Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.4, No.3, 121-126, 2017

Original Article

Utility of murine dendritic cell line DC2.4 for *in vitro* assay of skin-sensitization potential

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(Received May 30, 2017; Accepted May 31, 2017)

ABSTRACT — Animal tests, such as the local lymph node assay (LLNA), are the gold standard for assaying skin-sensitizing potential. However, because of concerns about animal welfare, extensive research has been conducted on the use of various cell lines, such as human leukemia cells, for *in vitro* assays of skin-sensitizing potential, but such assays have not replaced animal tests as stand-alone assays. Because Langerhans cells—a type of dendritic cell—are the main antigen-presenting cells in the epidermis and because they play a central role in the induction of allergic skin disorders, these cells may be useful for skin-sensitizing-potential assays. Here, we investigated the utility of the murine dendritic cell line DC2.4 for *in vitro* assay of the skin-sensitization potential of 2,4-dinitrochlorobenzene (DNCB), 2-mercaptobenzothiazole (MBT), and α-hexyl cinnamaldehyde (HCA), which are categorized as extremely, moderately, and weakly sensitizing, respectively, on the basis of LLNA results. DC2.4 cell viability decreased dose-dependently with increasing concentration upon treatment with each of the compounds for 24 hr; the DNCB, MBT, and HCA concentrations that resulted in 75% cell viability were 6.07, 120.14, and 118.70 μg/mL, respectively. At nontoxic concentrations (concentrations less than the 75% cell viability concentrations), these compounds dose-dependently upregulated the expression of both CD86 and CD54 on the surface of DC2.4 cells. Their potency decreased in the order DNCB > MBT > HCA, which agrees with the order indicated by the LLNA. These results suggest that DC2.4 cells may be a viable replacement for human leukemia cells in *in vitro* assays of skin-sensitization potential.

Key words: Dendritic cell, Allergic contact dermatitis, Local lymph node assay (LLNA), Human cell line activation test (h-CLAT), CD86, CD54

INTRODUCTION

The REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) regulation, which entered into force in the European Union in 2007, requires determination of the skin-sensitizing potential of chemical substances. The Organisation for Economic Co-operation and Development (OECD) adopted the murine local lymph node assay (LLNA; OECD Test Guideline No. 429; OECD, 2002) as a stand-alone test for skin sensitization. One of the merits of the LLNA is that it provides quantitative information on the sensitization potency of a test chemical in the form of an EC3 value (i.e., the estimated concentration required to induce a 3-fold increase in lymph node cell proliferation), which can be used for the risk assessment of finished products containing that chemical (Gerberick *et al.*, 2001; Griem *et al*., 2003; Api and Vey, 2008). The LLNA is currently the first-choice method for assessing sensitization potency (Angers-Loustau *et al*., 2011). However, owing to greater emphasis on animal welfare, the European Union adopted the 7th Amendment to the Cosmetic Directive in 2003, which banned the use of animal testing of cosmetic products and ingredients. Therefore, several non-animal skin sensitization

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test methods have recently been proposed (Basketter *et al*., 2005).

When the skin is exposed to a chemical allergen, the chemical is recognized by Langerhans cells, a type of dendritic cell (DC) that is the principal professional antigen-presenting cell in the epidermis and plays a key role in the development of allergic contact dermatitis. Following an encounter with a chemical allergen, DCs are activated and subsequently migrate from the skin to the draining lymph nodes, undergoing a maturation process during the journey (Kimber and Cumberbatch, 1992; Kimber *et al*., 2000). Mature DCs show upregulation of cell-surface markers such as CD54 and CD86, which in turn results in activation of naïve T-cells. This maturation process is what is detected in the human cell line activation test (h-CLAT) for skin-sensitizing potential, which uses the human monocytic leukemia cell line THP-1 (Ashikaga *et al*., 2006, 2010; Sakaguchi *et al*., 2006). This assay was designed to assess changes in cell-surface CD86 and CD54 expression by means of flow cytometry after 24 hr of exposure to a test chemical. When used to evaluate approximately 100 chemicals, this assay was found to have an accuracy of 84% relative to the LLNA (Ashikaga *et al*., 2010) and was thus adopted as OECD Test Guideline No. 442E in 2016. However, because the h-CLAT is not 100% accurate, it has not replaced the LLNA as a stand-alone assay. The inaccuracy has been attributed to two factors: (1) the fact that THP-1 is a human cell line rather than a mouse cell line (i.e. there is a species difference) and (2) the fact that, under normal culture conditions, THP-1 is a monocyte cell line not a DC line. Addressing these issues can be expected to improve the accuracy of cell-based *in vitro* assays relative to the LLNA.

