LOCAL LYMPH NODE ASSAY WITH NON-RADIOISOTOPE ALTERNATIVE ENDPOINTS

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ABSTRACT — The local lymph node assay has recently been accepted by regulatory agencies as a stand-alone alternate method for predicting allergic contact dermatitis. To compare the sensitivity of non-radioisotope methods with that of the standard assay, we determined if these modified methods would affect evaluation of sensitization potency. For this reason, we used 2,4-dinitrochlorobenzene (DNCB) and benzocaine for different sensitizing criteria.

Female CBA mice were treated for 3 days with a test compound or vehicle applied to each side of both ears. Bilateral auricular lymph node proliferative activity was assessed by the following endpoints with incorporation of ³H-methyl thymidine (3H-TdR), bromodeoxyuridine (BrdU) *in vivo*, and BrdU *ex vivo*, IL-2 production, and proliferating cell nuclear antigen (PCNA) expression. Ear thickness was also tested.

The strong sensitizer DNCB was detectable by any of the non-radioisotope endpoints as well as by radioisotope-dependent standard assay. On the other hand, when evaluating the weak sensitizer benzocaine, significant changes were evident in BrdU incorporation *ex vivo* and *in vivo*, and IL-2 production. We believe that these non-radioisotope methods can assess allergic contact dermatitis caused by chemicals even in the laboratory, where it can be difficult to handle radioisotopes.

KEY WORDS: Local lymph node assay (LLNA), Non-radioisotope method, Skin sensitizer, DNCB, Benzocaine

INTRODUCTION

The murine local lymph node assay (LLNA) is a new test method used to assess allergic contact dermatitis caused by chemicals, as first proposed by Kimber *et al.* in 1989. This method is already mentioned in the OECD Guideline 406 (1992) as a screening test. Since then, the method has been validated and accepted as a stand-alone method by regulatory agencies (Food and Drug Administration, Environmental Protection Agency, Occupational Safety and Health Administration and Consumer Product Safety Commission). Details of this validation and a review of associated work have been published (Chamberlain and Basketter, 1996; Basketter *et al.*, 1996, 1998; NIH Publication, 1999).

The LLNA standard method is as follows: 25 μ l/ear of the appropriate dilution of the test substance or vehicle alone was applied to the dorsum of both ears of female CBA mice daily for 3 consecutive days. Four days after the first application, mice were given, via the tail vein, phosphate-buffered saline (PBS) containing 3 H-methyl thymidine (3H-TdR). Five hours later, draining auricular lymph nodes were excised. Incorporation of 3H-TdR was measured using β -scintillation counting for each mouse. The stimulation index (SI) is the ratio of the mean dpm/mouse for the test substance treatment group versus the mean dpm/mouse for the vehicle treatment control group. The decision process to identify a positive response should include $SI \geq 3$, statistical significance and dose response information.

For decades, the guinea pig maximization test (GPMT) and the Buehler assay (BA) have been the standards used to assess the allergic contact dermatitis potential of chemicals. When compared with these methods, LLNA has several advantages in that it provides quantitative data, fewer animals are needed, color compounds can be evaluated, and much less time is needed. However, the weakness of LLNA is false negative results with weak sensitizers predicted by GPMT. Furthermore, the use of radioisotopes (3 H-methyl thymidine, 125 I-iododeoxyuridine) in LLNA standard protocol renders the method inconvenient for routine screening testing.

To avoid the use of radioactive labeling, many investigators have developed non-radioisotope methods in the LLNA. Boussiquet-Levoux *et al.* (1995) reported that a proliferating response of lymph node cells (LNC) in mice was assessed by immunohistochemistry, using bromodeoxyuridine (BrdU). The proliferation of LNC was also determined by the flow

cytometric (FCM) analysis of proliferating cell nuclear antigen (PCNA) (Kuhn *et al.*, 1995). Other than proliferating responses, several functional approaches were reported, including phenotypic analysis of LNC subpopulations: B220 positive cells (Sikorski *et al.*, 1996), CD3 positive cells and CD25 positive cells (De Silva *et al.*, 1993), CD62L^{lo}/CD44^{hi} double positive cells (Gerberick *et al.*, 1997, 1999a), CD86 positive cells, and I-A^k positive cells (Gerberick *et al.*, 1999b). These methods, based on the FCM analysis, could distinguish a contact allergen from irritants. Production of Th1 cytokines IL-2 (Hatao *et al.*, 1995), and IL-6 (Dearman *et al.*, 1994) were assessed, using a competitive reverse transcriptase-polymerase chain reaction (cRT-PCR) or enzyme-linked immunosorbent assay (ELISA). Cytokine production was increased by strong allergens. Hariya *et al.* (1999) reported that IL-2 production with lymph node weight and CD4 positive subset ratio in LNC improved sensitivity. Homey *et al.* (1998) and Vohr *et al.* (2000) presented an integrated model for differentiating skin reaction (IMDS), which assesses ear swelling and cell proliferation in lymph nodes.

