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B cell increases and *ex vivo* IL-2 production as secondary endpoints for the detection of sensitizers in non-radioisotopic local lymph node assay using flow cytometry

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ABSTRACT

Non-radioisotopic local lymph node assay (LLNA) using 5-bromo-2′-deoxyuridine (BrdU) with flow cytometry (FCM) is gaining attention since it is free from the regulatory issues in traditional LLNA (tLLNA) accompanying *in vivo* uses of radioisotope, ^{3}H-thymidine. However, there is also concern over compromised performance of non-radioisotopic LLNA, raising needs for additional endpoints to improve the accuracy. With the full 22 reference substances enlisted in OECD Test Guideline No. 429, we evaluated the performance of LLNA:BrdU-FCM along with the concomitant measurements of B/T cell ratio and *ex vivo* cytokine production from isolated lymph node cells (LNCs) to examine the utility of these markers as secondary endpoints. Mice (Balb/c, female) were topically treated with substances on both ears for 3 days and then, BrdU was intraperitoneally injected on day 5. After a day, lymph nodes were isolated and undergone FCM to determine BrdU incorporation and B/T cell sub-typing with $B220^{+}$ and $CD3e^{+}$. *Ex vivo* cytokine production by LNCs was measured such as IL-2, IL-4, IL-6, IL-12, IFN-γ, MCP-1, GM-CSF and TNFα. Mice treated with sensitizers showed preferential increases in B cell population and the selective production of IL-2, which matched well with the increases in BrdU incorporation. When compared with guinea pig or human data, BrdU incorporation, B cell increase and IL-2 production *ex vivo* could successfully identify sensitizers with the accuracy comparable to tLLNA, suggesting that these markers may be useful for improving the accuracy of LLNA:BrdU-FCM or as stand-alone non-radioisotopic endpoints.

1. Introduction

Skin contact with chemicals may lead to contact allergic dermatitis and other skin diseases. Therefore, an accurate evaluation of chemical-induced adverse effects on skin is important in the area of dermatotoxicology and for the development of safe pharmaceutical and industrial chemicals. The murine local lymph node assay (LLNA) is a validated model for the evaluation of contact allergic potential of chemicals, which has been endorsed by OECD (Organization for Economic Cooperation and Development) for an alternative to the conventionally used guinea pig tests (OECD_TG406, 1992). In traditional LLNA(tLLNA), sensitization response is examined through the measurement of proliferating lymphocytes with ^{3}H-thymidine uptake, but many countries have a strict regulation on the *in vivo* use of radioisotopes (Gerberick et al., 2002, 2007), hindering the dissemination of tLLNA. Recently, to avoid the *in vivo* use of ^{3}H-thymidine, many attempts have been made to introduce non-radioisotopic endpoints in LLNA. Among them, the measurement of 5-bromo-2′-deoxyuridine (BrdU) incorporation (Takeyoshi et al., 2001) is gathering a huge interest, owing to its mechanistic equivalency to tLLNA. For the measurement of BrdU incorporation into lymph node, several antibody-based assay methods are available including flow cytometric analysis (LLNA:BrdU-FCM) (Jung et al., 2010; Suda et al., 2002), immunohistochemistry (LLNA:BrdU-IHC) (Boussiquet-Leroux et al., 1995; Jung et al., 2010; Lee et al., 2002) and ELISA (LLNA:BrdU-ELISA) (Takeyoshi et al., 2001). Previously, with 7 reference substances, we compared the performance of LLNA:BrdU-FCM and LLNA:BrdU-IHC

Abbreviations: LLNA, local lymph node assay; tLLNA, traditional local lymph node assay; BrdU, 5-bromo-2′-deoxyuridine; FCM, flow cytometry.

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and demonstrated that LLNA:BrdU-FCM is superior in sensitivity which is comparable to tLLNA (Jung et al., 2010). However, there is a concern over low signal to noise ratios and consequently, compromised accuracy in non-radioisotopic LLNA (Takeyoshi et al., 2006), raising the needs for assessing the accuracy of LLNA:BrdU-FCM sufficiently with more reference substances.

