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Lipopolysaccharide interferes with the use of the human Cell Line Activation Test to determine the allergic potential of proteins

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Abstract

It was believed that high molecular weight molecules including proteins cannot penetrate the skin. However, protein penetration through disrupted/ruptured skin has been reported recently, thus carrying the potential for inducing an allergic response. We used the human Cell Line Activation Test (h-CLAT), an in vitro skin sensitization test, to assess the allergic potential of proteins by measuring levels of CD86 and CD54 in the human monocytic leukemia cell line THP-1. Six allergens including ovalbumin (OVA) and human serum albumin (HSA; negative control) upregulated CD86 and/or CD54; a false-positive result was obtained using HSA. This was caused by lipopolysaccharide (LPS) contamination. Naturally derived materials often include LPS at various concentrations and may influence protein induction of CD86 and CD54. Additionally, polymyxin B, an LPS inhibitor, could not completely overcome the effect of LPS. Therefore, if test proteins contain ≥ 0.1 EU/mL LPS, their allergenic potency will not be assessed accurately using h-CLAT. These data show that naturally occurring materials or those derived from living organisms should be evaluated for their LPS content. It is important to confirm the applicability of in vitro methods such as h-CLAT for assessing the allergenic potency of naturally occurring proteins; our findings can be a foundation for future studies.

Keywords: human serum albumin, ovalbumin, skin sensitization test, protein, human Cell Line Activation Test, lipopolysaccharide

Abbreviations

h-CLAT, human cell line activation test; OVA, ovalbumin; HSA, human serum albumin; LPS, lipopolysaccharide; OECD, Organization for Economic Cooperation and Development; DPRA, Direct Peptide Reactivity Assay; OVM, ovomucoid; LZY, lysozyme; BSA, bovine serum albumin; LAC, β -lactoglobulin; CAS, casein sodium salt; DNCB, 1-chloro-2,4-dinitrochlorobenzene; DMSO, dimethyl sulfoxide; ATCC, American Type Culture Collection; MFI, mean fluorescence intensity; RFI, relative fluorescence intensity; LAL, limulus amebocyte lysate; CSE, control standard endotoxin; WFI, water for injection; OD, optical density; EU, endotoxin unit; PMB; Polymyxin B; SD, standard deviation.

Introduction

It has been speculated that high molecular weight substances, including those above 500 Da, may not cause skin sensitization because they cannot penetrate the skin (Bos et al., 2000). However, recent cases have been reported in Japan wherein patients initially sensitized to hydrolyzed wheat protein (MW 35,000–50,000) via facial soap later experienced wheat-dependent anaphylaxis after ingesting normal wheat products. These reports show that high molecular weight substances including proteins can penetrate the skin when the barrier function of the skin or mucosa decreases, thereby potentially inducing serious allergic responses, such as anaphylaxis (Adachi et al., 2012; Matsunaga et al., 2015). There have been similar reports in the field of occupational allergy. In 1976, occupational protein contact dermatitis was reported in food handlers such as chefs (Hjorth and Roed-Pedersen., 1976). Recently, there have been some reports of protein-induced skin sensitization as it relates to allergies, for example to latex from rubber products, ovalbumin from eggs, parvalbumin from fish, and honey from bees (Hayes et al., 2000; Hsieh et al., 2003; Katayama et al., 2016; Yagami et al., 2015). Moreover, it has been reported that the skin is the most important route for the induction of IgE-mediated allergy (Smith Pease et al., 2002; Lack et al., 2003). Therefore, it is critical to assess the skin-sensitizing and allergenic potency of proteins when using naturally derived proteins for products such as cosmetics and toiletries. This necessitates the development of an in vitro test for assessing the allergenic potency of proteins.

Skin sensitization results in an immune response. Upon repeated skin exposure, certain chemicals can induce allergic contact dermatitis (ACD). The Organization for Economic Cooperation and Development (OECD) has adapted three testing methods as in vitro skin sensitization tests, which essentially replicate in vivo mechanisms. The Direct Peptide Reactivity

Assay (DPRA, TG442C) (Gerberick et al., 2004, 2007) is based on interactions between low molecular weight haptens and skin proteins. The ARE-Nrf2 luciferase test method (TG442D) (Natsch et al., 2007) is based on the activation of epidermal keratinocytes. Lastly, the human Cell Line Activation Test (h-CLAT, TG442E) is based on the activation of dermal dendritic cells. In particular, only h-CLAT assesses the response of antigen-presenting cells with important functions in the allergic response. The h-CLAT method measures changes in CD86 and/or CD54 marker expression in the human monocytic leukemia cell line THP-1 (Ashikaga et al., 2006; Sakaguchi et al., 2006). The concordance rate between h-CLAT results and those of the murine Local Lymph Node Assay (LLNA) is 84%, and the predictive performance of the h-CLAT for human skin sensitizing potential based on comparing the results with human data is 83% (Ashikaga et al., 2010; Nukada et al., 2011). Therefore h-CLAT is a reliable in vitro test for skin sensitization to low molecular weight chemicals.

In this study, we developed a test for detecting protein-induced skin sensitization based on the same markers and criteria used for h-CLAT because it is necessary to consider differences between humans and other animal species for assessing the allergenic potency of proteins (Ohsawa, 2009). We chose materials based on the Allergen Database for Food Safety (<http://allergen.nihs.go.jp/ADFS/>) in terms of their accessibility and solubility. Six proteins, ovalbumin (OVA), ovomucoid (OVM), lysozyme (LYZ), bovine serum albumin (BSA), β -lactoglobulin (LAC), and casein sodium (CAS), were used as allergens, and human serum albumin (HSA) was used as a negative control.

Materials and Methods

Reagents

OVA from hen egg white (A and B $\geq$ 98% purity, C $\geq$ 90% purity), 1-chloro-2,4-dinitrochlorobenzene (DNCB), trypsin inhibitor from chicken egg white type III-O (OVM), LYZ from chicken egg white ($\geq$ 98% purity), BSA (protease free, $\geq$ 98% purity), LAC from bovine milk ($\geq$ 90% purity), CAS salt from bovine milk, HSA (protease-free, $\geq$ 96% purity), Low-endotoxin ($\leq$ 1 EU/mg) HSA, lipopolysaccharide (LPS), and polymyxin B (PMB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan).