Eight member companies of the Japan Pharmaceutical Manufacturers Association (JPMA) collaboratively studied the following non-radioisotope methods: BrdU incorporation *ex vivo* and *in vivo*, IL-2 production, PCNA expression, and IMDS. These methods were compared with the radioisotope-dependent standard LLNA.

MATERIALS AND METHODS

Test materials

2,4-Dinitrochlorobenzene (DNCB) was obtained from Sigma Chemical Co., (St. Louis, MO, USA), and 4-amino benzoic acid ethyl ester (benzocaine) from Sigma or Tokyo Chemical Industry Co. (Tokyo, Japan). These materials were dissolved in 4:1 acetone/olive oil (AOO), at concentrations of 0.01%, 0.05%, 0.13%, 0.25% or 0.5% for DNCB, and 2.5%, 5% or 10% for benzocaine.

Animals

Female 7-8 week-old CBA mice were purchased from Charles River Japan (Atsugi, Japan). Only Laboratory H purchased animals from Bomoltgardt, Ry. (Denmark). All animals were used at age 8-9 weeks. Housing and feeding conditions were set according to the standard of each laboratory. Each group included 6 animals.

LLNA with non-radioisotope alternative endpoints.

Fig. 1. Schedules for various modified local lymph node assays.

Sensitization

Weighed mice were individually identified prior to dermal applications. For mice in the test substance treatment group, we applied 25 μ l/ear of the test substance to the dorsum of both ears of mice, and in the control group we applied the same volume of the vehicle alone to the dorsum of both ears. Treatment was given daily for 3 consecutive days (days 1-3).

Fig. 1 shows the schedule of each method.

LLNA (standard RI method)

On Day 5, mice were injected via the tail vein with 250 μ l of phosphate-buffered saline (PBS) containing 740 kBq of 3 H-methyl thymidine (3H-TdR, NEN Life Science Products, Inc., MA, USA). Five hours later, these mice were killed and bilateral draining lymph nodes for each animal were excised and pooled in PBS. Single cell suspensions of lymph node cells (LNC) were prepared by gentle mechanical separation through a 380-mesh nylon filter (Becton Dickinson Labware, NJ, USA). LNC were pelleted by centrifugation at $190 \times g$ for 10 min, washed twice with

an excess of PBS and precipitated with 3 ml of 5% trichloroacetic acid (TCA) overnight at 4°C. Pellets were resuspended in 1 ml TCA and transferred to 10 ml of scintillation fluid (Ultima Gold LLT, Packard Instrument Company, CT, USA). Incorporation of 3H-TdR was measured using a β -scintillation counter (LS 6000TA, Beckman, FL, USA) as disintegration per minute (dpm) for each mouse. This method was carried out in Laboratory A.

BrdU incorporation (*ex vivo*)

Cell proliferation ELISA system, version 2 (code RPN250, BIOTRAK™, Amersham Pharmacia Biotech, Tokyo, Japan) was used.

On Day 5, mice were killed and bilateral draining auricular lymph nodes were excised. Single cell suspensions were prepared for 5×10^6 cells/ml with 25 mM HEPES-RPMI 1640 medium (10% FBS, 100 μ g/ml penicillin, 100 U/ml streptomycin) in a 96-well microplate in a final volume of 100 μ l/well, and cultured for 18 hr (37°C, 5% CO₂). Then 100 mM BrdU labeling solution was added 10 μ l/well. Cells were cul-

tured for 20 hr (37°C, 5% CO₂) to incorporate the BrdU. Cells were washed 3 times with PBS, and dried (60°C, 1 hr). Cell fixative, including ethanol, was added at 200 µl/well, followed by incubation at room temperature for 30 min. After removing the fixative, blocking buffer (containing 1% BSA in 50 mM Tris-HCl, 150 mM NaCl, pH 7.4) was added at 200 µl/well. Peroxidase-labeled anti-BrdU antibody working solution was added at 100 µl/well, followed by incubation for 90 min at room temperature. Samples were washed 3 times with PBS, and tetramethylbenzidine (TMB) substrate was added at 100 µl/well, followed by incubation at room temperature for 20 min. To stop the reaction, 1 mol/L H₂SO₄ was added at 25 µl/well, and absorbance was determined in a plate reader at 450 nm within 5 min. This method was carried out in Laboratories B and C.

