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Data integration of non-animal tests for the development of a test battery to predict the skin sensitizing potential and potency of chemicals

Yuko Nukada, Masaaki Miyazawa *, Saitou Kazutoshi, Hitoshi Sakaguchi, Naohiro Nishiyama

Safety Science Research Laboratories, Kao Corporation, 2606 Akabane, Ichikai-Machi, Haga-Gun, Tochigi 321-3497, Japan

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ABSTRACT

Recent changes in regulatory restrictions and social views against animal testing have accelerated development of reliable alternative tests for predicting skin sensitizing potential and potency of many chemicals. Lately, a test battery integrated with different *in vitro* tests has been suggested as a better approach than just one *in vitro* test for replacing animal tests. In this study, we created a dataset of 101 test chemicals with LLNA, human cell line activation test (h-CLAT), direct peptide reactivity assay (DPRA) and *in silico* prediction system. The results of these tests were converted into scores of 0–2 and the sum of individual scores provided the accuracy of 85% and 71% for the potential and potency prediction, compared with LLNA. Likewise, the straightforward tiered system of h-CLAT and DPRA provided the accuracy of 86% and 73%. Additionally, the tiered system showed a higher sensitivity (96%) compared with h-CLAT alone, indicating that sensitizers would be detected with higher reliability in the tiered system. Our data not only demonstrates that h-CLAT can be part of a test battery with other methods but also supports the practical utility of a tiered system when h-CLAT and DPRA are the first screening methods for skin sensitization.

1. Introduction

Due to changes in public views and regulatory requirements, concerted efforts are now being made toward development and use of alternative *in vivo* tests. Allergic contact dermatitis resulting from skin sensitization, provoked by repeated skin contact with causative low molecular chemicals, is a significant environmental and occupational health concern (Kimber et al., 2011). Thus, development of *in vitro* tests to predict sensitizing chemicals will have a broad and major impact on the global public health and enable the industry to eliminate the use of experimental animals for assessing skin sensitization potential, which is important in the overall safety evaluation.

To cause skin sensitization, a chemical must be able to penetrate through the stratum corneum and form a conjugate with protein. Then, skin-resident dendritic cells (DCs) become activated once they recognize the complex, induce the dynamic changes in surface phenotype and function, and migrate into draining lymph nodes, where DCs present the complexes to T cells. Thus, several key events are required for acquisition of skin sensitization. Because skin penetrating potentials are predictable by physicochemical properties, numerous efforts for the development of *in vitro* tests that predict sensitizing potential of chemicals have been made focusing on the interaction of chemicals with proteins or DC activation. The direct peptide reactivity assay (DPRA) developed by Gerberick et al. (2004, 2007a) focuses on the rationale that a chemical's reactivity to proteins is the critical process on its ability to act as a skin sensitizer. Test chemicals are incubated with synthetic model peptides for 24 h and peptide depletions are monitored by HPLC. According to the extensive dataset of 82 test chemicals, the peptide-binding potentials assessed in DPRA correlated well with the *in vivo* skin sensitizing potentials determined by the local lymph node assay (LLNA), in which ^{3}H-thymidine uptake by lymph node cells is measured as the endpoint after topical application of test chemical to mice (Gerberick et al., 2007b). To test the induction mechanism for DC activation, various DC preparations (derived from peripheral blood) or cell line surrogates (e.g., THP-1 (a human monocytic leukemia cell line) and U937 (a human histiocytic lymphoma cell line)) with DC-like characteristic features have been examined for phenotypic markers of activated

Abbreviations: BN ITS, bayesian network integrated testing strategy; CV75, tested concentration providing a cell viability of 75% in the h-CLAT; DC, dendritic cell; DPRA, direct peptide binding assay; EC150, estimated concentration of RFI = 150 for CD86 in the h-CLAT; EC200, estimated concentration of RFI = 200 for CD54 in the h-CLAT; EC3, estimated concentration that produces a stimulation index of 3 in the LLNA; h-CLAT, human Cell Line Activation Test; LLNA, local lymph node assay; MFI, geometric mean fluorescence intensity; MIT, minimum induction threshold; RFI, relative fluorescence intensity.