Cell culture and maintenance

The human monocytic leukemia cell line THP-1 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI1640 (Gibco, Thermo Fisher Scientific Inc., Waltham, MA, USA) with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific Inc.), 0.05 mM 2-mercaptoethanol (Thermo Fisher Scientific Inc.), and 1% (v/v) penicillin-streptomycin solution (Thermo Fisher Scientific Inc.) at 37°C and 5% CO₂. Cells were routinely sub-cultured every 2–3 days at a density of 0.1–0.2 $\times$ 10⁶ cells/mL. Before the h-CLAT assay, cells were seeded at 0.1–0.2 $\times$ 10⁶ cells/mL and precultured for 48–72 h. The density of cells was not permitted to exceed 0.5 $\times$ 10⁶ cells/mL, and the passage number was under 30.

Preparation of test materials and treatments

Protein solutions of 0.01, 0.1, and 1% (w/v) were prepared in culture medium. Then, DNCB was dissolved in DMSO to obtain a concentration of 1.5 mg/mL. The DNCB/DMSO solution was then

diluted 250 times with culture medium. LPS (600,000 EU/mg) was dissolved in culture medium (1 mg/mL) and diluted to 0.00001–1000 EU/mL. Medium without chemicals and medium with DMSO only (250-fold dilution) were prepared as medium and solvent controls, respectively. Next, 500 μ L of each sample was added to 24-well plates, and 500 μ L of THP-1 cells was resuspended in fresh culture medium at a density of 2×10^6 cells/mL. Finally, 500 μ L of cell suspension was added to each well and exposed to the test materials.

Analysis of CD86 and CD54 expression and cell viability

CD86 and CD54 expression and cell viability were analyzed according to the protocol of h-CLAT (OECD TG442E). Cells treated with test samples for 12, 24, 48, and 72 h were collected into sample tubes. Cells were washed twice with 1 mL of phosphate-buffered saline (PBS) supplemented with 0.1% (w/v) BSA (Wako Pure Chemical Industries Ltd.). Supernatants were discarded, and the remaining cells were blocked with 600 μ L of blocking solution [0.01% (w/v) Globlins Cohn fraction II and III (Sigma–Aldrich)] at 4°C for 15 min. After blocking, cells were split into three aliquots of 200 μ L each (approximately 0.3×10^6 cells/tube) before staining with antibodies. Pre-mixed antibody solutions were prepared using either 6 μ L of anti-CD86 antibody (BD Pharmingen, San Diego, CA, USA) or 3 μ L of anti-CD54 antibody (Dako, Glostrup, Denmark), and 3 μ L of FITC-labeled mouse IgG1 (Dako) was added to 44 μ L or 47 μ L of PBS with 0.1% BSA, respectively, to a final volume of 50 μ L. The entire 50 μ L of pre-mixed antibody solution was added to each cell pellet. After gentle mixing by hand, cells were incubated at 4°C for 30 min in the dark. After staining with antibodies, cells were washed twice with 200 μ L of PBS supplemented with 0.1% (w/v) BSA and re-suspended in a final volume of 400 μ L. The cell suspensions were transferred to a 5-mL tube through a cell strainer. Before flow cytometry acquisition, 10 μ L of propidium iodide (PI) solution [25 μ g/mL (Wako Pure Chemical Industries

Ltd.) in PBS] was added to obtain a final concentration of 0.625 µg/mL in each tube.

Data analysis and criteria

The expression of cell surface antigens was analyzed using flow cytometry with a FACSCalibur (Becton Dickinson, San Jose, CA, USA). The FITC acquisition channel (FL-1) and the PI acquisition channel (FL-3) were adjusted for optimal detection of the FITC fluorescence signal and the PI fluorescence signal, respectively. Dead cells were gated-out by staining with PI. A total of 10,000 living cells were assessed. The mean fluorescence intensity (MFI) of viable cells and viability for each sample were determined. The relative fluorescence intensity (RFI) was used as an indicator of CD86 and CD54 expression and was calculated as follows for each concentration of every test material:

$$\text{RFI (\%)} = \frac{\text{MFI of test material-treated cells} - \text{MFI of test material-treated isotype control cells}}{\text{MFI of solvent-treated control cells} - \text{MFI of solvent-treated isotype control cells}} \times 100$$

MFI = Geometric mean fluorescence intensity

For each concentration of test material, cell viability was recorded from the isotype control cells.

The positive criteria were adapted from the h-CLAT criteria (CD86 ≥ 150% and/or CD54 ≥ 200%).

Determination of LPS concentrations in test proteins

LPS concentrations were determined using the limulus amoebocyte lysate (LAL) assay (Pyrochrome® reagent with Glucashield® buffer, Seikagaku Corporation, Tokyo, Japan) in accordance with the manufacturer's instructions. Control Standard Endotoxin (CSE; Seikagaku Corporation) was dissolved in sterile water for injection (WFI; Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) to obtain final concentrations of 0.0625, 0.125, 0.25, 0.5, 1.0, and 2.0 EU/mL. Each test protein was diluted with WFI appropriately. Pyrochrome® reagent was reconstituted in the dark with 3.2 mL Glucashield® buffer and incubated for at least 5 min at room temperature

(approximately 26°C). For each CSE, test sample, WFI sample (as negative control), and positive product control sample, 50 µL was transferred into a 96-well plate, and 50 µL of Pyrochrome® reagent with Glucashield® buffer was added. After agitation for 1 min, samples were incubated for 30 min at 37°C. Thereafter, 25 µL of 50% acetic acid was added to each well to terminate the reaction, and optical density (OD) was determined using the EnVision Multilabel Plate Reader (PerkinElmer, Inc., Waltham, MA, USA) at 405 nm. A standard curve was formed per the OD readings against standard endotoxin concentrations, and the means of the endotoxin concentrations of test proteins (EU/mg) were determined from three experiments.