BrdU incorporation (*in vivo*)

After the third application of the test substance or vehicle, the ALZET micro-osmotic pump (model 1007D, ALZA Co., Palo Alto, CA, USA), filled with bromodeoxyuridine (BrdU, Sigma) solution, was implanted subcutaneously in the abdomen. BrdU was dissolved with 50% dimethyl sulfoxide in physiological saline at 150 or 300 mg/ml. On Day 8 or 9, bilateral draining auricular lymph nodes were excised and single cell suspensions of LNC were prepared by gentle mechanical disaggregation. LNC were suspended in Dulbecco's minimum essential medium (DMEM) with 1% FCS added. Cells were then washed in DMEM (centrifuge 500×g for 15 min 2 times). After the first washing, the cell suspension was filtered through a cell strainer. Anti-Fc receptor antibody was added to the cell suspension in 200 µl DMEM, mixed and then stored at room temperature for 5 min. In one laboratory, fluorescence-labeled anti-B220 antibody (clone: RA3-6B2, Pharmingen, San Diego, CA, USA) or anti-CD3 antibody (clone: 145-2C11, Pharmingen) was added. In another laboratory, fluorescence-labeled anti-B220 antibody (clone: RA3-6B2, Pharmingen), anti-CD4 antibody (clone: RM4-5, Pharmingen) or anti-CD8 antibody (clone: 53-6.7, Pharmingen) was added and the preparation stored on ice in the dark for 30 min. Cells were then washed twice in DMEM. A pellet loosened by vortex was suspended in iced 250 µl saline. A few drops of iced 95% ethanol were added and the tubes were thoroughly shaken. The treatment was repeated until 600 µl of ethanol had been added. After incubation at 4°C for 30 min, the preparation was centrifuged at 3000×g for 3 min, the supernatant was

aspirated, and cells were resuspended with 100 µl PBS. Cells were fixed and permeabilized in PBS containing 1% paraformaldehyde and 0.01% Tween 20 at room temperature for 30 min. Cells were then washed twice in PBS (centrifuge 3000×g for 3 min). BrdU was then detected as described (Penit and Vasseur, 1993). Two hundred µl of 50 U/ml DNase/IMDM was added and the preparation was stored for 10 min. Cells were then placed in a 96-well plate. Fluorescence-labeled anti-BrdU antibody (clone: B44, Becton Dickinson, San Jose, CA, USA) was added to each well and the preparation was stored at 4°C for 20 min. Cells were then washed twice in PBS. Immunofluorescence was analyzed using a FACScan flow cytometer (Becton Dickinson) and the Cell Quest program. This method was carried out in Laboratories D and E.

PCNA expression

On Day 5, mice were killed and bilateral draining auricular lymph nodes were excised. LNC suspensions were added to the test tube (1×10⁶ cells/tube) which was centrifuged at 425×g for 5 min. Pellets were suspended with 500 µl of 1% paraformaldehyde, and kept at room temperature for 5 min. One ml of ice-cold PBS was added to each tube, centrifuged and the supernatant was aspirated. Pellets were resuspended with 1 ml of cold methanol (−20°C), and kept at room temperature for 10 min. Each tube was centrifuged and the supernatant was aspirated. These test tubes were centrifuged twice with 0.1% Triton X100. Fifty µl of FITC-conjugated anti-PCNA antibody (25 µg/ml in 0.1% Triton X100, clone: 19F4, Boehringer-Mannheim Biochemicals, Germany) was added and the preparation kept at room temperature for 15 min protected from light. Cells were washed twice in 0.1% Triton X100. Labeled cells were analyzed using a flow cytometer (EPICS XL, BECKMAN COULTER, Florida, USA). This method was carried out in Laboratory F.

