

Prediction of preservative sensitization potential using surface marker CD86 and/or CD54 expression on human cell line, THP-1

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Abstract Preservatives are important components in many products, but have a history of purported allergy. Several assays [e.g., guinea pig maximization test (GPMT), local lymph node assay (LLNA)] are used to evaluate allergy potential of preservatives. We recently developed the human Cell Line Activation Test (h-CLAT), an in vitro skin sensitization test using human THP-1 cells. This test evaluates the augmentation of CD86 and CD54 expression, which are key events in the sensitization process, as an indicator of allergy following treatment with test chemical. Earlier, we found that a sub-toxic concentration was needed for the up-regulation of surface marker expression. In this study, we further evaluate the capability of h-CLAT to predict allergy potential using eight preservatives. Cytotoxicity was determined using propidium iodide with flow cytometry analysis and five doses that produce a 95, 85, 75, 65, and 50% cell viability were selected. If a material did not have any cytotoxicity at the highest technical dose (HTD), five doses are set using serial 1.3 dilutions of the HTD. The test materials used were six known allergic preservatives (e.g., methylchloroisothiazolinone/methylisothiazolinone, formaldehyde), and two non-allergic preservatives (methylparaben and 4-hydroxybenzoic acid). All allergic preservatives augmented CD86 and/or CD54 expression, indicating h-CLAT correctly identified the allergens. No augmentation was observed with the

non-allergic preservatives; also correctly identified by h-CLAT. In addition, we report two threshold concentrations that may be used to categorize skin sensitization potency like the LLNA estimated concentration that yield a three-fold stimulation (EC3) value. These corresponding values are the estimated concentration which gives a relative fluorescence intensity (RFI) = 150 for CD86 and an RFI = 200 for CD54. These data suggest that h-CLAT, using THP-1 cells, may be able to predict the allergy potential of preservatives and possibility classify the potency of an allergen.

Keywords Preservative · THP-1 · h-CLAT · CD86 · CD54 · In vitro skin sensitization test

Abbreviations

CV75	Cell viability 75% concentration
DCs	Dendritic cells
DMSO	Dimethyl sulfoxide
DNCB	2,4-Dinitrochlorobenzene
EC3	Estimated concentration that yield a three-fold stimulation
FcR	Fc receptor
GPMT	Guinea pig maximization test
h-CLAT	Human Cell Line Activation Test
HPC	Hematopoietic progenitor cell
HTD	Highest technical dose
LC	Langerhans cell
LLNA	Local lymph node assay
mAb	Monoclonal antibody
MCI/MI	Methylchloroisothiazolinone/ methylisothiazolinone
MDBGN	Methyldibromo gutaronitrile

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MFI	Mean fluorescence intensity
MHC class II	Class II major histocompatibility complex antigen
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
PBMC	Peripheral blood mononuclear cell
PI	Propidium iodide
RFI	Relative fluorescence intensity
SDS	Sodium dodecyl sulfate

Introduction

Preservatives are an indispensable material to prevent bacterial and fungal contamination of consumer products. Although indispensable, preservatives have the potential to be sensitizers and are common allergens in cosmetics and toiletries [27]. In the 1980s, an outbreak of contact dermatitis was observed in many European countries due to the introduction of methylchloroisothiazolinone/methylisothiazolinone (MCI/MI) [17]. In the mid-1980s, methylidibromo glutaronitrile (MDBGN) was introduced to the European market as a replacement for MCI/MI. However, a significant increase in contact dermatitis has also been reported with the introduction of MDBGN in Europe [28]. Fortunately, the outbreaks were contained and new regulations were instituted that limit the use of these specific preservatives. To launch a new preservative, evaluation of the skin sensitization potential is one important component of the overall safety assessment process. Until now, guinea pig models were the main methods used for prediction of skin sensitization. Recently, an alternate method, the local lymph node assay (LLNA), has become a popular method. Several investigators have shown the LLNA to be a useful alternative skin sensitization test [6]. Using the LLNA method, the above preservatives (MCI/MI and MDBGN) were predicted as extreme and moderate sensitizers, respectively. But more recently, the seventh amendment of the EU requires in vitro testing methods to be developed for skin sensitization and a greater emphasis is being placed on non-animal skin sensitization tests due to animal welfare concerns. Since LLNA is not a replacement of animal testing, but rather a reduction and refinement of animal testing, an alternative non-animal testing protocol for skin sensitization is still needed.

In order to meet the recent regulatory changes, more non-animal testing methods are being developed. Some researchers have evaluated cell based methods

using human CD14⁺ peripheral blood mononuclear cell (PBMC), CD34⁺ hematopoietic progenitor cell (HPC), and some human cell lines in the development of an alternative skin sensitization test (1, 2, 3, 4, 9, 10, 11, 16, 20, 24, 25, 26, 29). Most of these methods focus on evaluating the cellular changes of dendritic-like cells (DCs) involved in the sensitization process. During this process, immature DCs, e.g., Langerhans cells—the allergen presenting cells, travel to secondary lymphoid organs to sensitize naïve T cells. The immature DCs, along the way, become mature dendritic cells with substantial phenotypic changes that include augmentation of various intercellular adhesion molecules, co-stimulatory molecules, and major histocompatibility complex (MHC) class II antigens (i.e., CD54, CD86, HLA-DR antigen, and other markers) [18]. In the experiments using human blood cells, some allergens such as 2,4-dinitrochlorobenzene (DNCB), but not irritants such as sodium dodecyl sulfate (SDS), significantly increased the surface expression of CD54 (an intercellular adhesion molecule), CD86 (a co-stimulatory molecule), and HLA-DR antigen [2].