Preparation of and treatment with PMB

PMB was dissolved in sterile WFI to obtain a concentration of 1 mg/mL, and it was then diluted with culture medium to a final concentration of 4 µg/mL. This solution was added to the LPS solution (0.04, 0.4, or 4 EU/mL) at a ratio of 1:1. The mixed solution was incubated for 30 min at room temperature (approximately 26°C). Next, 500 µL of each mixed solution containing PMB (2 µg/mL) and LPS (0.02, 0.2, or 2 EU/mL) was transferred to a 24-well plate. THP-1 cells were re-suspended in fresh culture medium at a density of 2×10^6 cells/mL, and 500 µL of cell suspension was added to each well.

Statistical analysis

In this study, cell-based assays were performed three times, and representative results are shown. The data presented are expressed as the mean $\pm$ standard deviation (SD). The criteria for h-CLAT (CD86 $\geq$ 150% and/or CD54 $\geq$ 200%) were used to evaluate the results according to the protocol of h-CLAT (TG442E). The statistical significance of differences in the RFIs of CD86 and CD54 between LPS-treated THP-1 cells and LPS+PMB-treated THP-1 cells was evaluated using

paired Student's t tests ($p < 0.05$).

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Results

Effect of exposure time and protein concentration on CD86 and CD54 expression

We used OVA to determine the optimal treatment conditions for adapting h-CLAT for the assessment of proteins. We then tested three concentrations, i.e., 0.01, 0.1, and 1% (w/v), following 12, 24, 48, and 72 h of exposure and found that OVA treatment upregulated CD86 and CD54 beyond the h-CLAT criteria ($CD86 \geq 50\%$ and/or $CD54 \geq 200\%$) (Fig. 1A, 1B). CD86 expression was highest at 48 h, while CD54 expression was highest at 12 h and decreased over time. Moreover, at the 24 h exposure time suggested by h-CLAT, OVA-induced CD54 expression was very high and was approximately 20 times that induced by DNCB (h-CLAT positive control).

Evaluation of proteins via modified h-CLAT

Based on the above conditions, we evaluated seven proteins (OVA, OVM, LYZ, LAC, CAS, BSA, and HAS) at three concentrations following 24 h of exposure (Fig. 1A, 1B). Consequently, OVA, OVM, LYZ, LAC, and CAS upregulated CD86 beyond the h-CLAT criteria established for chemicals; however, BSA and HAS did not (Fig. 2A). Furthermore, all proteins except BSA upregulated CD54 beyond the assay criteria (Fig. 2B).

Effect of LPS on HSA-induced CD54 expression

As shown in Fig. 2B, HSA augmented CD54 expression beyond the assay criteria, indicating a false-positive result. We speculated that this was caused by contaminants in reagents. Therefore, we used low endotoxin (i.e., low LPS) HSA ($\leq 1\text{EU/mg}$; Low HSA; negative control) to investigate the upregulation of CD54 and compared the results with that of Normal HSA. Neither Normal HSA nor Low HSA affected CD86 expression (Fig. 3A). In contrast, Normal HSA upregulated

CD54 expression, whereas Low HSA did not (Fig. 3B).

Determination of LPS levels in test proteins

The results in Fig. 3B suggest that LPS enhances HSA-induced CD54 expression, suggesting that the presence of endotoxin (i.e., LPS) has a significant effect on the results of h-CLAT. Thus, we determined the levels of LPS in the test proteins. Table 1 shows the amount of LPS in endotoxin units (EU) in each milligram of protein. LAC contained the most LPS at 3917 EU/mg, followed by OVA at 401 EU/mg and CAS at 124 EU/mg. LYZ contained the least LPS at 0.234 EU/mg. Therefore, naturally occurring proteins appear to be frequently contaminated with varying levels of LPS.

Effect of lot or grade of OVA on CD86 and CD54 expression

The results in Table 1 show that levels of LPS contamination in reagents differ among different types of protein. In addition, we investigated whether there were differences in CD86 and CD54 expression and in levels of LPS among lots or grades of the same reagent (OVA). The amount of LPS varied among three lots (A1, A2, A3 $\geq$ 98% purity) and grades (B $\geq$ 98% purity, C $\geq$ 90% purity) of OVA (Table 2). Moreover, the purity of the OVA did not correlate with the observed LPS levels. The three lots and grades of OVA were used at concentrations of 0.01, 0.1, and 1% and exposed to cells for 24 h. Every lot and grade of OVA upregulated CD86 and CD54 expression beyond the criteria (Fig. 4A, 4B). Furthermore, CD54 expression was correlated with the LPS level in each reagent (Table 2), though CD86 expression did not.

Effect of PMB on CD86 and CD54 expression induced by LPS

We used PMB, an LPS inhibitor, to attempt to overcome the effect of LPS on CD86 and CD54

expression. PMB was added to LPS solutions (final concentrations of 0.01, 0.1, and 1.0 EU/mL) at 1 µg/mL, a concentration which is not toxic to cells and does not affect CD86 or CD54 expression. When added to the LPS solutions, PMB inhibited CD86 and CD54 expression. However, the effect of inhibition on CD86 and CD54 expression levels under the criteria of h-CLAT was observed for CD86 expression with 0.1 EU/mL and CD54 with 0.01 EU/mL (Fig. 5A, 5B).

Effect of LPS treatment on CD86 and CD54 expression

As the effect of PMB was limited (Fig. 5A, 5B) and as it is important to know the limitations of the application of the modified h-CLAT for assessing test materials that include LPS, we evaluated CD86 and CD54 expression and cell viability by varying the levels of LPS in the test protein solutions. The concentration dependency increased between 0.01 and 10 EU/mL LPS for CD86 and between 0.01 and 1000 EU/mL for CD54. These data show that 0.1 EU/mL LPS upregulated CD86 and CD54 to levels beyond the criteria of h-CLAT (Fig. 6A, 6B). In addition, there were no changes in CD86 and CD54 expression over at LPS levels of 1000–40,000 EU/mL (data not shown).

Discussion

In order to adapt h-CLAT for the assessment of the allergenic potency of proteins, we investigated the optimal exposure time and concentration using OVA. The tested concentrations were 0.01, 0.1, and 1% for in vivo tests for protein antigenicity (Itagaki et al., 1991). CD86 and CD54 expression was upregulated beyond the h-CLAT criteria used for chemicals upon treatment with 0.01, 0.1, and 1% (CD86 $\geq$ 150% and/or CD54 $\geq$ 200%). CD86 and CD54 expression levels were highest at 48 h and 12 h, respectively. In addition, an increase in the treatment time increased the aggregation of cells and their adherence to the 24-well plate. Therefore, physical scraping or chemical detachment may affect the cell-surface expression of CD86 and CD54. Thus, the optimal treatment time was determined to be 24 h, which is the same as that used in the h-CLAT protocol for chemicals, because the effect of adhesion was low and the CD86 and CD54 expression was adequate.