* Corresponding author. Tel.: +81 285 68 7342; fax: +81 285 68 7452.
E-mail address: miyazawa.masaaki@kao.co.jp (M. Miyazawa).

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DC (e.g., CD86 and CD54). We have recently developed the human cell line activation test (h-CLAT) to assess the changes of CD86 and CD54 expression on THP-1 cells by flow cytometry after a 24 h exposure to test chemical (Sakaguchi et al., 2006; Ashikaga et al., 2010). The h-CLAT displayed an overall accuracy of 84% to the LLNA data of 100 chemicals (Ashikaga et al., 2010). Currently, the DPRA and h-CLAT are formally undergoing pre-validation by the European Centre for the Validation of Alternative Methods (ECVAM). In addition, some commercially available *in silico* models such as DEREK (Sanderson and Earnshaw, 1991) and TIMES (Dimitrov et al., 2005) have been also developed based on the Structure Activity Relationship (SAR).

However, each single *in vitro* test cannot serve as a stand-alone assay to fully replace animal testing such as the LLNA, because the *in vitro* assay is not designed to assess all the chemical, physicochemical, biological properties that are required for a given chemical to cause allergic contact dermatitis. Recently, several battery approaches of *in vitro* tests focusing on the key steps of skin sensitization process and/or *in silico* models have been proposed for the complete replacement of the LLNA. The original integration framework proposed by Jowsey et al. (2006) was the scoring system, weighing the evidence from SAR, penetration, peptide reactivity, and dendritic cell and T-cell activation to evaluate the sensitizing potential as well as the relative potency. Natsch et al. (2009) proposed the scoring integration system based on the chemical dataset from two *in vitro* tests and an *in silico* model. Jaworska et al. (2011) also developed a Bayesian Network Integrated Testing Strategy (BN ITS) based on the information for bioavailability and the dataset from three *in vitro* tests and an *in silico* model. In the present study, we created a 101 chemical dataset of the h-CLAT, DPRA, and DEREK for data integration based on published and newly generated data. We then applied the original concept by Jowsey et al. (2006) on these data as well as developed a test battery system for optimizing the prediction of skin sensitizing potential and the relative potency. To the best of our knowledge, this is the first report documenting any impact of h-CLAT on the ITS for a large-scale chemical dataset.

2. Materials and methods

2.1. Test chemicals

We selected a total of 101 test chemicals based on their skin sensitizing potential reported in the literature (Gerberick et al., 2005, 2007a; Ashikaga et al., 2010), diverse chemical structures, and availability from commercial sources. This collection represented a good distribution of 9 extreme, 15 strong, 30 moderate, and 22 weak sensitizers along with 25 non-sensitizers, as summarized in Table 1. All tested chemicals were purchased at highest possible purity from Sigma–Aldrich (St. Louis, MO, USA), except for lillial and 2-hydroxypropyl methacrylate (Wako Pure Chemical Industries, Osaka, Japan), tetrachlorosalicylanilide (Eastman Kodak Company, Rochester, NY, USA), methyldibromo glutaronitrile (Alfa Aesar, Ward Hill, MA, USA), and 5-methyl-2,3-hexandione (Penta MFG, Livingston, NJ, USA).