DC2.4 cells, a line of immature DCs, have dendritic morphology; they express DC-specific markers, major histocompatibility complex molecules, and costimulatory molecules and have phagocytic activity as well as antigen-presenting capacity (Shen *et al*., 1997). In addition, many researchers have used these cells as DCs in immunological research (Fang *et al*., 2010; Hayashi *et al*., 2007; Kato *et al*., 2010; Okada *et al*., 2001; Takekoshi *et al*., 2010). In this study, we evaluated the utility of DC2.4 for a cell-based *in vitro* assay for skin-sensitization potential. Specifically, we assessed whether DC2.4 cells could be used to determine the skin-sensitization potential and potency classification of three test chemicals by analyzing changes in the expression of cell-surface markers by means of flow cytometry.

MATERIALS AND METHODS

Test chemicals

2,4-Dinitrochlorobenzene (DNCB), 2-mercaptobenzothiazole (MBT), and α-hexyl cinnamaldehyde (HCA) were obtained from Wako Pure Chemical Industries (Tokyo, Japan) and used as test chemicals. All the chemicals were dissolved in dimethyl sulfoxide (DMSO, Wako).

Cell culture

DC2.4 cells, a murine DC line (Shen *et al*., 1997), were kindly provided by Dr. Kenneth Rock (University of Massachusetts Medical Center, Worcester, MA, USA). The cells were grown in RPMI1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% heat-inactivated fetal calf serum, 0.1 mM modified Eagle medium nonessential amino acid solution (Nacalai Tesque), and 0.05 mM 2-mercaptoethanol (Nacalai Tesque). The cells were maintained at 37°C in a humidified incubator under 5% CO_2. They were grown to confluence, and the medium was changed every 2 or 3 days.

To determine the effects of the test compounds on cell viability and expression of cell-surface markers, we treated the cells with vehicle alone (0.1% DMSO) or with various concentrations of the test compounds in 0.1% DMSO. In control experiments, treatment with 0.1% DMSO did not alter cell viability or expression of cell-surface markers.

Cytotoxicity tests

DC2.4 cells (2.5×10^4 cells/100 μL/well) were cultured in 96-well plates (NUNC/Thermo Fisher Scientific, Grand Island, NY, USA). After 24 hr of culture, the cells were treated with various concentrations of the test compounds for another 24 hr. At the endpoint of each treatment, 10 μL of a mixture of 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1, Dojindo Laboratories, Kumamoto, Japan) and 1-methoxy-5-methyl-phenazinium methylsulfate (1-methoxy PMS, Dojindo Laboratories) in 20 mM 2-[4-(2-Hydroxyethyl)-1-piperazinyl]ethanesulfonic Acid (HEPES) buffer (pH 7.4) was added to each well (final concentrations: WST-1, 0.5 mM; 1-methoxy PMS, 0.02 mM). The cells were incubated at 37°C for an additional 3 hr, and then the absorbance of each well was measured at 450 nm, with the reference wavelength at 655 nm, using a microplate reader (Nippon Bio-Rad Laboratories, Tokyo, Japan).

The CV75 value—that is, the concentration at which 75% of the DC2.4 cells survived (25% cytotoxicity)—

was calculated by log-linear interpolation as described in OECD Test Guideline No. 442E (h-CLAT) by means of the following equation:

$$\log CV75 = [(75 - c) \times \log d - (75 - a) \times \log b]/(a - c)$$

where a is the minimum cell viability > 75% in the testing groups, c is the maximum cell viability < 75% in the testing groups, and b and d are the concentrations at which the cell viabilities were a and c, respectively.