We evaluated six allergenic proteins (OVA, OVM, LYZ, LAC, CAS, and BSA) and HSA as a negative control. All proteins except for BSA and HSA upregulated CD86; however, all except BSA upregulated CD54. CD54 is expressed in cells other than monocytes and plays a role in cell adhesion and inflammation (Lawson et al., 2009; Rosette et al., 2005; Van de Stolpe et al., 1996; Zimmerman et al., 2008). The induced levels of CD54 were very high in all cases except for BSA and OVM, with percentages ranging from a few thousand to tens of thousands, while percentages induced by chemicals (known as skin sensitizers) range from a few hundred to the thousands (Nukada et al., 2012; Sakaguchi et al., 2009, 2010). Moreover, CD54 expression varied widely among the three experiments. Therefore, the mechanism underlying the induction of CD54 expression by proteins may differ from that underlying chemical induction.

We hypothesized that the increase in CD54 expression may be influenced by impurities in the

test proteins because CD54 expression induced by HSA varied widely. Proteins commercially available as reagents for scientific research are isolated and purified by electrophoresis, chromatography, or ultrafiltration. Naturally occurring proteins are frequently contaminated with endotoxins (i.e., LPS). LPS is present in varying concentrations everywhere, e.g., in water and air, and LPS exhibits activity even in very small amounts (from picograms to nanograms). Moreover, it is very difficult to eliminate or inactivate LPS because LPS persists if bacteria are heat-inactivated (Tanamoto et al., 2008). There are no reports on the allergenic potency of LPS. However, it is known that LPS can activate an innate immune response by promoting the production of cytokines, e.g., IL-1, IL-6, and TNF- α , in monocytes or macrophages (Guha et al., 2001). In addition, LPS was reported to upregulate CD86 and CD54 in THP-1 cells used in h-CLAT (Miyazawa et al., 2007). Furthermore, THP-1 cells express TLR-4, an LPS receptor, and CD14 for LPS recognition (Wang et al., 2009). Therefore, it is important to consider the effect of LPS on in vitro methods including h-CLAT by considering the immune responses of cultured cell lines as endpoints. Thus, we used a low-endotoxin reagent (Low HSA, ≤ 1 EU/mg) and showed that the false-positive result for HSA may be caused by LPS.

We then measured the LPS concentrations of the test proteins. Our findings that each protein was contaminated with varying amounts of LPS and the amount of LPS detected per milligram of OVA were concurrent with the findings of a previous study (Boverhof et al., 2010). However, there was no correspondence between expression levels of CD86 and CD54 and the amount of LPS in each protein sample. For example, when comparing LYZ (0.234 EU/mg) with OVM (2.82 EU/mg), CD86 and CD54 expression was induced to a higher extent by 1% LYZ than by 1% OVM. Moreover, BSA (12.5 EU/mg) did not affect CD86 and CD54 expression. HSA (21.3 EU/mg) only enhanced CD54 expression. This implies that not only the effect of LPS but also that of each

protein can be determined when using CD86 and CD54 expression as markers. However, BSA may be able to adsorb LPS and inhibit its effect on THP-1 cells because BSA did not affect CD86 and CD54 expression.

Moreover, we investigated the differences in the amount of LPS among lots and grades of OVA and found approximately 2—20-fold differences among lots and grades. However, the purity of the OVA did not correlate with observed LPS levels. Hence, LPS content should be evaluated for each lot and the effects of LPS should be considered for assessment for the allergenic potency of naturally occurring proteins. Furthermore, the expression levels of CD54 but not CD86 were correlated with the amount of LPS in three lots and grades of OVA. This suggests that CD54 may be more affected by LPS than CD86. Therefore, h-CLAT using CD54 expression as a marker may be a potentially useful tool for detecting LPS in test substances.

Our results suggested that LPS has a substantial effect on the results of h-CLAT. Therefore, we attempted to eliminate the effect of LPS in the test proteins. Ways of overcoming the effect of thermostable LPS mainly involve the use of LPS inhibitors such as PMB or anti Toll-like receptor-4 (TLR-4) antibody and column chromatography (Magalhaes et al., 2007; Petsch et al., 2000). However, anti-TLR-4 antibody would not have been an appropriate inhibitor of LPS in this study because it regulates skin sensitization pathways (Ainscough et al., 2013; Honda et al., 2013). In addition, adsorption removal by affinity chromatography is a laborious and difficult method for reducing LPS to levels below the minimum limit of detection of h-CLAT because they are limited in their adsorption capacity. Removal by two-phase detergent-based extraction shows good adsorption capacity (Teodorowicz et al., 2017), but we must consider the cytotoxicity of the eluate and the effect of the eluate on immune responses in cells, such as CD86 and CD54 expression in THP-1 cells. Furthermore, the elimination of LPS from test materials in every test

is rather unrealistic, as raw materials are usually contaminated with LPS. Hence, in this study, we used PMB as an LPS inhibitor. PMB is an antibiotic synthesized from naturally occurring peptides, and it inhibits the effect of LPS by binding to its lipid moiety, the major active site of LPS (Morrison et al., 1976). PMB has been reported to inhibit the production of cytokines, e.g., TNF- α , in THP-1 cells (Cardoso et al., 2007; Song et al., 2000). We used PMB at 1 μ g/mL to eliminate any effects on h-CLAT. Consequently, PMB downregulated CD86 and CD54 expression in THP-1 cells to levels below the h-CLAT criteria for chemicals, at LPS levels of 0.1 and 0.01 EU/mL, respectively. These data show that 1 μ g/mL of PMB cannot completely inhibit CD86 and CD54 expression induced by 0.2–4000 EU/mg LPS in the test proteins. This suggests that the complete exclusion of the effect of LPS in the test proteins in h-CLAT would be very difficult using PMB.