We proposed a new in vitro skin sensitization test (calling it—the human Cell Line Activation Test or h-CLAT) using a human monocytic leukemia cell line, THP-1 cells [4]. In the h-CLAT, we evaluate the alteration of CD86/CD54 expression on the surface of THP-1 cells after a 24 h exposure to test chemicals based on the above mechanism. Using this procedure, we obtained a good correlation between h-CLAT and in vivo guinea pig data for skin sensitization potential in an earlier study [24]. The h-CLAT correctly identified the six known allergens (e.g., DNCB) and the three non-allergens (e.g., SDS) tested. The promising results lead us to further evaluate other classes of materials as well optimize the assay.

In the current study, we evaluated eight preservatives that had been classified for their skin sensitization potential by LLNA and/or guinea pig method. For allergic preservatives, we chose MCI/MI, formaldehyde, and MDBGN that were classified as an extreme, strong, and moderate sensitizer by LLNA and reported as high “European average” sensitivity rates [28]. Three other allergic preservatives (imidazolidinyl urea, diazolidinyl urea, and 2-bromo-2-nitropropane-1,3-diol) were selected because they were formaldehyde-releasing preservatives. For non-allergic preservatives, methylparaben and 4-hydroxybenzoic acid were selected because they were not classified as a sensitizer by LLNA [14, 22]. Our goal was to evaluate whether the h-CLAT could predict the allergy potential of these preservatives and whether we could establish a categorization scale for skin sensitization potential. In

addition, suitable test dose settings and appropriate criteria were evaluated.

Materials and methods

Cells and medium

THP-1 cells from American Type Culture Collection (ATCC, Manassas, VA, USA) were cultured in RPMI-1640 (Invitrogen Corp., Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; JRH Biosciences, Lenexa, KS, USA), and 0.05 mM 2-mercaptoethanol and 1% of antibiotic-antimycotic (Invitrogen Corp., Carlsbad, CA, USA). Passage number of the THP-1 cells was monitored and new colonies were set up every 2 months in order to minimize variability. Cell morphology and replication rate remained constant during and between the two-month periods.

Chemicals

Allergens employed in the experiment were: 5-chloro-2-methyl-4-isothiazolin-3-one (methylchloroisothiazolinone/methylisothiazolinone; MCI/MI), formaldehyde (FA), 1,2-dibromo-2,4-dicyanobutane (methylidibromoglutaronitrile; MDBGN), imidazolidinyl urea (IU), diazolidinyl urea (DU), and 2-bromo-2-nitropropane-1,3-diol (BN). The non-allergens were methyl-4-hydroxybenzoate (methylparaben; MP) and 4-hydroxybenzoic acid (4-HBA). All tested chemicals except MDBGN were purchased from Sigma–Aldrich (St. Louis, MO, USA). MDBGN was purchased from Lancaster (Morecambe, UK). MCI/MI, FA, IU, DU, and BN were first solubilized in saline, while MDBGN, MP, and 4-HBA were solubilized in dimethyl sulfoxide (DMSO). The final concentration of DMSO in culture media was always less than 0.2%. Globlins Cohn fraction II and III was obtained from SIGMA-Aldrich as an Fc receptor (FcR) blocking agent.

Pre-study cytotoxicity test (PI assay)

THP-1 cells were cultured in 24-well plates (1.0×10^6 cells/1 mL/well) or 96-well plates (1.6×10^5 cells/160 μ L/well) with various concentrations of each chemical for 24 h. Cells were washed twice, then stained with propidium iodide (PI, 0.625 μ g/mL as the final concentration), and cell viability was measured by using flow cytometry. Total events for living cell counting were 10,000. At least two independent experiments

were done and the mean cell viability value of each dose was used to calculate the concentration, which produced 95, 85, 75, 65, and 50% cell viability (respectively, CV95, CV85, CV75, CV65, and CV50). If a chemical did not have any cytotoxicity at the highest technical dose (HTD), five doses are set using serial 1.3 dilutions of the HTD. The HTD is set at 1,000 μ g/mL due to the maximum solubilization of a chemical in DMSO and the maximum dose of DMSO in cell culture medium. The cell viability determined in the pre-study cytotoxicity test was termed the “Estimated Cell Viability”. Cytotoxicity was also measured in conjunction with measurement of the relative fluorescence intensity (RFI), as calculated from mean fluorescence intensity (MFI). The cell viability determined during the measurement of surface marker expression was termed “Actual Cell Viability” and was compared to the “Estimated Cell Viability” or CV95, CV85, CV75, etc. See section entitled “Flow cytometric analysis and mAbs” for method.