Flow cytometry

DC2.4 cells (1.5×10^5 cells/1 mL/well) were cultured in 24-well plates (Corning Incorporated, Corning, NY, USA). After 24 hr of culture, the cells were treated with various concentrations of the test compounds for 24 hr. At the endpoint of each treatment, tripsinized cells were harvested and washed with phosphate-buffered saline, and then resuspended in 0.1 mL of phosphate-buffered saline containing 2% fetal calf serum. The cells were blocked with Fc Block (Japan Becton Dickinson Bioscience, Tokyo, Japan) for 15 min at 4°C and then incubated with biotin-conjugated hamster anti-mouse CD54 antibody (Japan Becton Dickinson Bioscience) or biotin-conjugated rat anti-mouse CD86 antibody (Japan Becton Dickinson Bioscience) for 30 min at 4°C. After incubation, the cells were washed again with, resuspended in 0.1 mL of phosphate-buffered saline containing 2% fetal calf serum, and incubated with allophycocyanin (APC)–streptavidin (Japan Becton Dickinson Bioscience) for 30 min at 4°C. For flow cytometry, propidium iodide (100 μg/mL) was added to discriminate dead cells. Stained cells were collected with a flow cytometer (BD FACSVerse, Japan Becton Dickinson Bioscience), and data were analyzed with the FACS Suite software (Japan Becton Dickinson Bioscience).

Geometric means of the raw data were used to calculate relative fluorescence intensity (RFI), an indicator of CD86 and CD54 expression, as follows:

$$RFI\ (\%) = 100 \times (\text{MFI of chemical-treated cells})/(\text{MFI of vehicle-only cells})$$

where MFI is geometric mean fluorescence intensity.

Statistics

Data were analyzed by means of Dunnett's multiple comparisons test (SPSS Software, Chicago, IL, USA). Control and treatment group data were always obtained from equal numbers of replicate experiments, and experiments were performed independently at least twice. A P value of < 0.01 was considered to indicate statistical significance.

RESULTS AND DISCUSSION

We used DNCB, MBT, and HCA, which are categorized by the LLNA as extremely, moderately, and weakly sensitizing, respectively (Table 1), to investigate the utility of the murine DC line DC2.4 in assaying skin sensitization *in vitro*. To confirm the nontoxic concentration ranges of the test compounds in DC2.4 cells, we performed

Table 1. Test chemicals used

Chemical name (CAS No.)	Potency category (EC3 from LLNA[1])	Dose (μg/mL)	MFI2		RFI2		Cell viability (%)[2]
			CD54	CD86	CD54	CD86	
2,4-Dinitrochlorobenzene (97-00-7)	Extreme (0.05 %)	0	2498 ± 230	2498 ± 230	100 ± 9.2	100 ± 9.2	95.5 ± 1.0
		1.5	2950 ± 465	2971 ± 212	118 ± 19	119 ± 8.5	94.1 ± 1.0
		3	4192 ± 94	3559 ± 335	168 ± 3.8*	142 ± 13*	92.2 ± 0.5
		6	5127 ± 109	4181 ± 103	205 ± 4.4*	167 ± 4.1*	76.9 ± 1.6
2-Mercaptobenzothiazole (149-30-4)	Moderate (1.7 %)	0	2651 ± 56	3461 ± 32	100 ± 2.1	100 ± 0.9	97.2 ± 0.5
		17.5	2709 ± 32	3399 ± 106	102 ± 1.2	98 ± 3.1	96.2 ± 1.2
		35	3188 ± 52	4436 ± 164	120 ± 2.0*	128 ± 4.7*	95.0 ± 0.3
		70	3534 ± 173	5114 ± 53	133 ± 6.5*	148 ± 1.5*	81.5 ± 1.4
α-Hexyl cinnamaldehyde (101-86-0)	Weak (11 %)	0	3212 ± 124	4514 ± 385	100 ± 3.9	100 ± 8.5	94.2 ± 0.3
		25	2986 ± 167	5161 ± 351	93 ± 5.2	114 ± 7.8	92.5 ± 1.2
		50	3342 ± 38	5305 ± 186	104 ± 1.2	118 ± 4.1*	92.6 ± 1.1
		100	4804 ± 274	6546 ± 42	150 ± 8.5*	145 ± 0.9*	85.0 ± 1.6