Moreover, we investigated the effect of LPS alone on h-CLAT to determine the range of the application of this test for naturally occurring proteins contaminated with varying levels of LPS. We measured CD86 and CD54 expression and cell viability based on the suggested LPS levels in the test proteins. We found that 0.1 EU/mL LPS induced CD86 and CD54 expression beyond the criteria for h-CLAT, implying that if the test proteins contained over 0.1 EU/mL LPS, their allergenic potency would not be assessed accurately. Moreover, this is not exclusive to only naturally occurring proteins but also should be considered for plant extracts and other naturally occurring substances (Nakae et al., 2015).

Therefore, according to this study, h-CLAT is useful for assessing the allergenic potency of proteins containing less than 0.1 EU/mL LPS if CD86 expression is not inhibited by the addition of PMB (1 μ g/mL). Otherwise, h-CLAT is useful for assessing the allergenic potency of proteins containing less than 0.01 EU/mL LPS if CD54 expression is not inhibited by the addition of PMB

(1 µg/mL).

Conclusion

In this study, we attempted to adapt h-CLAT for use in assessing the allergenic potency of proteins and to determine the effect of LPS in the test proteins on CD86 and CD54 expression. Each protein was contaminated with varying amounts of LPS, and LPS may have contributed to the induction of CD54 expression by HSA, a negative control. Moreover, we revealed that if test proteins contain over 0.1 EU/mL LPS, their allergenic potency will not be assessed accurately using this method. Therefore, it is important to measure LPS levels and confirm the applicability of h-CLAT for assessing the allergenic potency of naturally occurring proteins. In the future, we aim to determine the optimal test conditions or endpoints and methods of exposing proteins to cells without the effect of LPS.

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Table 1. Amount of LPS per milligram of each test protein.

Name of proteins	LPS [EU/mg]	SD
Lysozyme (LYZ)	0.234	0.091
Ovomucoid (OVM)	2.82	1.47
Bovine serum albumin (BSA)	12.5	5.36
Human serum albumin (HSA)	21.3	9.04
Casein sodium (CAS)	124	52.0
Ovalbumin (OVA)	401	23.6
β -lactoglobulin (LAC)	3917*	—

Data show the mean endotoxin concentrations of all test proteins (endotoxin units; EU/mg) and SD calculated from three experiments. *The concentration of LAC is derived from two experiments, as there was insufficient reagent from the same lot for an additional experiment.

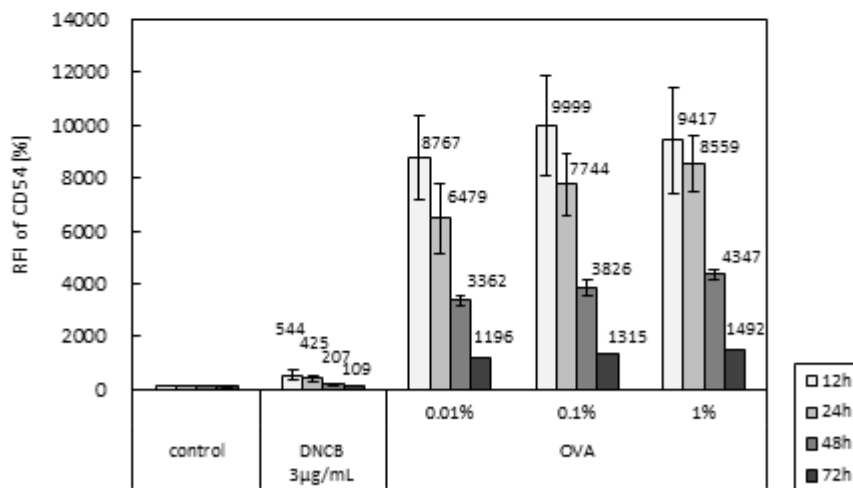
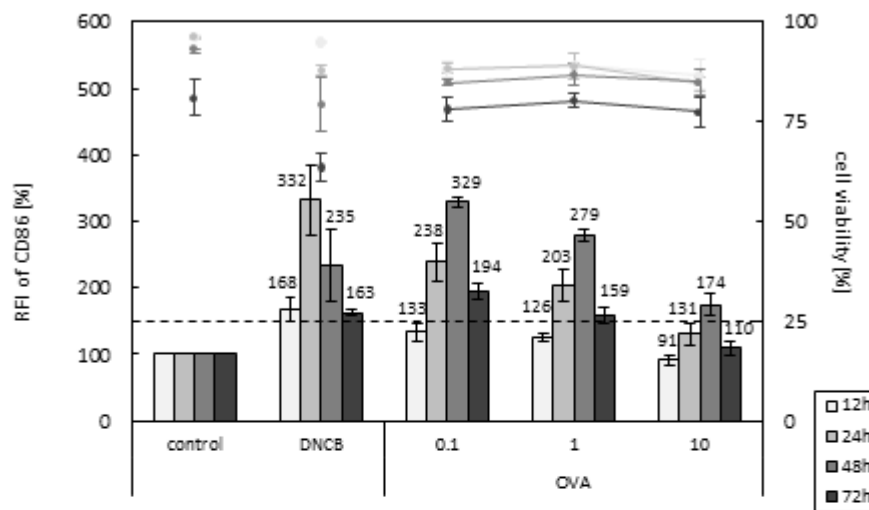
Table 2. Amount of LPS per milligram among lots and grades of OVA.

OVA		LPS [EU/mg]	SD
A ($\geq 98\%$ purity)	1	2852	231
	2	657	37.6
	3	315	15.8
B ($\geq 98\%$ purity)		141	15.0
C ($\geq 90\%$ purity)		2191	101

Data show the mean endotoxin concentrations for all test proteins (endotoxin unit; EU/mg) and SD calculated from three experiments.

Fig.1

1A



1B

Fig. 1 Effect of exposure time and concentration of ovalbumin (OVA) on CD86 and CD54 expression.

Fig. 1 Effect of exposure time and concentration of ovalbumin (OVA) on CD86 and CD54 expression. THP-1 cells were treated with OVA for 12, 24, 48, and 72 h. After exposure, (A) CD86 expression, cell viability, and (B) CD54 expression were quantified using flow cytometry. Mean values of RFI $\pm$ SD for three individual experiments are shown.