Chemical treatment of THP-1

THP-1 cells were plated at 1×10^6 cells/mL and treated for 24 h with five concentrations of each chemical; concentrations, except for 4-HBA, were based on the estimated cell viability of 95% (CV95), 85% (CV85), 75% (CV75), 65% (CV65), and 50% (CV50). Since the HTD for 4-HBA (1,000 μ g/mL in DMSO vehicle) was not cytotoxic, the concentrations evaluated were 1,000, 769, 592, 455 and 350 μ g/mL (1.3 serial dilutions of the HTD). The test concentrations used for each test chemical are listed in Table 1. Once treated, FcR blocking procedure was conducted: 0.01% of Globlins Cohn fractions II and III were added for 10 min on 4°C. Cells were then stained with antibodies. At least three independent experiments were done for each concentration.

Flow cytometric analysis and mAbs

After treatment with chemicals for 24 h, the fluorescence intensities of the THP-1 cell surface markers were analyzed by flow cytometry (FACS Calibur Cell Quest, Becton Dickinson, San Jose, CA, USA). Cell staining was performed using the following FITC-conjugated monoclonal antibodies (mAbs): anti-human CD54 (clone; 6.5B5) from DAKO (Glostrup, Denmark), anti-human CD86 (clone; Fun-1) from BD-PharMingen (San Diego, CA, USA); FITC labeled-mouse IgG1 (clone; DAK-G01) from DAKO. Using the manufacturer’s recommended dilutions, cells were incubated with the above mAbs at 6 μ L/ 3×10^5 cells/50 μ L for the

Table 1 The test concentrations (µg/mL) used for each test chemical

	CV95	CV85	CV75	CV65	CV50
MCI/MI (act.)	1.9	2.4	3.0	3.8	4.7
Formaldehyde (act.)	3.5	6.1	6.8	7.7	9.1
MDBGN	4	8	9	10	13
Imidazolidinyl Urea	31	35	39	44	53
Diazolidinyl urea	16	18	20	23	27
2-Bromo-2-nitropropane-1, 3-diol	5	15	20	26	34
Methylparaben	88	283	373	492	608
4-Hydroxybenzoic acid*	350	455	592	769	1000

THP-1 cells were treated with five concentrations based on cytotoxicity or HTD. In the case of cytotoxicity, CV95, CV85, CV75, CV65 and CV50 (µg/mL), were estimated to produce 95, 85, 75, 65, and 50% cell viability, respectively, for each chemical except 4-HBA. An active 1.5% solution of MCI/MI and an active 37% solution of formaldehyde were used. The active test concentrations are shown in this table. The HTD was achieved with 4-HBA. In this case (and denoted with “*”), the five doses for 4-HBA were based on serial 1.3 dilutions of 1,000 µg/mL

anti-human CD86 mAb, and $3 \mu\text{L}/3 \times 10^5$ cells/50 µL for the anti-human CD54 mAb. Also FITC labeled-mouse IgG1 as an isotype control was used at a dilution of $3 \mu\text{L}/3 \times 10^5$ cells/50 µL. These were treated for 30 min at 4°C. After washing and resuspending with PBS supplemented with 0.1% bovine serum albumin (BSA), cell viability was determined using a PI solution (0.625 µg/mL) and flow cytometry analysis. After which, dead cells were gated out and mean fluorescence intensity was recorded. A total of 10,000 living cells were analyzed. When the cell viability for each independent experiment was less than 50%, Relative Fluorescence Intensity (RFI) was not calculated for that independent experiment. The reason is that the diffuse labeling cytoplasmic structures that occur due to cell membrane destruction will interfere with fluorescent measurements [8]. RFI was used as an indicator of CD86 and CD54 expression and was calculated by the following formula:

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

where MFI is the (Geometric) Mean fluorescence intensity.

Three independent experiments were carried out using five doses in each. The five doses were either based on cytotoxicity (CV95, CV85, CV75, CV65, and CV50) or on 1.3 serial dilutions of the HTD (1,000 µg/mL). Mean RFI values and cell viability (SD was calculated) values were calculated from these three independent experiments. Cell viability determined using the flow cytometry analysis was called “Actual Cell Viability”.

Statistics

The *t*-test was used to establish a statistical significance between mean fluorescent intensity for vehicle control cells and mean fluorescent intensity for chemical treated cells. Increases in fluorescent intensity corresponded to an up-regulation of CD86 and CD54 expression. *P*-value less than or equal to 0.05 were considered statistically significant.