In the same context, many efforts have been made to develop additional endpoints to improve the accuracy of non-radioisotopic LLNA. Allergen-treatment induces a significant increase in lymphocyte population (Gerberick et al., 1999, 2002; Inman, 1966). Both B cell and T cell populations are known to increase but a more specific and prominent expansion is observed with B cell population in response to sensitizers when compared with irritants. Flow cytometric analysis of the positivity of B220, an isoform of a transmembrane protein expressed on B cells that assists the activation of the cells, can provide a specific marker to measure B cell expansion (Gerberick et al., 2002). Fortunately, flow cytometry method allows an analysis of multiple parameters at one scope (Bilsland et al., 2006), which enables the simultaneous measurement of both the immunophenotyping of lymph node cells (LNCs) and BrdU incorporation.

Contact allergens provoke the production of pro-inflammatory cytokines (Dearman et al., 1996a,b, 2003; Ku et al., 2008; Manetz et al., 2001; Van Och et al., 2002; Vandebriel et al., 2000). The development of immune reaction by skin sensitizers is orchestrated by the activity of lymphocytes and their cytokine production. In skin sensitization, the major role is played by the cytokines secreted by T_H1 cells that include IL-2 and IFN-γ but significant changes are also observed with other pro-inflammatory cytokines and anti-inflammatory cytokines such as IL-4, IL-6, IL-10, GM-CSF, MCP-1 and TNFα (Azam et al., 2005; Dearman et al., 2003; He et al., 2001; Ku et al., 2008). Therefore, cytokine production *ex vivo* from the isolated LNCs from draining lymph node has been frequently examined to determine sensitizers and to discriminate from irritants or non-sensitizers. However, detailed evaluation and comparison of *ex vivo* cytokine production with other endpoints of LLNA using a sufficiently large number of chemicals has not been done to our best knowledge.

In this study, we performed LLNA:BrdU-FCM with 22 recommended reference substances (18 minimum and 4 optional) composed of 13 sensitizers, 5 non-sensitizer, 3 false positives and 1 false negative enlisted in OECD test guideline No. 429 (OECD_TG429, 2010) to evaluate its performance. In addition, *ex vivo* cytokine assay and flow cytometric measurement of B/T cell population in LNCs were conducted which could be done with the surplus LNCs without sacrificing extra animals. We evaluated the performance of respective endpoints and compared them with tLLNA and real human data to provide an insight into the performance of these methods in the evaluation of sensitization potentials.

2. Material and methods

2.1. Chemicals and reagents

A total of 22 reference substances were selected from OECD test guideline No. 429 (OECD_TG429, 2010) to include 13 sensitizers, 5 non-sensitizers and 4 optional substances. The details and tested concentrations of the 22 reference substances tested are presented in Table 1. Selected 3 concentrations appropriately include the known LLNA EC3 values for sensitizers that were free from systemic toxicity and/or excessive local skin irritation. For compounds without known EC3 (negative) value, we checked the irritation potential in preliminary test with a concentration range that starts from the maximum soluble concentration to find the concentrations free from excessive irritation as advised in OECD TG429. BrdU was dissolved in phosphate-buffered saline (PBS) at a concentration of 10 mg/ml. Phycoerythrin (PE) conjugated rat monoclonal anti-mouse CD45R/B220$^+$ and APC conjugated hamster monoclonal anti-mouse CD3e$^+$ were purchased from BD Pharmingen™(BD Biosciences, Franklin Lakes, NJ) The following cytokine ELISA kit were purchased from Becton Dickinson ((Mississauga, Canada): Mouse IL-2, Mouse IL-4, Mouse IL-6, Mouse IL-12, Mouse IFN-γ, Mouse TNFα, Mouse GM-CSF and Mouse MCP-1).

2.2. Animal and experimental protocol

Both the animal care and study protocol employed were in accordance with Institutional Animal Care and Use Committee (IACUC) of Amorepacific R&D center. They were kept under controlled conditions of temperature (23 ± 3 °C) and relative humidity (50 ± 10%) with alternating 12 h light and dark. Throughout the study, animals had *ad libitum* access to tab water. Female Balb/c mice (7–8 weeks old, body weight 18–22 g) were purchased from OrientBio (Seoul, Korea), and were used in all experiments. After they were kept for at least 1 week on laboratory solid diet (Purina Co., Korea), all animals were used at the age of 8–9 weeks. This study was performed according to the method of Jung et al. (2010) with minor modifications. Groups of mice (N = 4 or 6) were treated with 25 μl of the test substances in vehicle or vehicle alone on the back of both ears daily for 3 consecutive days (Days 1–3). On Day 5, mice were intraperitoneally injected with BrdU and were sacrificed after a day. After sacrifice, auricular lymph nodes were isolated, weighed and undergone lymphocyte preparation. After bilateral auricular lymph nodes were pooled on individual basis, lymph node cells (LNCs) were prepared by disaggregation through 70 μm mesh (BD Biosciences, Franklin Lakes, NJ) in 1 ml PBS. The LNCs were counted using a hemacytometer after stained with trypan blue.