Abbreviations: LLNA, local lymph node assay; MFI, geometric mean fluorescence intensity; RFI, relative fluorescence intensity.
[1]; LLNA data for the test chemicals is adapted from Nukada *et al.* 2012.
[2]; Results are expressed as the mean ± 1 S.D. of triplicate cultures.
*; $P < 0.01$ vs RFI of control group (0 μg/mL), Dunnett's multiple comparisons test.

WST-1 cytotoxicity tests. We found that cel1 viability decreased dose-dependently with increasing concentration for all three compounds (Fig. 1); the CV75 values for DNCB, MBT, and HCA were 6.07, 120.14, and 118.70 μg/mL, respectively.

To assess the responsiveness of DC2.4 to the three chemicals, we treated the cells with the chemicals at concentrations below the CV75 value for 24 hr and then analyzed CD54 and CD86 expression on the cell surface by means of flow cytometry (Fig. 2). For both markers and all three compounds, we found that the MFI values increased dose-dependently with increasing chemical concentration relative to the MFI values for DMSO-treated cells (Table 1). The RFI values for both markers and all three compounds also increased dose-dependently with increasing concentration (Fig. 3). Cells treated with DNCB at 3 or 6 μg/mL and cells treated with MBT at 35 or 70 μg/mL exhibited significant increases ($P < 0.01$) in the RFI values of both CD54 and CD86. Cells treated with HCA at 100 μg/mL also exhibited significant increases ($P < 0.01$) in the RFI values of both CD54 and CD86, whereas HCA at 50 μg/mL significantly ($P < 0.01$) upregulated the RFI of CD86 but not that of CD54. At the test concentrations, treatment with DNCB, MBT, and HCA resulted in cell viability values ranging from 76.9% to 96.2% (Table 1). These results indicate that DC2.4 cells could detect skin sensitization by the test chemicals at nontoxic concentration ranges and that the potency of the chemicals decreased in the order DNCB ≫ MBT > HCA, which is consistent with the order indicated by the LLNA.

THP-1 cells, which are used for OECD Test Guideline No. 442E (h-CLAT), are human monocytic leukemia cells that can be DC precursors. Berges *et al*. (2005) reported that THP-1 cells differentiate rapidly into mature DCs when cultured in serum-free medium containing granulocyte-macrophage colony-stimulating factor, tumor necrosis factor-α, and ionomycin. THP-1-derived DCs are highly pure and monotypic, and they display the morphologic, phenotypic, molecular, and functional properties of DCs generated from human donor-derived monocytes or $CD34^+$ hematopoietic progenitor cells. In addition, THP-1 can differentiate into immature DCs even in culture media supplemented with serum (Berges *et al*., 2005). However, THP-1-derived immature DCs never differentiate into mature DCs, even when the culture medium is supplemented with typical DC differentiation agents, such as lipopolysaccharide, tumor necrosis factor-α, prostaglandin E2, interferon-α, interferon-γ, interleukin-6, interleukin-1β, and CD40 ligand, either alone or in various combinations. These results indicate that special culture conditions are necessary for differentiation of THP-1 cells into mature DCs, even though the cells are potential DC precursors. In contrast, under normal culture conditions, THP-1 cells readily differentiate into macrophages, but not into DCs, in response to stimuli such as phorbol-12-myristate-13-acetate and 1,25-dihydroxyvitamin D_3 (Schwende *et al*., 1996). These results suggest that, under normal culture conditions, THP-1 cells may be inadequate as a DC model in *in vitro* assays of skin-sensitization potential because these cells tend to differentiate into macrophages rather than DCs.