Results

Predicted performance for cell viability by PI assay

Five doses were used for each chemical. These doses were selected from the dose response curve obtained in the pre-study cytotoxicity test (CV95, CV85, CV75, CV65, and CV50 that provided a 95, 85, 75, 65, and

50% cell viability, respectively, or 1.3 serial dilutions of the HTD). The Actual Cell Viability was obtained during the concurrent measurement of surface marker expression in the definitive study as described in the Materials and Methods section. Table 2 shows the mean value of the Actual Cell Viability and gives the percent of viable cells expected to be present (Estimated Cell Viability) when exposed to the same concentration of chemical. When the cell viability for the Estimated Cell Viability was compared to the Actual Cell Viability, a good correlation in viability

Table 2 Cell viability estimated from the pre-study cytotoxicity assay (Estimated Cell Viability) or obtained from the definitive study while concurrently assessing marker expression (Actual Cell Viability) is given for all chemicals except 4-HBA

Estimated cell viability (%)	CV95 95	CV85 85	CV75 75	CV65 65	CV50 50
Actual cell viability (%)					
Mean of seven chemicals	96.3 ± 0.7	90.7 ± 6.8	81.8 ± 13.9	60.6 ± 25.6	33.0 ± 23.1
MCI/MI	96.5 ± 0.7	93.5 ± 3.7	78.4 ± 15.2	37.8 ± 19.2	17.3 ± 11.1
FA	96.4 ± 0.6	80.9 ± 10.5	61.8 ± 7.7	35.8 ± 2.5	5.9 ± 3.8
MDBGN	96.5 ± 1.1	92.6 ± 1.8	90.3 ± 1.1	85.8 ± 3.7	64.2 ± 10.0
IU	96.0 ± 0.5	95.3 ± 0.9	92.0 ± 1.8	79.9 ± 11.4	29.9 ± 18.8
DU	96.5 ± 0.4	96.2 ± 0.4	94.8 ± 1.0	93.1 ± 1.6	55.9 ± 25.0
BN	96.2 ± 0.8	93.1 ± 1.7	85.3 ± 6.1	53.3 ± 16.5	38.1 ± 2.2
MP	95.7 ± 0.9	83.4 ± 1.9	70.1 ± 4.6	38.5 ± 12.8	19.8 ± 8.7

Since 4-HBA was not cytotoxic, the data for 4-HBA is not included in this table. Cell viability determined before treatments with test chemicals (Estimated Cell Viability) using the PI assay is compared to the cell viability determined concurrently with surface marker expression following treatment with test chemicals (Actual Cell Viability). In both cases, cell viability was determined using flow cytometry analysis. The Actual Cell Viability of each test concentration is shown as the mean and SD for each of the seven chemicals tested

was observed with the higher concentrations (e.g., CV95 and CV85). Cell viability became more variable as the cytotoxicity of the chemical became more evident. The significant difference between the Estimated Cell Viability and Actual Cell Viability at the CV50 exposure may be the result of interference with fluorescent measurements due to increased diffuse labeling cytoplasmic structures known to occur with higher cell destruction. Overall, there was a tendency that the higher cell viability gave better reproducible results. As such, the SD value was larger when cell viability was lower. A successful test (i.e., good cell viability) was observed for all chemicals with three or four doses (CV95, CV85, CV75, and CV65), except for MDBGN where all five doses showed over 50% cell viability.

The alteration of CD86 expression

Calculated RFI values for CD86 expression following a 24 h treatment are shown in Fig. 1a. All allergic preservatives showed augmentation in a dose-dependent manner. Except for MCI/MI and DU, all allergic preservatives augmented RFI to an RFI of 200 or more. Statistical significance ($P < 0.05$) was observed in the up-regulation of the surface cell marker for a few doses for several allergens (CV85 and CV75 for MCI/MI; CV85 and CV75 for FA; CV85, CV75, CV65 and CV50 for MDBGN; and CV65 for IU). Although a statistical significance was not obtained with the other doses, a trend in augmentation is noted for all the allergens. On the other hand, MP and 4-HBA, which were negative in the LLNA, did not augment significantly RFI at any tested dose ($P > 0.05$) and did not show a trend in augmentation. When cell viability and RFI levels were compared for each replicate of the allergic preserva-

tives (MP and 4-HBA are not included), CD86 expression was augmented above our RFI criteria of an RFI ≥ 150 with a cell viability of 51–95% and augmented above our RFI criteria of an RFI ≥ 200 with a cell viability of 51–92% (Table 3). Interestingly, significant augmentation of CD86 expression was not likely to occur when cell viability was above 95%. Augmentation of surface marker expression appeared to be greater with a lower cell viability range; greatest augmentation for each allergic preservative was observed in the cell viability range of 65–84%. In order to reach the RFI criteria above 200, a lower cell viability than the one observed with an RFI of 150 was needed.

