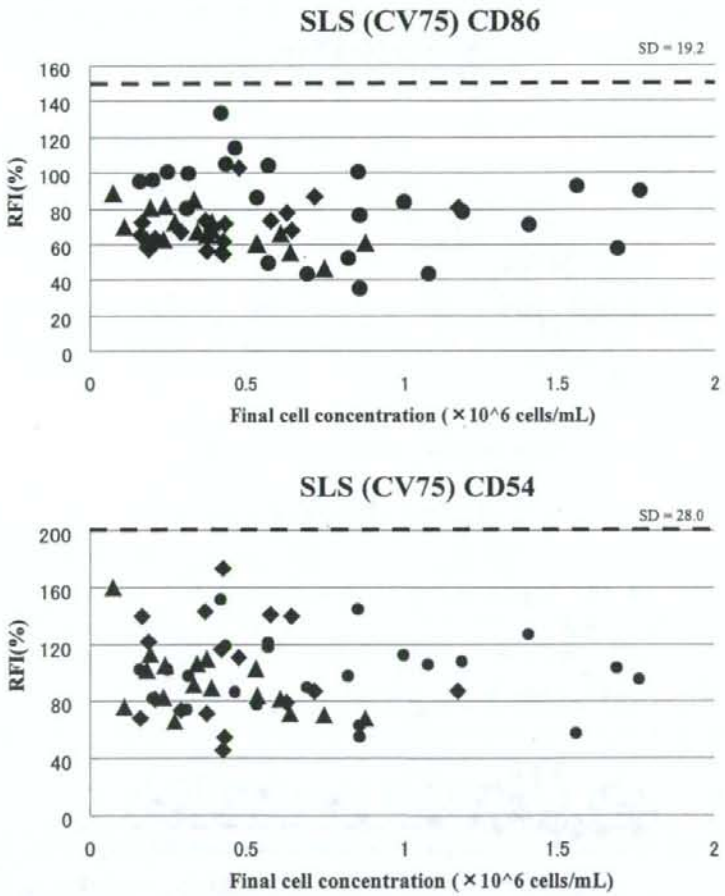

Fig. 1(e, f)
The relation between final cell concentration (x10^6 cells/mL) in pre-culture and RFI values (%)
Final cell concentration in pre-culture and RFI values of three laboratories for each chemical are shown. Fig. 1(e) is for SLS CD86. The total number of experiments is 58. Standard deviation of RFI is shown above each figure. Small dotted line shows positive criteria of CD86 or CD54. In the figures, CV75 indicates the test doses using for h-CLAT, does not indicate the concentration obtained the cytotoxicity assay in Table 1.

●: the data of Lab. E; ◆: the data of Lab. F; ▲: the data of Lab. G

About CD86 and CD54 RFI values induced by CV75 of DNCB (Fig. 1(a) and 1(b)) and 5 μg/mL of DNCB (Fig. 1(g) and 1(h)), only low RFI values tended to be observed as final cell concentration in pre-culture became higher. But the significant correlation between final cell concentration in pre-culture and RFI values was not observed. The coefficient of determination (R^2) was only 0.13 in Fig. 1(a), 0.23 in Fig. 1(b), 0.14 in Fig. 1(g), 0.25 in Fig. 1(h). A similar tendency was also shown in Fig. 1(d) for CD54 RFI induced by CV75 of Ni. But obvious difference in RFI values between Lab. G and other laboratories was seen and this difference was statistically significant. The other chemicals did not show this tendency.

We also evaluated the relation between cell proliferation rate and RFI values. But there was not any tendency in all chemicals (data not shown).

Discussion

Ashikaga *et al.* have already examined h-CLAT conditions for each experimental step including pre-culture and optimized the protocol. They studied the effect of pre-culture time on CD86 expres-