Fig.2

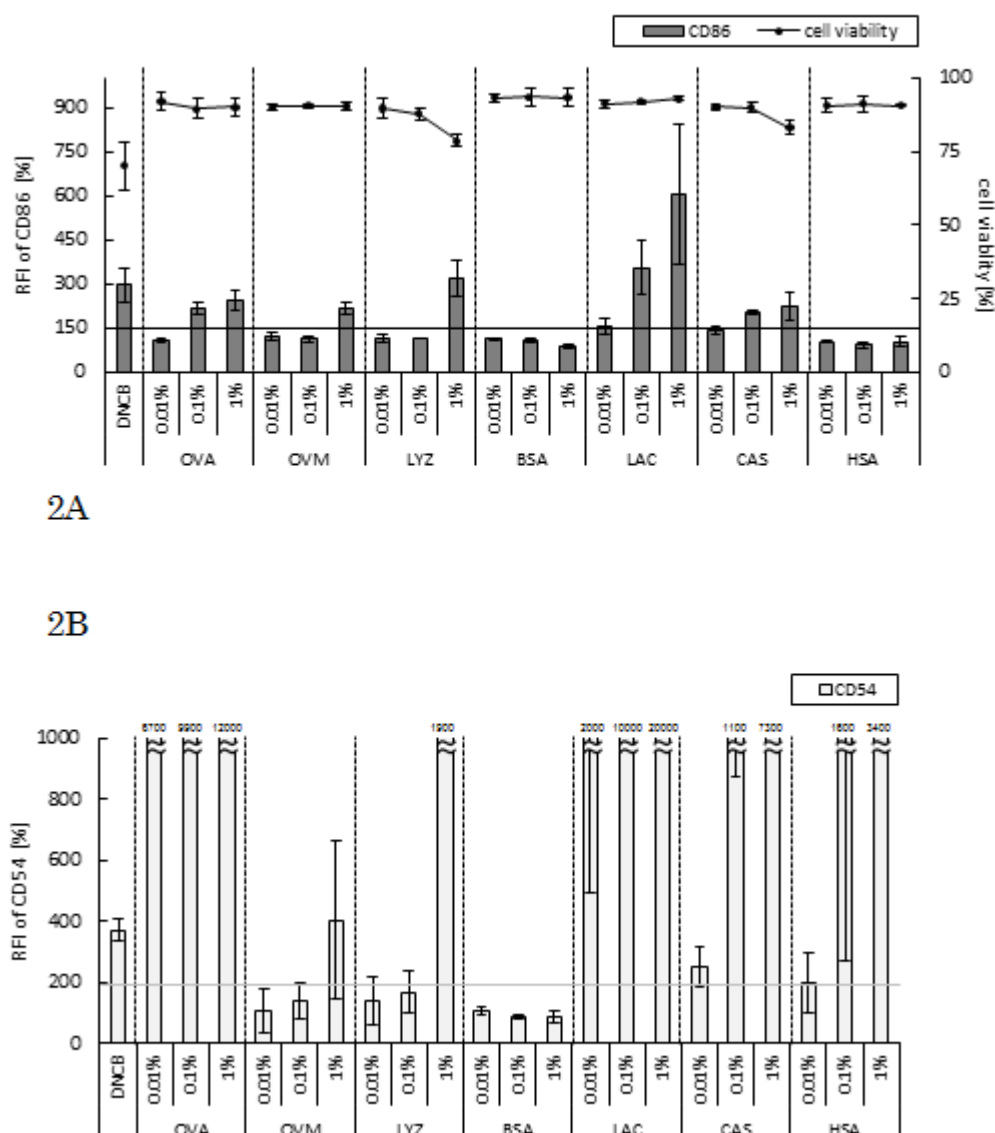
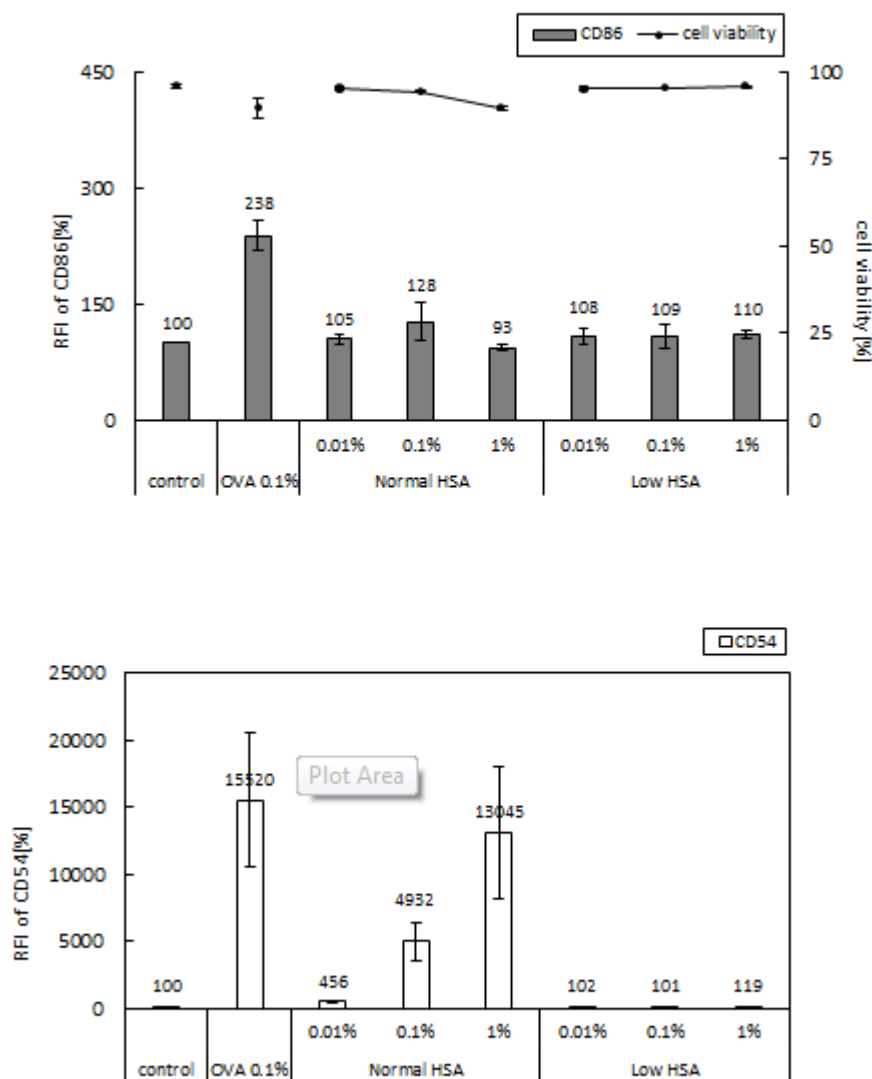


Fig. 2 CD86 and CD54 expression for test proteins.