The alteration of CD54 expression

The RFI values of CD54 expression after 24 h treatment are shown in Fig. 1b. All allergic preservatives showed augmentation in a dose-dependent manner and augmented RFI over 150 except for MCI/MI. FA, MDBGN, IU, and BN caused an up-regulation of RFI values greater than 200. The CV75 dose for FA and CV50 dose for MDBGN were statistical significant ($P < 0.05$). Although a statistical significance was not obtained with the other doses, a trend in augmentation is noted for all the allergens. For MP, RFI value was augmented over 150 at the dose of CV85 only, but the increase was not dose-dependent and not statistically significant ($P > 0.05$). 4-HBA did not show a trend in augmentation. When cell viability and RFI levels were compared for each replicate of allergic preservatives (excluding MP and 4-HBA), CD54 expression was augmented above our RFI criteria of an RFI ≥ 150 in the cell viability range of 51–95% and augmented above our RFI criteria of an RFI ≥ 200 with a cell

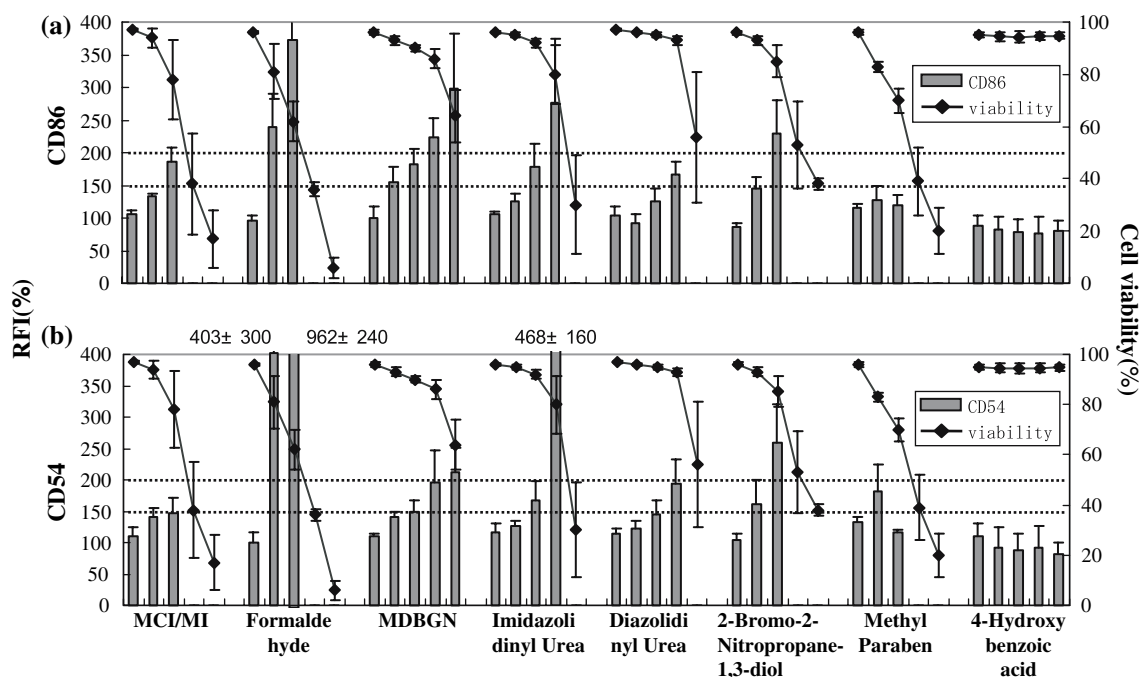


Fig. 1 The effect of allergic and non-allergic preservatives on THP-1 cell expression of CD86 (a) and CD54 (b) is shown. The cells were treated with five concentrations for each preservative. After treatment, the expression of CD86 (a) and CD54 (b) were

measured by flow cytometry and RFIs were calculated (indicated by columns in figure). The lines across the graph indicate RFI values of 150 and 200. The line graphs represent Actual Cell Viability

Table 3 THP-1 cell viability for each independent trial is listed when RFI value was over 150 or 200

	CD86	CD54
MCI/MI	60, 62, 82* , 92	82, 89, 92*
FA	56, 58, 71* , 69, 85, 89	56, 58, 71* , 69, 85, 89
MDBGN	54, 65* , 74, 82 , 85, 89, 90, 90, 92 , 95	54, 65, 74, 82* , 90, 90
IU	51, 67* , 84 , 89, 90, 92	51* , 67, 84, 89 , 90, 92
DU	84* , 91, 93	84* , 91 , 93, 95, 95
BN	72* , 79, 85 , 91, 92, 94	72* , 79, 85, 91, 92

The cell viability was from the independent data of three replicates for each of the six allergens (total number of data points possible is 15: five doses $\times$ three replicates). The left column was the cell viability for the RFI values of CD86 $\geq$ 150 and the right column was the cell viability for the RFI values of CD54 $\geq$ 150. The bold character represents the cell viability at the dose that the RFI value was more than 200. The cell viability with asterisks showed the greatest RFI value. Data for MP and 4-HBA are not provided since they were not considered allergens

viability of 51–92% (Table 3). A higher cell viability ($>95\%$) did not further increase surface marker expression. This was similar to the situation with CD86 expression. The optimum surface marker expression for each allergic preservative was observed with lower cell viability (51–92%). FA, DU, and BN showed greatest augmentation of both CD86 and CD54 at the same cell viability.