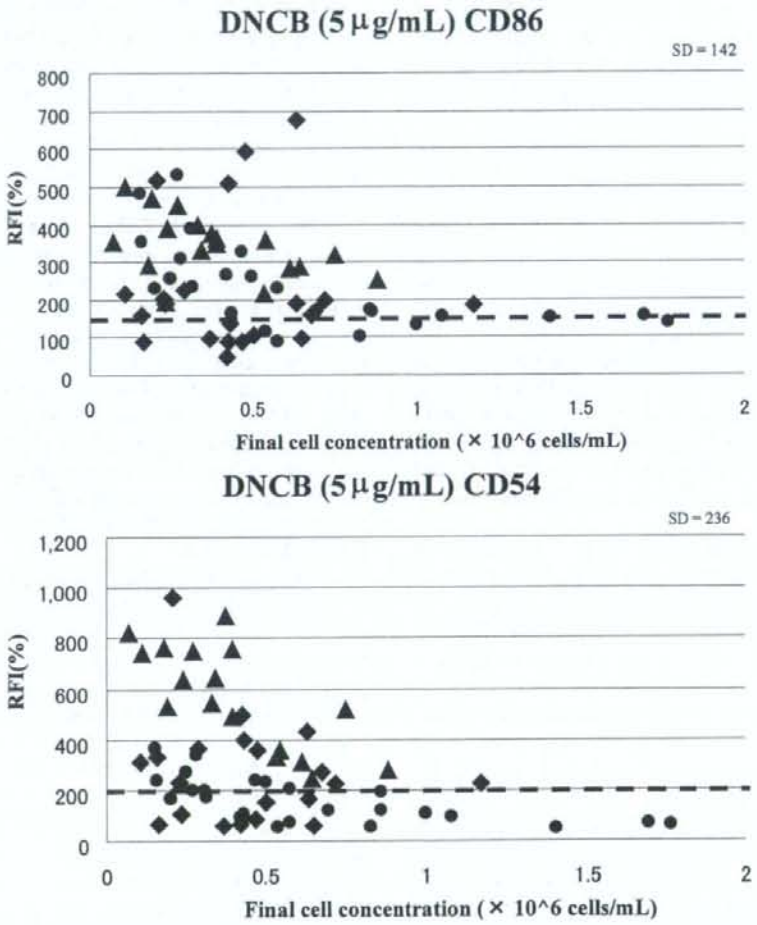

Fig. 1(g, h)
The relation between final cell concentration (x10^6 cells/mL) in pre-culture and RFI values (%)
Final cell concentration in pre-culture and RFI values of three laboratories for each chemical are shown. Fig. 1(g) is for 5μg/mL of DNCB CD86 and 1(h) is for 5μg/mL of DNCB CD54. The total number of experiments is 63. Standard deviation of RFI is shown above each figure. Small dotted line shows positive criteria of CD86 or CD54. In the figures, CV75 indicates the test doses using for h-CLAT, does not indicate the concentration obtained the cytotoxicity assay in Table 1.

●: the data of Lab. E; ◆: the data of Lab. F; ▲: the data of Lab. G

sion by 3 μg/mL DNCB and showed that 48 h and 72 h pre-cultivation induced maximum CD86 expression. From this result, they defined the protocol that THP-1 cells are seeded at between $0.1x10^6$ and $0.2x10^6$ cells/mL and pre-cultured for 48 h or 72 h before treated with a test chemical. In the study they used only one initial cell concentration of $0.4x10^6$ cells/mL and only one chemical of DNCB, so have not investigated factors such as the effect of initial cell concentration the protocol defines, the result of CD54 expression and the result by other chemicals.

Therefore, we investigated effects of pre-culture conditions on the test results of not only CD86 RFI value but also CD54 RFI value in detail using three chemicals and changing initial cell concentration and culture time in this study.

Some of CV75 values obtained at third week were much different from our previous test data. We could not use these CV75 values for the study and decided some test doses referring our previous data. Kosaka *et al.* (2008) also indicated that CV75 values of DNCB in some cells lots obtained at third week were larger than that at fourth week. The values at third week were too toxic for h-CLAT and they re-examined PI assay at fourth week and used this new CV75 in their study. In this study it is thought that the cells at third week, particularly in Lab. F, had not yet been stable completely. It is considered that it is important to use the cells in stable conditions for cytotoxicity assay and h-CLAT.