2.3. Flow cytometry analysis – BrdU incorporation

The LNCs (1.5×10^6/ml) were centrifuged (500 g) for 5 min in PBS and re-suspended for fixation and permeabilization step, according to the instruction manual of BrdU Flow kits (BD Pharmingen™, Franklin Lakes, NJ). Then LNCs were permeabilized using Cytoperm plus buffer, which contains 10% DMSO. After DNA was denatured by incubating in DNase for 1 h, LNCs were washed, and incubated with FITC conjugated anti-BrdU antibody at a dilution of 1:50 for 20 min at RT in the dark. Cells were washed once more and then re-suspended in 20 μl of 7-AAD solution to label DNA. Ten thousand 7-AAD expressing cells were gated, and the number of the cells expressing BrdU was analyzed using BD FACSCalibur™ system. Similar to tLLNA, LLNA:BrdU-FCM adopts a stimulation index (SI) value to distinguish skin sensitizers from non-sensitizers. The SI in the LLNA: BrdU-FCM is the ratio of the mean number of LNCs with incorporated BrdU from mice in each of the test substance dose groups to the mean number of LNCs with incorporated BrdU from mice in the vehicle control group.

2.4. Flow cytometry analysis – B cell and T cell

The LNCs (1.5×10^6/ml) were centrifuged (500 g) for 5 min in PBS and re-suspended in staining buffer. For double staining, LNCs were incubated with 5 μl of with hamster anti-mouse CD3e and rat anti-mouse CD45R/B220 in staining buffer for 15 min at 4 °C, and washed again. Ten thousand cells were gated, and the number of the cells expressing CD45R/B220 and CD3e was analyzed using BD FACSCalibur™ system. The SI was defined as the increase in total B cells in the substance-treated group relative to that in the vehicle control group. An SI of 3 was defined as the cut-off value to identify sensitizers.

2.5. Measurement of cytokines (ELISA assays)

Isolated lymphocytes were cultured in RPMI 1640 (Sigma), 10% fetal bovine serum (Invitrogen), and 100 μg/ml-100 U/ml penicillin–streptomycin. Cell suspensions (5×10^6 cells/ml) were seeded into a collagen-coated 96-well tissue culture plate and cultured at 37 °C in 5% CO_2 for 24 h. For cytokine assay, samples from culture supernatants (100 μl) were used. The concentrations of cytokines were determined in duplicate using commercial Opt EIA Mouse set according to the manufacturer's instructions (BD Bioscience, Mississauga, Canada). OD was measured at 450 nm using a FLEX station3 (Molecular Devices, Sunnyvale, CA). Results were expressed as means ± standard deviation (SD).

2.6. Statistics

The EC3 value is defined as the estimated concentration that yields an SI value of 3. Among three methods of estimating EC3 values from the SI values, we used linear interpolation method (Basketter et al., 1999). The mean EC3 was estimated and classified into the appropriate chemical category (Gerberick et al., 2004). Finally, the sensitivity, specificity and accuracy were calculated as the measure of relevance on the basis of the mean EC3 in order to assess the concordance of the LLNA:BrdU-FCM and LLNA:B-cell results with tLLNA or Guinea Pig Maximization Test/Buehler Test results (ICCVAM, 1999; OECD_TG429, 2010). The statistical significant of the differences between groups was determined by the one way analysis of the variance (ANOVA). In the ANOVA, when significant differences were detected, Dunnett's method as a *post hoc* test was used to compare treatment groups with the appropriate vehicle control group. All the statistical analyses were performed using SPSS (Statistical Package for the Social Sciences, version 13, SPSS Inc., Chicago, IL, USA) and $P < 0.05$ and $P < 0.01$ was considered significant.