Accuracy

For this study, accuracy is the agreement between the published in vivo (LLNA or guinea pig) data for the tested chemicals and the classification of the chemical

in the h-CLAT. The RFI values obtained in the h-CLAT for the allergens and non-allergen were reviewed in order to set criteria for each chemical. Based on the data, the threshold for categorization as either an allergen (positive) or non-allergen (negative) was defined as augmentation of CD86 and/or CD54 above an RFI value of 150 or 200 for any concentration tested. Table 4 describes the overall accuracy obtained when either one or both markers are used at either criterion level, or when the combination of both markers and criterion are used. When the criterion was set at an RFI value of 150 or more, the accuracy was 100% (8/8) if CD86 expression is used and 75% (6/8) if we only use CD54 expression. The lower accuracy with

Table 4 The accuracy (concordance rate between LLNA or guinea pig data and h-CLAT findings) for CD86 and CD54 surface markers at our selected RFI criteria is shown

Marker	Criteria of RFI	Accuracy (%)
CD86	≥150	100.0
	≥200	75.0
CD54	≥150	75.0
	≥200	75.0
Battery	CD86 or CD54 ≥ 150	87.5
	CD86 or CD54 ≥ 200	75.0
	CD86 ≥ 150 or CD54 ≥ 200	100.0
	CD86 ≥ 200 or CD54 ≥ 150	75.0

For CD86, RFI ≥ 150 gives a 100% accuracy, which is better than at an RFI ≥ 200. For CD54, the accuracy did not differ with either RFI criteria; RFI ≥ 150 or 200. Overall, CD86 with a criterion of RFI ≥ 150, and the combination of CD86 with a criterion of RFI ≥ 150 or CD54 with a criterion of RFI ≥ 200 had the highest accuracy; 100%

the CD54 expression was the result of a false negative (i.e., MCI/MI) and a false positive (i.e., MP). When the criterion was set at an RFI value of 200 or more, the accuracy was 75% (6/8) for CD86 expression due the false negative observed for MCI/MI and DU. For CD54 expression, the accuracy for a criterion of greater than 200 was 75% (6/8; both MCI/MI and DU were false negative). The false positive result for MP when using CD54 expression disappeared once the criterion changed from an RFI value of 150 to an RFI value of 200. And when both markers were used to calculate the accuracy, an RFI ≥ 150 for CD86 or an RFI ≥ 200 for CD54 resulted in an accuracy of 100%.

The relationship between EC3 (LLNA) and EC150 (CD86) and EC200 (CD54)

We calculated the estimated concentration required to induce a threshold positive response: RFI = 150 for CD86 and RFI = 200 for CD54. The former was the Estimated Concentration of 150 (EC150 (CD86)) and the latter was the Estimated Concentration of 200 (EC200 (CD54)). We listed the EC150 (CD86) and EC200 (CD54) in Table 5. The LLNA's estimated concentration three-fold stimulation index (EC3) values for MCI/MI, FA, MDBGN, and IU have been reported. For DU and BN, no data was available. For the remaining two chemicals, MP and 4-HBA were determined to be non-allergens. The EC3 values are also listed in Table 5. When we compared the h-CLAT EC150 (CD86) or EC200 (CD54) values to the LLNA EC3 values for the four chemicals with data, both assay methods provided the same outcome: the potency order for each chemical was the same.

Table 5 EC150 and EC200 (μg/mL) values for CD86 and CD54, respectively, are given for each preservative tested

Chemical name	CD86 EC150	CD54 EC200	EC3 of LLNA
MCI/MI	2.6	–	0.005 ^a
Formaldehyde	4.5	4.3	0.61 ^b
MDBGN	7.6	11.1	1.3 ^c
Imidazolidinyl uca	37.1	39.9	24 ^b
Diazolidinyl Urea	21.4	–	No data
2-Bromo-2-ntropropane-1, 3-diols	15.1	16.8	No data
Methylparaben	–	–	Non allergen ^d
4-Hydroxybenzoic acid	–	–	Non allergen ^b

EC150 is the effective concentration of test chemical required to produce a 1.5-fold (150%) increase in RFI value. EC200 is the concentration of a two-fold RFI value (200%) increase. EC3 values from LLNA are also added in this table if the value exists. MP and 4-HBA were determined to be non-allergens

^a Ref. [13]

^b Ref. [14]

^c Ref. [7]

^d Ref. [22]

Discussion

Preservatives are one of the key ingredients in many personal care products, including cosmetic and toiletries. Although some of the preservatives have been found to have a sensitization potential, early evaluation of the skin sensitization potential of a preservative is critical in the development of a new preservative. Recently, the LLNA as part of a quantitative risk assessment process was proposed as an early evaluation tool for allergy potential [12]. The LLNA is a useful method, but it will still be necessary to develop a non-animal method in view of the seventh amendment to the Cosmetic Directive in the European Union. As a result, several non-animal skin sensitization test methods have been proposed [5]. Since the mechanism of skin sensitization is very complex, duplication of the full process in vitro would be very difficult. For this reason, the development of an in vitro method should consider or address the potential dermal penetration of a chemical, the protein/peptide interaction, and the initiation of an antigen-specific immune response [5].