Unlike THP-1 cells, the DC2.4 cell line is well established as an immature DC cell line in mice (Shen *et al*., 1997). This cell line has already been used as a DC model by many immunology researchers. For example, DC2.4 cells have been used to investigate the maturation of DCs regulated by transcriptional factor E2F1 in human and mouse (Fang *et al*., 2010). In addition, THP-1 fails to detect skin sensitization by HCA in the h-CLAT assay (Nukada *et al*., 2012), whereas we found that DC2.4 cells responded positively to HCA. Consequently, DC2.4 cells may be a better DC model than human leukemia cell lines and may have potential for use in *in vitro* skin-sensitization assays instead of THP-1. Future studies are needed to

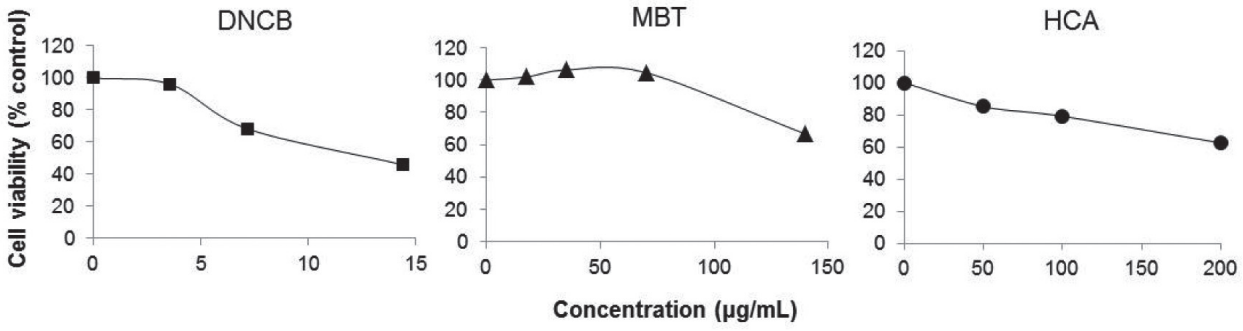

Fig. 1. Results of cytotoxicity tests of 2,4-dinitrochlorobenzene (DNCB), 2-mercaptobenzothiazole (MBT), and α-hexyl cinnamaldehyde (HCA) against DC2.4 cells. Results are expressed as means for duplicate cultures.