IL-2 production

On Day 4, mice were killed and bilateral auricular lymph nodes were excised. LNC were suspended in RPMI 1640 medium supplemented with 10% FCS, 25 mM HEPES, 100 µg/ml penicillin and 100 U/ml streptomycin at the concentration of 2×10⁶ cells/ml. An aliquot of 200 µl of LNC suspension was seeded into each well of a 96-well tissue culture plate. Concanavalin A (ConA, Sigma) was added to each well at the final concentration of 2 µg/ml. LNC were cultured for 24 hr (37°C, 5% CO₂). The amount of IL-2 released

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into the supernatant was measured using an IL-2 ELISA kit (Endogen Inc., MA, USA). This method was carried out in Laboratory G.

Flow cytometric (FCM) analysis of lymphocyte subsets

On Day 5, mice were killed and bilateral auricular lymph nodes were excised. Single cell suspensions were prepared for each group. The following fluorescence-labeled monoclonal antibodies were used to detect lymphocyte subpopulations. Anti-mouse CD3 antibody (clone: 145-2C11, Pharmingen) was used for detection of mature T lymphocytes, anti-mouse CD4 antibody (clone: RM4-4, Pharmingen) for detection of T helper cells, anti-mouse CD8 antibody (clone: 53-6.7, Pharmingen) for detection of T suppressor/cytotoxic cells, anti-mouse B220 antibody (clone: RA3-6B2, Pharmingen) for detection of B lymphocytes, and anti-mouse CD25 antibody (clone: 7D4, Pharmingen) for detection of activated T and B lymphocytes. Labeled cells were analyzed using a FACScan flow cytometer (Becton Dickinson). This method was carried out in Laboratory G.

Integrated model for differentiating skin reaction (IMDS)

Before initial treatment (Day 1) and the day after the final treatment (Day 4), ear thickness was measured and mean ear swelling was calculated. On Day 4, bilateral auricular lymph nodes were removed and pooled for each individual animal. After weighing the lymph nodes, single cell suspensions were prepared for each mouse. Using these suspensions, LNC were counted, using an automatic cell counter, and cellularity index was calculated. In addition, cells were labeled by fluorescence-labeled anti-B220 antibody (clone: RA3-6B2, Pharmingen), anti-CD69 antibody (clone: H1.2F3, Pharmingen), which is expressed on very early activated lymphocytes, and anti-I-A antibody (clone: M5/114, Boehringer-Mannheim Biochemicals), which reacts with the mouse MHC Class II molecule. FCM analysis was performed using FACScan equipment (Becton Dickinson). To differentiate between allergic and irritant potential, Differentiation Indices (DI) were calculated, as described (Homey *et al.*, 1998), using the following formulas. This method was carried out in Laboratory H.

$$\% \text{ of maximal increase in lymph node cell count index} = \frac{\text{LN index} - 1}{4} \times 100$$

$$\% \text{ of maximal ear swelling} = \frac{\text{ear swelling (mm)}}{15 \times 0.01 \text{ (mm)}} \times 100$$

$$\text{DI} = \frac{\% \text{ of maximal increase in lymph node cell count index}}{\% \text{ of maximal ear swelling}}$$

DI < 1 $\equiv$ irritant potential DI > 1 $\equiv$ allergic potential

Cellularity

Seven laboratories calculated cellularity indices from comparisons of cell counts of each test substance treatment group and the control AOO treatment group. In Laboratories B, C and D, cells were counted manually using a hemocytometer. In Laboratories E, F, G and H, cells were counted using an automatic cell counter.

Statistical analysis

For each method, the stimulation index (SI) was the ratio of the mean of actual measurements for each test substance versus that of the AOO control group. Each test substance treatment group and the AOO treatment control group were compared by Dunnett's multiple comparison test (Dunnett, 1955) using the SAS program.

RESULTS

LLNA (standard RI method)

The results are shown in Table 1. SIs of DNCB were 1.31, 1.99, 10.87, 90.86 and 145.43 in the 0.01%, 0.05%, 0.13%, 0.25% and 0.5% groups, respectively. Dose-dependent increases were evident, and statistically significant increases were observed in the 0.25% and 0.5% groups. On the other hand, SIs of benzocaine were 1.66, 0.83 and 2.45 in the 2.5%, 5% and 10% groups, respectively. Dose-dependent increases and statistically significant increases were not observed.

