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Comparison of flow cytometry and immunohistochemistry in non-radioisotopic murine lymph node assay using bromodeoxyuridine

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ABSTRACT

Non-radioisotopic local lymph node assay (LLNA) employing 5-bromo-2′-deoxyuridine (BrdU) with flow cytometry (FACS) or immunohistochemistry (IHC) is gaining attention due to a regulatory issue of using radioisotope, ^{3}H-thymidine, *in vivo* in traditional LLNA. In this study, to compare the performance of these non-radioisotopic endpoints, 7 chemicals with known sensitizing potencies were examined in LLNA. Mice were topically treated with chemicals or vehicle on both ears for 3 days. After intraperitoneal injection of BrdU, bilateral lymph nodes were isolated separately and undergone respectively, FACS or IHC to determine BrdU incorporated lymph node cells (LNCs). Weight and histology of treated ears were also examined to evaluate chemical-induced edema and irritation. Both FACS and IHC could successively identify the skin sensitizers from non-sensitizers. Comparison of FACS and IHC with traditional LLNA revealed that FACS has a higher sensitivity although both assays produced comparable sensitivity and performance to traditional LLNA. In conclusion, non-radioisotopic LLNA using FACS and IHC can successfully detect sensitizers with a good correlation to traditional LLNA. Notably, FACS showed almost equivalent sensitivity and accuracy to traditional LLNA.

1. Introduction

Mouse local lymph node assay (LLNA) is a validated alternative method for testing the sensitization potential of chemicals, replacing the conventional guinea pig maximization tests (Farrell et al., 2009). This method offers important advantages over guinea pig assays for the animal welfare in terms of reduction and refinement without deteriorating the assay quality, leading to a wide acceptance of LLNA as a representative alternative method for the identification of chemicals with a skin sensitizing potential.

In LLNA, the skin sensitization potential is determined by measuring lymphocyte proliferation in the draining auricular lymph nodes in response to the treated chemicals (Gerberick et al., 2007). Radiolabeling of proliferating lymphocytes using ^{3}H-thymidine has been commonly employed, however many countries have a strict regulation limiting the introduction of radioisotopes into animal *in vivo*, mandating a heavy ventilation, filtering and completely isolated animal housing facilities. Accordingly, traditional LLNA has not been generalized widely, raising an urgent need for the development and the validation of non-radioisotopic endpoints in LLNA.

Recently, to avoid the use of ^{3}H-thymidine *in vivo* and to introduce non-radioisotopic endpoints in LLNA, many attempts have been made. These include the examination of the phenotype of proliferating lymphocyte subsets (Gerberick et al., 1999), the indirect estimation of lymphocyte proliferation by measuring intracellular ATP content of lymph nodes (Idehara et al., 2008) and the assay of *ex vivo* cytokines production by draining lymph node cells (LNCs) (Dearman et al., 1999, 1994; Ku et al., 2008; van den Berg et al., 2005). Of these approaches, the measurement of 5-bromo-2′-deoxyuridine (BrdU) incorporation into DNA (Takeyoshi et al., 2001) is gathering a huge interest, owing to its similarity to the conventional LLNA method employing ^{3}H-thymidine.

BrdU is a non-radioisotopic analog of thymidine, which is incorporated into DNA during the S-phase of the cell cycle. It has substituted ^{3}H-thymidine labeling in many biological assays measuring cellular proliferation (Porstmann et al., 1985). To measure BrdU incorporation into lymph node, many antibody-based assay methods are available including flow cytometric analysis (FACS) (Suda et al., 2002), immunohistochemical staining (IHC)

Abbreviations: LLNA, local lymph node assay; FACS, flow cytometry; PPD, *p*-phenylenediamine; DNCB, 2,4-dinitrochlorobenzene; IS, isopropylalcohol; SLS, sodium lauryl sulfate; BrdU, 5-bromo-2′-deoxyuridine; IHC, immunohistochemistry; HCA, hexylcinnamaldehyde.

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Table 1
Tested chemicals and their reported sensitization potency.

Substance name (abbreviation)	Vehicle	Potency category	EC3[a] (%)	Test concentration (%)
2,4-Dinitrochlorobenzene (DNCB)	AOO	Extreme	0.04	0.01, 0.1, 0.25, 0.5
4-Phenylenediamine (PPD)	AOO	Strong	0.11	0.1, 1, 3
Isoeugenol	AOO	Moderate	1.5	2.5, 5, 10
Hexylcinnamaldehyde (HCA)	AOO	Moderate	9.9	5, 10, 25
Eugenol	AOO	Weak	10.1	5, 10, 25
Isopropylalcohol (IS)	AOO	Negative	–	50
Sodium lauryl sulfate (SLS)	50% ethanol /DMF	Moderate (false positive)	8.1	5, 10, 15

[a] Values from ICCVAM 2009, Recommended Performance Standards: Murine Local Lymph Node Assay (2009, NIH Publication No. 09-7357).