Fig. 2 CD86 and CD54 expression induced by test proteins. THP-1 cells were treated with test proteins at concentrations of 0.01, 0.1, and 1% for 24 h, and their (A) CD86 cell surface expression, cell viability, and (B) CD54 cell surface expression were analyzed using flow cytometry. Data are shown as relative fluorescence intensity (% of control). Mean values of RFI $\pm$ SD for three individual experiments are shown. Black and gray lines indicate the criteria for h-CLAT (CD86 $\geq$ 150%, CD54 $\geq$ 200%).

Fig.3

3A

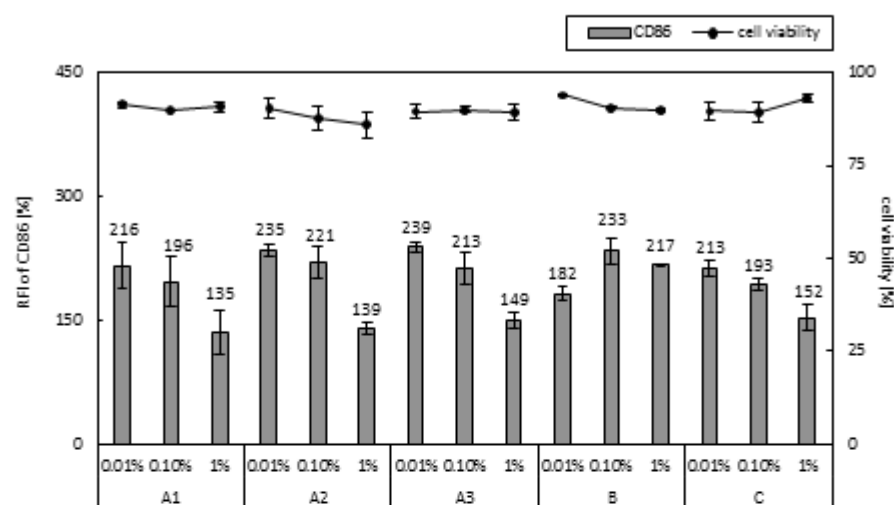


3B

Fig. 3 Effect of endotoxin dose in human serum albumin (HSA) on CD86 and CD54 expression. THP-1 cells were treated with Normal HSA and Low HSA for 24 h. Low HSA included under 1 EU/mg of endotoxin (i.e., LPS). Furthermore, (A) CD86 expression, cell viability, and (B) CD54 expression were quantified using flow cytometry. Data are shown as relative fluorescence intensity (% of control). Mean values of RFI $\pm$ SD for three individual experiments are shown. Lines indicate the criteria for h-CLAT.

Fig. 4

4A



4B

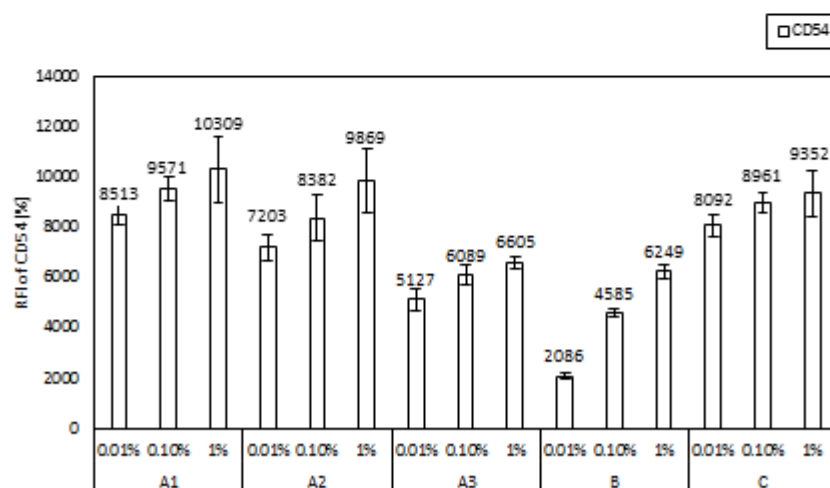
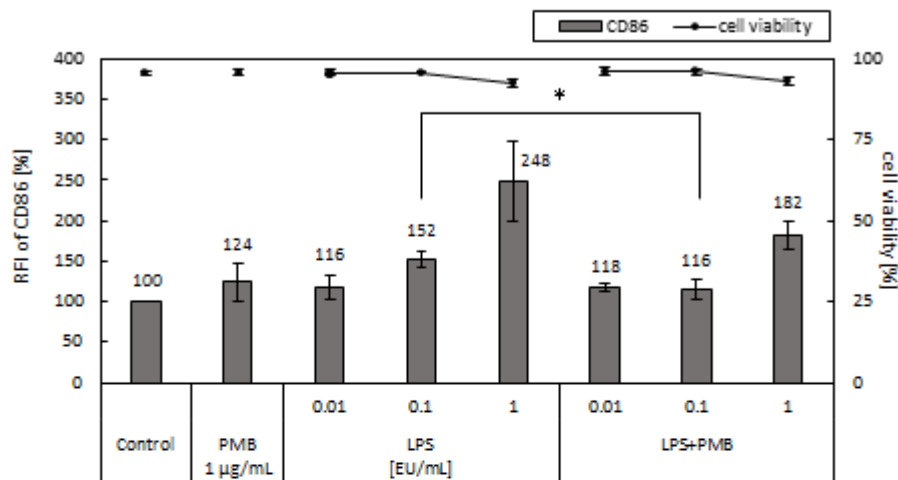


Fig. 4 Effect of lot and grade of OVA on CD86 and CD54 expression. THP-1 cells were cultured with three lots (A1, A2, A3, $\geq 98\%$ purity) and grades (B $\geq 98\%$ purity, C $\geq 90\%$ purity) of OVA at concentrations of 0.01, 0.1, or 1% for 24 h. (A) CD86 expression, cell viability, and (B) CD54 expression were measured by flow cytometry. Data are shown as relative fluorescence intensity (% of control). Mean values of RFI $\pm$ SD for three individual experiments are shown.