BrdU incorporation (*ex vivo*)

The results are shown in Table 1. In the two laboratories, SIs of the 0.25% DNCB group were 4.14 and 2.47. However, at concentrations lower than 0.25% DNCB, contact hypersensitivity-eliciting potential was not detected. As to benzocaine, there was no significant change between the benzocaine groups and AOO group in one laboratory. In another laboratory, statistically significant increases were observed in the 5% and 10% benzocaine groups.

Table 1. Stimulation indices determined by various modified local lymph node assays.

Substance	3H-TdR incorporation		BrdU incorporation (<i>ex vivo</i>)-1		BrdU incorporation (<i>ex vivo</i>)-2	
	(dpm)	Index	O.D. _{450 nm}	Index	O.D. _{450 nm}	Index
AOO	94.5 ± 17.8	1.00	0.16 ± 0.02	1.00	0.15 ± 0.05	1.00
0.01% DNCB	124.6 ± 55.5	1.31	0.18 ± 0.03	1.15	0.17 ± 0.03	1.20
0.05% DNCB	188.4 ± 74.3	1.99	0.25 ± 0.07	1.50	0.19 ± 0.05	1.34
0.13% DNCB	1673 ± 1186	10.87	—	—	—	—
0.25% DNCB	10890.4 ± 214415 **	90.86	0.68 ± 0.09 **	4.14	0.36 ± 0.18 **	2.47
0.50% DNCB	17431 ± 1227 **	145.43	—	—	—	—
AOO	119.9 ± 22.3	1.00	0.16 ± 0.02	1.00	0.15 ± 0.05	1.00
2.50% Benzocaine	199.4 ± 217.6	1.66	0.19 ± 0.12	1.18	0.21 ± 0.03	1.48
5.00% Benzocaine	99.4 ± 28.8	0.83	0.12 ± 0.02	0.72	0.26 ± 0.01 **	1.81
10.00% Benzocaine	293.8 ± 343.8	2.45	—	—	0.24 ± 0.05 **	1.68

Substance	BrdU incorporation (<i>in vivo</i>)-1		BrdU incorporation (<i>in vivo</i>)-2		PCNA expression	
	Number of cells incorporating BrdU (×10 ⁴)	Index	Number of cells incorporating BrdU (×10 ⁴)	Index	Number of PCNA-positive cells (×10 ⁴)	Index
AOO	13.2 ± 5.7	1.00	54.4 ± 13.3	1.00	2.1 ± 0.7	1.00
0.01% DNCB	31.7 ± 20.6	2.40	65.8 ± 18.1	1.21	3.3 ± 0.7	1.59
0.05% DNCB	60.9 ± 30.2	4.62	87.5 ± 22.9	1.61	5.3 ± 2.3	2.51
0.13% DNCB	—	—	—	—	—	—
0.25% DNCB	494.4 ± 245.8 **	37.50	494.0 ± 75.9 **	9.08	71.8 ± 10.3 **	34.17
0.50% DNCB	—	—	—	—	—	—
AOO	13.2 ± 5.7	1.00	43.1 ± 8.6	1.00	2.1 ± 0.7	1.00
2.50% Benzocaine	19.2 ± 9.14	1.46	87.1 ± 27.6 *	2.02	3.4 ± 0.8	1.63
5.00% Benzocaine	25.4 ± 14.6	1.93	122.9 ± 48.4 **	2.85	2.4 ± 0.4	1.12
10.00% Benzocaine	18.6 ± 10.4	1.41	96.7 ± 23.1 *	2.24	3.3 ± 0.6	1.56

Substance	IL-2 production		Lymph node weight		Ear swelling	DI (IMDS)
	(pg/ml)	Index	(mg)	Index	Index	
AOO	1529 ± 783	1.00	4.69 ± 0.27 (4.38 ± 0.87)	1.00		
0.01% DNCB	2104 ± 693	1.38	4.92 ± 0.40	1.05	1.01	1.08
0.05% DNCB	2562 ± 1253	1.68	4.71 ± 0.41	1.00	0.99	n.c.
0.13% DNCB	—	—	(5.29 ± 1.25)	1.21	1.02	2.73
0.25% DNCB	18475 ± 6922 **	12.08	(6.80 ± 1.16)	1.55	1.01	7.50
0.50% DNCB	—	—	(8.98 ± 2.99 ***)	2.05	1.17	2.79
AOO	1529 ± 783	1.00	4.38 ± 0.87	1.00		
2.50% Benzocaine	2978 ± 1631	1.95	5.45 ± 0.65 *	1.25	1.05	1.07
5.00% Benzocaine	4668 ± 1556 **	3.05	4.04 ± 0.66	0.93	1.05	n.c.
10.00% Benzocaine	2164 ± 1260	1.42	5.56 ± 0.79 *	1.28	0.99	1.86