2.2. human Cell Line Activation Test (h-CLAT)

The DC like cell activation assay using THP-1 cells, termed h-CLAT, was performed as described by Ashikaga et al. (2010). Briefly, THP-1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). THP-1 cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (MP Biomedicals, Irvine, CA, USA), 0.05 mM 2-mercaptoethanol, and 1% (v/v) antibiotic–antimycotic (Invitrogen). Test chemicals were dissolved in saline or dimethyl sulfoxide (DMSO). The final DMSO concentration in the assay medium did not exceed 0.2%. For the cytotoxicity testing, THP-1 cells (1×10^6 cells/mL/well) were treated with a 2-fold serial dilution of eight concentrations of test chemical in 24 well plates and incubated for 24 h. The cells were washed twice with staining buffer, PBS containing 0.1% BSA (Sigma–Aldrich), stained with propidium iodide (PI), and analyzed by flow cytometry, FACS Calibur (Becton Dickinson, San Jose, CA, USA) and CELLQUEST software. The concentration resulting in 75% cell viability, termed CV75, was calculated based on the analysis of viable cells. In the main experiment, cells were incubated for 24 h with test chemicals at eight concentrations of a 1.2-fold serial dilution starting at $1.2 \times$ CV75. Then, the cells were resuspended with staining buffer containing 0.01% globlins Cohn fraction II/III (Sigma–Aldrich) for the Fc receptor-blocking and incubated for 15 min at 4 °C. Cells were stained for 30 min at 4 °C with the following fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies: mouse IgG1 (clone: DAK-G01), anti-human CD54 (clone: 6.5B5) from Dako (Glostrup, Denmark), and anti-human CD86 (clone: Fun-1) from BD Pharmingen (San Diego, CA, USA). After washing and resuspending, the cells were stained with PI and the fluorescence intensity of the viable cells was analyzed by flow cytometry. For all tested chemicals including the chemicals taken from the previous work, the above experiment was repeated three times. Each experiment was considered an individual replicate. Based on these experiments, the relative fluorescence intensity (RFI) of CD86 and CD54 was determined at more than 50% of cell viability as reported by Nukada et al. (2011). If the RFI of CD86 or CD54 was greater than 150% or 200% at any dose in at least two out of three experiments, the test chemical was judged as a sensitizer. Otherwise, it was considered a non-sensitizer. From the dose-dependency curves of three experiments, the median concentration inducing 150% of CD86 RFI or 200% of CD54 RFI (EC150 or EC200) was calculated like EC3 value determination in the LLNA. The resulting EC value was defined as minimal induction threshold (MIT).

2.3. Direct peptide reactivity assay (DPRA)

An *in chemico* method for reactivity of a test chemical with model peptides was developed by Gerberick et al. (2004, 2007a). Briefly, model heptapeptides of cysteine (Ac–RFAACAA–COOH) and lysine (Ac–RFAAKAA–COOH) were obtained from Synbiosci (Livermore, CA, USA). 1.25 mM peptide stock solution prepared in buffer and a 100 mM test chemical stock solution prepared in either acetonitrile (Sigma) or DMSO/acetonitrile were added to 100 mM sodium phosphate buffer (pH 7.5) for the cysteine peptide or 100 mM ammonium acetate buffer (pH 10.2) for the lysine peptide. The final reaction, containing 0.5 mM of the peptide and 5 (for cysteine) or 25 mM (for lysine) of test chemical was mixed in triplicate and incubated in the dark for 24 h at 25 °C. The free peptide was quantified by reverse phase HPLC on a Waters Alliance 2695 system (Waters Corporation, Milford, MA, USA) on a Zorbax SB-C18 column (3.5 μm, 100×2.1 mm) with UV detection at 220 nm (Waters 996 PDA detector). For each model peptide, percent depletion was determined based on the decrease in non-reacted peptide concentration in the sample relative to the average concentration measured in the control. When the mean peptide depletion of cysteine and lysine peptide was greater than 6.376% (Gerberick et al., 2007a), the test chemical was judged as a sensitizer. The value of 6.376% mean peptide depletion was found by Gerberick et al. (2007a) to distinguish between a non-sensitizer (when mean peptide depletion was below 6.376%) and a sensitizer (when mean peptide depletion was above 6.376%) If the mean peptide depletion was found to be below 6.376% in our study, the chemical was judged to be a non-sensitizer. For all tested chemi-