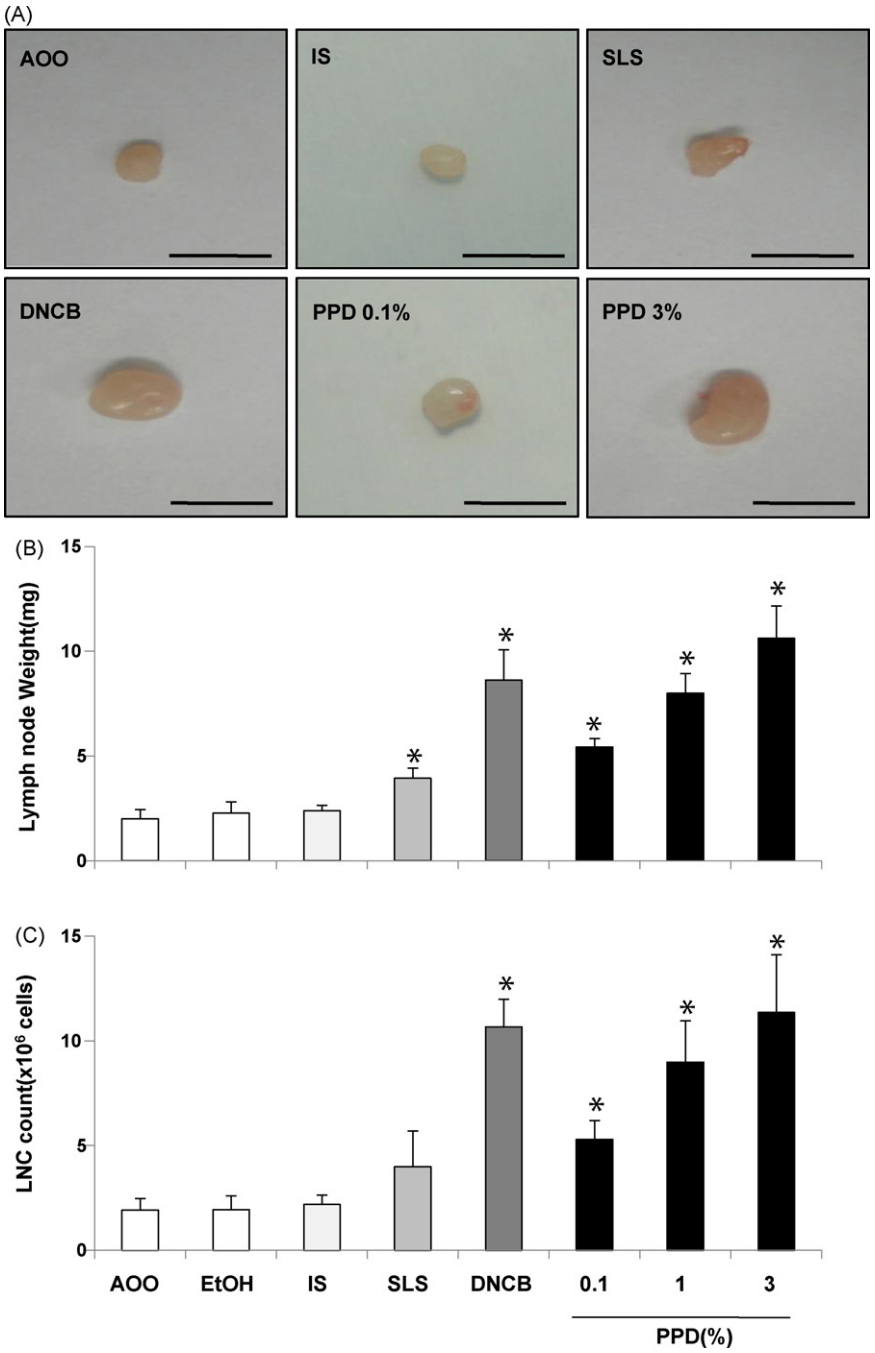

Fig. 1. Changes in auricular lymph node weights and the number of LNCs (lymph node cells). (A) Representative photograph of auricular lymph node from the treated mouse. Bar indicates 5 mm. (B) After mice were treated with 0.1, 1 and 3% PPD, 0.25% DNCB, 15% SLS or 50% IS for 3 days, auricular lymph node was collected on Day 5 and weighed. * Significant difference from vehicle group, $p < 0.05$, values are mean ± S.D. ($N = 5$ or 6). (C) The number of LNC ($\times 10^6$ cells) in an auricular lymph node from treated mice.