Recently Langerhans cell (LC)-like dendritic cells, which are derived from human PBMC [1, 2, 9, 10, 25, 26] or from CD34+ HPC obtained from human cord blood [11, 20], have been shown to provide a promising tool for identifying allergy potency in vitro. These cells act as surrogates for Langerhans cells. The Langerhans cell is the key cell type involved in the sensitization process [18]. Thus, the above methods measured

changes in surface intracellular adhesion molecules and co-stimulatory molecule expression (e.g. CD86 or CD54) as markers of Langerhans cells activation following exposure to known human allergens. However, it is difficult to obtain the appropriate number of dendritic cells from human PBMC or cord blood. In addition, other technical issues associated with maintaining the dendritic cells derived from human PBMC and cord blood (e.g., more involved cell culture procedures) and donor-to-donor variability are associated with these sources of LC-like cells.

To address these points, we have developed an in vitro skin sensitization test using a human monocytic leukemia cell line (THP-1 cells) and a human myeloid leukemia cell line (KG-1 cells) [29]. Again, our assay and the previous investigators' methods base the assays on alteration of CD86/CD54 surface expression on a dendritic-like cell, which is a key event in the sensitization process. Also, an in vitro testing assay that evaluated the alteration of CD86 expression and internalization of MHC class II using THP-1 cells was also reported [3]. More recently, we optimized the in vitro skin sensitization test, which used THP-1 cells and a human histiocytic lymphoma cell line (U937 cells), and reported that the assay was a useful method with a high correlation to in vivo data [4, 24]. Furthermore, we found that a certain level of cell cytotoxicity was needed for up-regulation of CD86 and CD54 expression following treatment with allergens.

In our previous protocol [24], a methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay was used to assess cytotoxicity prior to assessment of cell surface marker expression. In later protocols, cell viability was measured using flow cytometry with PI concurrently with cell surface marker expression. The latter set of experiments revealed that cell viability played a role in cell surface marker expression. In our current study, a cytotoxicity test using PI was carried out beforehand in order to estimate the concentration at which the probability of cell viability becomes 50, 65, 75, 85, and 95%. In addition, cell viability was measured concurrently with cell surface marker expression. As shown in Table 2, the cell viability assessment using Estimated Cell Viability (pre-study cytotoxicity) prior to cell surface marker expression correlated well in the higher doses with the cell viability measurements taken concurrently with determination of CD86/CD54 expression (Actual Cell Viability). The results indicate that a pre-study cytotoxicity assessment is appropriate for setting the test concentrations for the definitive study. In comparing to the previous method using MTT to the results obtained in this study, the PI method was more reliable and easier to use. These data suggest that the

PI method would provide a more useful protocol for dose setting levels prior to initiating the definitive study and for obtaining a more accurate measure of cell viability compared to a pre-study with MTT.

Increases in dendritic cell surface antigen expression (e.g. CD86 or CD54) is proposed to be an indication of the sensitization process, which largely involve the activation of the LC following exposure to known allergens. In the evaluation of CD86 expression, the RFI values of all allergic preservatives were up regulated over 150 in a concentration dependent manner (Fig. 1a). For CD54 expression (Fig. 1b), all allergic preservatives, except MCI/MI and DU, augmented RFI values over 200 in a concentration dependent manner. On the other hand, MP and 4-HBA, which were classified as non-sensitizers in the LLNA, did not augment CD86 expression over an RFI value of 150 or CD54 expression over an RFI value of 200. In looking at the data for the preservatives, an RFI value ≥ 150 for CD86 expression would correctly identify all the preservative allergens (Table 4). At the moment, this RFI value would then be used as the criterion that would distinguish between an allergen and non-allergen. However, we still need to further validate and optimize the method in order to more adequately determine proper criteria. For example, earlier we reported that allergens do not uniformly augment the same marker [24]. In some cases, we saw one allergen augment CD86 and not CD54, while a different allergen would augment CD54 and not CD86. Therefore, both CD86 and CD54 markers may be needed to correctly predict an allergen using the h-CLAT. When we use both CD86 and CD54 markers, the combination of an RFI value ≥ 150 for CD86 and/or an RFI value ≥ 200 for CD54 is a good criterion to use for identifying allergic and non-allergic preservatives.

A successful in vitro assay would need to correctly identify an allergen and differentiate between an allergen and non-allergen. In our earlier study, no increase in CD86 and CD54 expression using THP-1 cells was noted with formalin, a potent sensitizer [29]. In the current study, we do observe the augmentation. The observed difference, we believe, was due to differences in protocol procedures (e.g., the use of a different clone of the CD86 and CD54 antibody; different test doses). However, other researchers were not able to correctly identify a couple of known allergens with their assay. Cytokine-induced PBMC-DC and KG-1 cells were used to evaluate MCI/MI [16]. MCI/MI increased expression of CD86 to 194% of untreated control only in KG-1 cells and not PBMC-DC. In addition, formalin did not induce the augmentation of CD86 expression using cytokine-induced PBMC-DC

[19]. MCI/MI and formaldehyde consistently sensitize *in vivo*. The failure to detect these potent chemical allergens suggests a lack or limitation in sensitivity of PBMC-DC. On the other hand, the two cell lines, THP-1 and KG-1, correctly predicted these preservatives as allergens, which suggest that the human cell lines may have a greater sensitivity for these preservatives.