Each value represents the mean ± S.D. (—: Not examined). In the lymph node weight, (the mean ± S.D.) represents data, which were obtained using the same method on another day. ×10⁴ represents multiplication of the number of cells by 10000. Index is the ratio of the mean of actual measurements for each test substance treatment group against that of the AOO treatment control group. Each test substance treatment group and the AOO treatment control group were compared by Dunnett's multiple comparison.

*: p<0.05, **: p<0.01, ***: p<0.001.

—: not examined, n.c.: not calculated.

BrdU incorporation (*in vivo*)

The results are shown in Table 1. In one laboratory, the BrdU positive cell count indices of DNCB on Day 8 were 2.40, 4.62 and 37.50 in the 0.01%, 0.05% and 0.25% groups, respectively. In another laboratory, indices on Day 9 were 1.21, 1.61 and 9.08 in the 0.01%, 0.05% and 0.25% groups, respectively. In both of these laboratories, dose-dependent increases were evident, and a statistically significant increase was observed in the 0.25% group. As to benzocaine, in the former laboratory, BrdU positive cells did not increase. The indices of benzocaine were below 2 and dose-dependent increases were nil. In the latter laboratory, benzocaine induced statistically significant increases in the number of BrdU positive cells at all three concentrations, with no dose dependence.

In FCM analysis, BrdU and B220 or CD3; 2 color staining (Table 2), some differences in distribution of increasing cells were found in the 0.25% DNCB group. Cell count indices for 'BrdU+/CD3+' and 'BrdU-/CD3+' were 38.69 and 9.65 (cell % indices: 2.46 and 0.66), respectively. Cell count indices for 'BrdU+/B220+' and 'BrdU-/B220+' were 21.21 and 26.12 (cell % indices: 1.67 and 1.94), respectively. It was assumed that increasing cells that were BrdU negative had proliferated before the pump was implanted or delivered to the auricular lymph nodes. Table 3 shows the results of 2-color staining with BrdU and B220 or CD4 or CD8 in the DNCB group. The cell count index of 'BrdU+/CD4+' and 'BrdU+/CD8+' was 8.47, and that of 'BrdU+/B220+' was 6.71 in the 0.25% group.

PCNA expression

The results are shown in Table 1. SIs of the DNCB groups (at concentrations of 0.01%, 0.05% and 0.25%) were 1.59, 2.51 and 34.17, respectively. At concentration of 0.25%, there was a significant increase. SIs of the benzocaine groups (at concentrations of 2.5%, 5% and 10%) were 1.63, 1.12 and 1.56, respectively. There were no significant differences between the benzocaine groups and the AOO group.

IL-2 production

The results are shown in Table 1. SIs of the DNCB groups (at concentrations of 0.01%, 0.05% and 0.25%) were 1.38, 1.68 and 12.08, respectively. At concentration of 0.25%, the increase was significant. SIs of benzocaine groups (at concentrations of 2.5%, 5% and 10%) were 1.95, 3.05 and 1.42, respectively. At a concentration of 5%, the increase was significant.

FCM analysis of lymphocyte subsets

The results are shown in Table 4. The percentages of B220 positive cells and CD25+ cells increased dose-dependently in the DNCB groups. On the other hand, the percentages of CD3+ cells and CD4+ cells decreased, and those of CD8+ cells were unchanged. Lymphocyte subpopulations were unchanged in the benzocaine groups.

Integrated model for differentiating skin reaction (IMDS)

In the laboratory where IMDS was measured, DNCB showed a dose-dependent increase in LN weight (Table 1) and cell count indices (see Cellularity-7 in Table 6). Indices were 1.21, 1.55, 2.05 for weight and 1.71, 2.95, 3.52 for cell count in the 0.13%, 0.25% and 0.5% groups, respectively. In ear swelling index, a slight increase was found at a concentration of 0.5%, but not so at 0.13% or 0.25%. DIs of DNCB were 2.73, 7.50 and 2.79 (lower due to increased ear swelling [cf. METHOD]) in the 0.13%, 0.25% and 0.5% groups, respectively. Since DI showed values > 1 for all concentrations tested, DNCB was an allergic compound. In FCM analysis, increases of B220+/I-A+ and CD69+/I-A+ cells were observed in the DNCB groups (Table 5). In addition, at 0.5%, ear swelling index showed a slight increase. Therefore, the weak irritant potential of DNCB at higher concentrations was determined using this test system as well.