Fig. 5

5A



5B

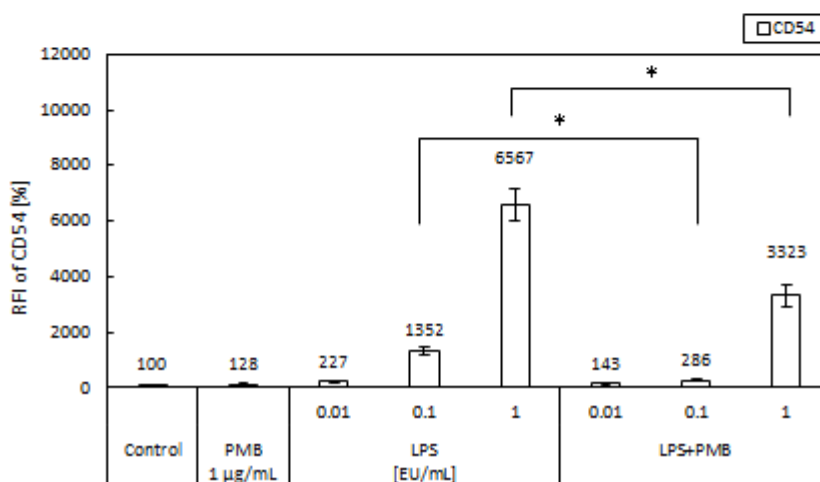
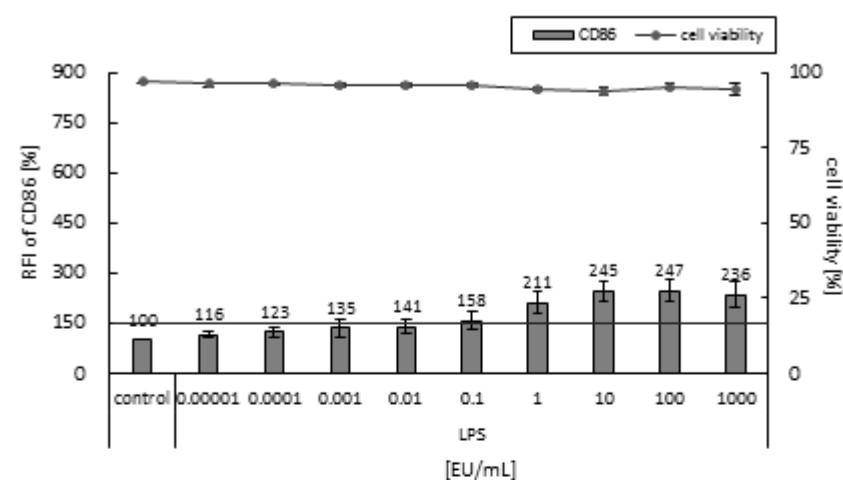


Fig. 5 Effect of Polymyxin B (PMB) on the CD86 and CD54 expression induced by lipopolysaccharide (LPS).

Fig. 5 Effect of polymyxin B (PMB) on CD86 and CD54 expression induced by lipopolysaccharide (LPS). PMB was diluted with culture medium to 4 µg/mL. LPS was diluted with culture medium to 0.04, 0.4, or 4 EU/mL. PMB solution was added to LPS solution at a ratio of 1:1. The mixture was incubated for 30 min at room temperature, and THP-1 cells were treated with this mixture containing PMB (final concentration, 1 µg/mL) and LPS (final concentration, 0.01, 0.1, or 1 EU/mL) for 24 h. (A) CD86 expression, cell viability, and (B) CD54 expression were evaluated using flow cytometry. Data are shown as relative fluorescence intensity (% of control). Mean

values of RFI $\pm$ SD for three individual experiments are shown. Statistically significant differences between corresponding dilutions of LPS-treated and LPS+PMB-treated preparations are shown (* $p < 0.05$).

Fig. 6



6A

6B

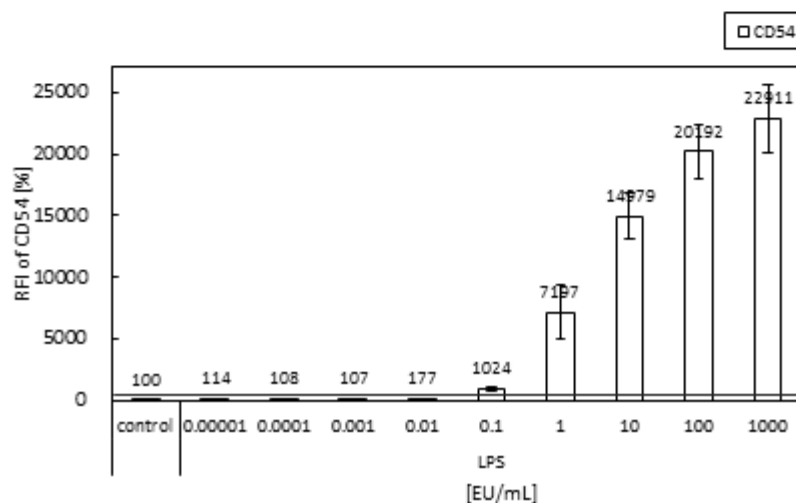


Fig. 6 Effect of LPS treatment on CD86 and CD54 expression. LPS was diluted with culture medium to 0.00001–1000 EU/mL, and THP-1 cells were treated with the LPS solution for 24 h. (A) CD86 expression, cell viability, and (B) CD54 expression were measured by flow cytometry. Data are shown as relative fluorescence intensity (% of control). Mean values of RFI $\pm$ SD for

three individual experiments are shown.

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