A more refined assay may also result in better results. Our previous findings [24] demonstrated that a certain level of cytotoxicity was needed in the h-CLAT for better identifying the test chemicals. Based on this knowledge, we set up five test concentrations, which would produce cell viability from 50 to 95%. As we show in Table 1, all allergic preservatives except 4-HBA had a narrow dose range for a cell viability of 50–95%. For MCI/MI and BN, three concentrations resulted in cell viability above 70% but only one concentration (CV75) met one of the criteria (CD86 was above 150 for MCI/MI, and CD86 was above 150 and CD54 above 200 for BN). Only one concentration was also noted to give an RFI value above the threshold mark for DU, but here four concentrations resulted in cell viability above 80%. These data highlight the importance of selecting the appropriate test concentration in order to correctly predict the allergy potential of test chemicals.

Depending on the chemical, the suitable cell viability for augmentation of CD86 or CD54 expression varied as we show in Table 3. However, augmentation of CD86 and CD54 expression consistently occurred when cell viability was between 65 and 90%. From our selected five doses, correct prediction of allergens was mainly seen with CV75 or CV65, the estimated dose that caused 75% or 65% cell viability. Other researchers also showed that CD86 expression in PBMC and/or human cell lines increased when cell viability was affected. PBMC-DC treated with concentrations of dinitrobenzenesulfonic acid (DNBS) that induced 10–35% cytotoxicity [15] or a 15% decrease in cell viability [23] augmented CD86 expression. In the case of using U-937 cells, a >70% cell viability increased expression of CD86 [21]. These data suggest that some cytotoxicity is needed in order to enhance CD86 and CD54 expression and to make this human cell-based *in vitro* method a useful tool in assessing skin sensitization. As a result, we will incorporate one of these two estimated doses (CV65 or CV75) into the h-CLAT. Further understanding of the association between CD86/CD54 expression and cytotoxicity would make this assay a more robust test.

Another critical refinement to the assay is the selection of the criterion for identifying an allergen and differentiating allergens and non-allergens. A statisti-

cal analysis of the mean fluorescent intensities for each concentration tested found only a few concentrations to be statistically significant compared to vehicle control. Statistical significance was observed for each chemical predominantly with either the CV75 or CV85. Again, this finding supports the observation that some cytotoxicity enhances CD86 and CD54 expression. Cytotoxicity may also be responsible for the higher variability at the higher concentrations, which would result in larger standard deviations leading to non-significance. Further evaluation of the h-CLAT using the refined protocol and a larger number of independent experiments may provide insight into the non-statistical differences. However, evaluation of the RFI data shows a good dose response and an increasing trend in RFI intensity with those chemicals known to be allergens.

The use of RFI to identify and distinguish between known allergens and non-allergens seems appropriate due the fact that known allergens increased RFI values while non-allergens did not consistently increase RFI values. In our previous report, we set two tentative criteria: $\text{RFI} \geq 150$ or ≥ 200 [24]. In this study, we compared these criteria and found a 100% accuracy when we used $\text{RFI} \geq 150$ for CD86 and/or $\text{RFI} \geq 200$ for CD54. In another *in vitro* skin sensitization test that used human cell lines, CD86 expression of 120% or more, above control cells, was needed to declare a “positive” [21]. These studies and the current findings suggest that our method, h-CLAT, using THP-1 cells may be able to predict the allergy potential of allergens. In order to fully validate the h-CLAT as a useful *in vitro* skin sensitization model for the prediction of contact allergens including preservatives, more chemicals, both allergens and non-allergens, need to be assessed. In addition, the method will need to undergo evaluation by other laboratories and the method will need further optimization (e.g., final criteria).

In addition to identifying an allergen, our method may be used to classify the skin sensitization potency of a chemical. In the LLNA, the EC3 value is the estimated concentration that causes an stimulation index of 3-fold increase. This value is used for rank classification of allergens in the LLNA. Using a similar approach, we established an estimated concentration (EC) that would reach an $\text{RFI} \geq 150$ for CD86 and an $\text{RFI} \geq 200$ for CD54. These values would be the EC150 for CD86 and EC200 for CD54 and would be used for classification of the skin sensitization potential of a chemical in the h-CLAT. Once we had the h-CLAT potency values, we compared the h-CLAT EC150 (CD86) or EC200 (CD54) to the EC3 values from the LLNA for the same chemicals. Currently, we had only four chemicals with

LLNA data. In terms of the potencies, the order for each chemical was the same in both methods (Table 5). This means that there is a possibility that the h-CLAT method may classify allergy potency of a chemical. Since these values are influenced by the extent of cytotoxicity, it is necessary to evaluate the role of cytotoxicity in the allergy potential of the chemical.

Based on our current and former findings [24], we think that the h-CLAT may be a useful in vitro skin sensitization test for evaluating various chemicals including preservatives. We need to further confirm the usefulness of the h-CLAT by advancing the validation of this method to the next phase (i.e., multiple institution assessment, wider chemical selection) and to further discern the possibility of a rank classification using h-CLAT.

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