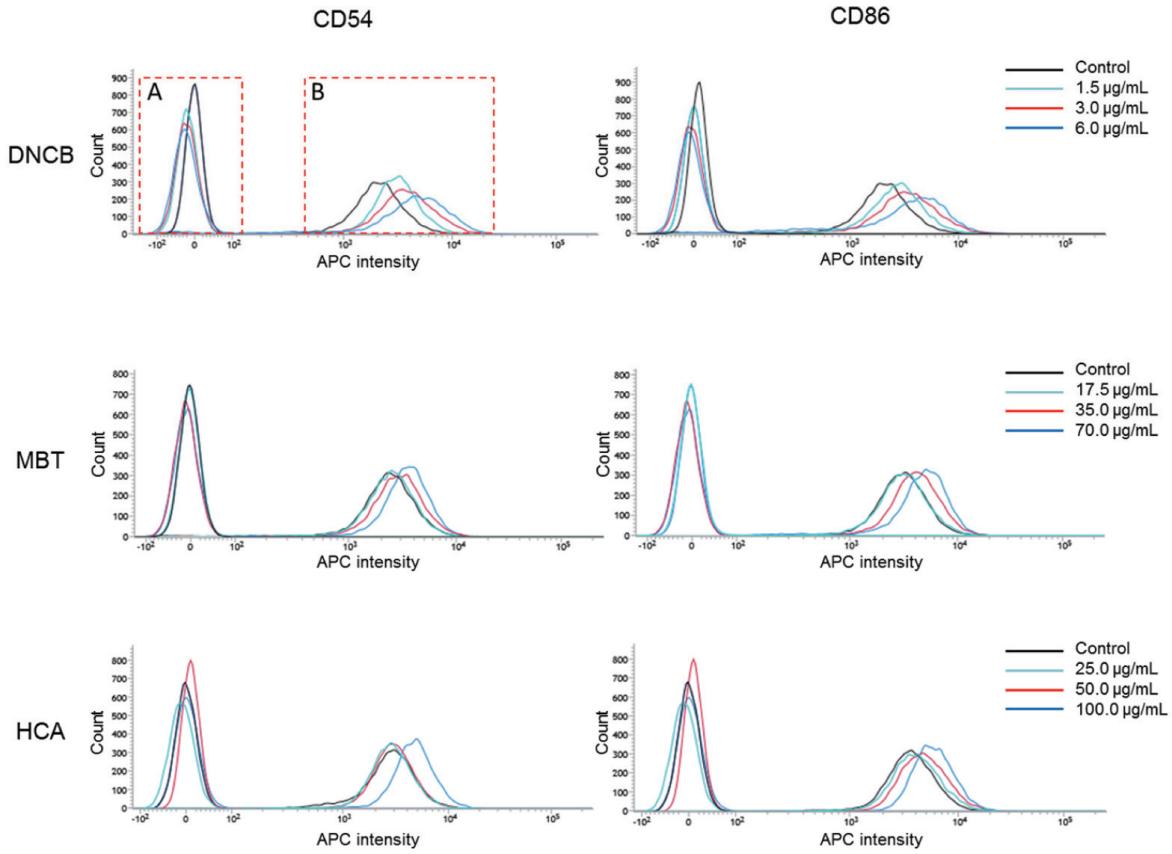

Fig. 2. Histograms showing CD54 and CD86 expression after treatment with 2,4-dinitrochlorobenzene (DNCB), 2-mercaptobenzothiazole (MBT), or α-hexyl cinnamaldehyde (HCA). DC2.4 cells were cultured for 24 hr and then treated with DMSO (control), DNCB, MBT, or HCA. After 24 hr, CD54 and CD86 expression levels on the cell surface were measured by means of flow cytometry, staining without (A) or with (B) each biotin-conjugated specific antibody. The figure shows representative data from three independent experiments. The figure shows representative data from three independent experiments.

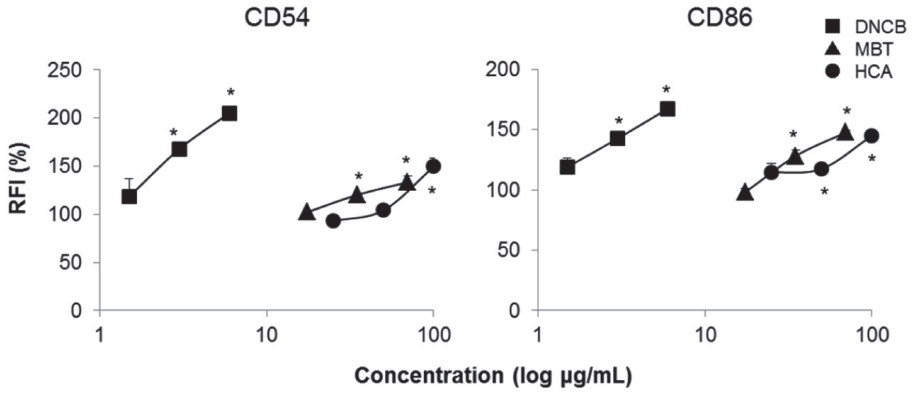

Fig. 3. Effect of treatment with 2,4-dinitrochlorobenzene (DNCB), 2-mercaptobenzothiazole (MBT), or α-hexyl cinnamaldehyde (HCA) on CD54 and CD86. Changes in CD86 and CD54 expression after treatment with the three chemicals for 24 hr are indicated as relative fluorescence intensity (RFI) values. RFI values were calculated from the geometric means of the raw data obtained by flow cytometry (see section "Flow cytometry" and Fig. 2). Results are expressed as means and standard deviations for triplicate cultures. *: $P < 0.01$ vs. RFI of control, as indicated by Dunnett's multiple comparisons test.

confirm the utility of DC2.4 for this purpose.

ACKNOWLEDGMENTS

This research was supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (nos. 16K15150 and 16K00558) and the FY2017 Strategic International Collaborative Research Program (SICORP) of the Japan Science and Technology (JST) Agency.

Conflict of interest---- The authors declare that there is no conflict of interest.

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