All laboratories almost correctly evaluated the skin sensitizing potential of DNCB, Ni and SLS in this study. But the predictivity of 5 μg/mL of DNCB was not so good in Lab. E and F. The test dose for CV75 of DNCB at each laboratory was 3.8 μg/mL for Lab. E, 2.5 μg/mL for Lab. F and 4.6 μg/mL for Lab. G, and the average cell viabilities for 5 μg/mL of DNCB in Lab. E and F were same as 69.2% although that of Lab. G was 75.2%. The difference of average cell viability between Lab. G and the others was statistically significant. These average viabilities did not include the data when cell viability was below 50%, and the viability did not reached 50% 3 times of 27 tests in Lab. E, 4 times of 25 tests in Lab. F and never of 18 tests in Lab. G. The total number of experiments for 5.0 μg/mL of DNCB in Lab. F was 25, not 27 because Lab. F couldn't obtain the data of DNCB twice with the instrumental trouble. These data indicated clearly that DNCB showed stronger cytotoxicity to the cells in Lab. E and F compared to the cell in Lab. G. Sakaguchi *et al.* (in press) described that positive response of DNCB was obtained at the range of 70-87% cell viability in their article. It is thought that one of the reasons of this low predictivity in Lab. E and F was that the concentration of 5.0μg/mL DNCB was too toxic to the cells and not appropriate test dose to assess the sensitizing potential for these laboratories. Now it is considered that we have a need to re-evaluate whether 5.0 μg/mL of DNCB is an appropriate concentration for positive control.

Statistically significant difference of CD54 average RFI values by sensitizers between Lab. G and the other laboratories were shown in this study. It is supposed that there was something different in cell conditions between Lab. G and the others, but we could not clarify this different cell condition at all and further investigations will be necessary in order to explain the reason for this difference.

The cells in Lab. E showed rapid proliferation among these laboratories through the study and statistically significant difference of average cell number at the end of pre-culture between Lab. E and the other laboratories were observed in some pre-culture conditions. But the significant differences of predictivity of sensitization potential, average RFI values, cell viabilities and CD86 and CD54 RFI values between Lab. E and the others were not shown. These results suggested that the cell proliferation rate during pre-culture have little effect on assessing the sensitizing potential of chemicals in h-CLAT.

From the whole data we tested on CV75 or 5.0 μg/mL of DNCB in this study, it was observed that only lower CD86 and CD54 RFI values tend to be obtained as the initial cell concentration in pre-culture becomes higher and pre-culture time becomes longer, in other words, as the final cell concentration in pre-culture becomes higher. Whenever final cell concentration in pre-culture was over $1.0x10^6$ cells/mL, it means the cells were in over-growth conditions, both CD 86 and CD54 RFI values for CV75 or 5.0 μg/mL of DNCB were low and never exceeded 300%. It was indicated that high RFI values for CV75 or 5.0 μg/mL of DNCB are obtained only when cell density at the end of pre-culture is not high. But the correlation between final cell concentration in pre-culture and RFI values was not observed. This reason is that the low response to DNCB were sometimes seen when cell density at the end of pre-culture was low.

Therefore it was thought that low cell density during pre-culture is necessary for keeping effective response to allergens, but it is not a sufficient condition for good response. It is well known that one of the other factors that effects this response is cell viability in h-CLAT (Ashikaga *et. al.*, 2002; Yoshida *et al.*, 2003; Sakaguchi *et al.*, 2006).

The product information sheet for THP-1 cells by ATCC shows that the cells should be maintained at between $0.5x10^5$ cells/mL and $0.8x10^6$ cells/mL, and do not allow the cell concentration to exceed $1.0x10^6$ cells/mL. It was thought that the high cell density, there were some cases during pre-culture in this study, is an abnormal culture condition for the cells. Although this product information sheet also mentions that the general doubling time of THP-1 is approximately 26 h, the cells indicated the variable of cell doubling time in the present study. The cells seeded at $0.2x10^6$ cells/mL and cultivated for 72 h according to the h-CLAT protocol were sometimes in over-growth condition. Charrad *et al.* (2002) and Berges *et al.* (2005) reported that terminal differentiation of acute myeloid leukemia cells is related to the inhibition of proliferation. The condition with the high cell density, in which cell growth is partially inhibited, might cause this different reactivity. However it was not made clear in this study why the response to sensitizers decreased in such conditions especially at the points of cell maturation and differentiation. Further study is required to resolve this phenomenon.

For maintaining the response of THP-1 cells to allergens and distinguishing allergens and non-allergens more clearly, THP-1 cells should be avoided being in over-growth conditions during pre-culture although cultivation with test chemicals in over-growth conditions are required in h-CLAT. Therefore a supplementary experimental condition about pre-culture for h-CLAT that final cell concentration in pre-culture must not exceed $1.0x10^6$ cells/mL was defined.

Acknowledgements

This study was supported by a Grant-in-Aid from Ministry of Health, Labor and Welfare.

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