Benzocaine at concentrations of 2.5% and 10% showed significant increases in LN weight. Indices were 1.25, 0.93, 1.28 for weight and 1.28, 1.03, 1.47 for cell count in the 2.5%, 5% and 10% groups, respectively. There were no changes in ear swelling index at any concentration used. Since DI at 10% was 1.86, it was suggested that the response after treatment of benzocaine was caused by its potential as an allergic substance.

Cellularity

The results are shown in Table 6. In all laboratories, statistically significance increases were observed in the 0.25% DNCB group. As for benzocaine, in two laboratories, statistically significance increases were observed (5% group in one laboratory and the 2.5%, 5% and 10% groups in another laboratory).

DISCUSSION

In the study presented here, non-radioisotope methods were compared with the radioisotope-depen-

Table 2. Flow cytometric analysis for BrdU incorporation in lymph node cells-1.

Substance	BrdU+/B220+				BrdU+/CD3+			
	(%)	Index	($\times 10^4$)	Index	(%)	Index	($\times 10^4$)	Index
AOO	1.69 $\pm$ 1.06	1.00	3.39 $\pm$ 2.62	1.00	6.05 $\pm$ 2.40	1.00	11.03 $\pm$ 4.51	1.00
0.01% DNCB	1.59 $\pm$ 0.52	0.94	5.64 $\pm$ 2.84	1.66	5.72 $\pm$ 1.57	0.95	24.32 $\pm$ 22.00	2.20
0.05% DNCB	1.37 $\pm$ 0.48	0.81	8.33 $\pm$ 2.32	2.46	7.24 $\pm$ 3.25	1.20	51.75 $\pm$ 40.56	4.69
0.25% DNCB	2.82 $\pm$ 0.73 *	1.67	71.91 $\pm$ 32.81**	21.21	14.90 $\pm$ 2.73 **	2.46	426.79 $\pm$ 292.57 **	38.69
2.50% Benzocaine	1.26 $\pm$ 0.27	0.75	4.62 $\pm$ 2.11	1.36	4.17 $\pm$ 1.05	0.69	15.93 $\pm$ 8.23	1.44
5.00% Benzocaine	1.21 $\pm$ 0.31	0.72	5.30 $\pm$ 1.65	1.56	4.79 $\pm$ 3.33	0.79	22.34 $\pm$ 15.01	2.03
10.00% Benzocaine	1.34 $\pm$ 0.51	0.79	3.55 $\pm$ 0.86	1.05	6.65 $\pm$ 2.04	1.10	19.85 $\pm$ 10.53	1.80

Substance	BrdU-/B220+				BrdU-/CD3+			
	(%)	Index	($\times 10^4$)	Index	(%)	Index	($\times 10^4$)	Index
AOO	16.89 $\pm$ 6.67	1.00	34.83 $\pm$ 25.03	1.00	74.53 $\pm$ 4.60	1.00	142.34 $\pm$ 37.51	1.00
0.01% DNCB	23.76 $\pm$ 4.35 *	1.41	97.00 $\pm$ 61.17	2.78	69.07 $\pm$ 2.83	0.93	276.69 $\pm$ 187.89	1.94
0.05% DNCB	21.17 $\pm$ 1.98	1.25	137.01 $\pm$ 45.44	3.93	67.54 $\pm$ 4.77 *	0.91	448.89 $\pm$ 176.92	3.15
0.25% DNCB	32.73 $\pm$ 3.29 **	1.94	909.78 $\pm$ 537.46 **	26.12	48.83 $\pm$ 2.98 **	0.66	1373.37 $\pm$ 849.51 **	9.65
2.50% Benzocaine	17.54 $\pm$ 1.07	1.04	65.67 $\pm$ 27.77	1.91	76.86 $\pm$ 4.71	1.03	284.38 $\pm$ 115.63	2.00
5.00% Benzocaine	17.29 $\pm$ 5.02	1.02	90.81 $\pm$ 66.19	2.61	74.57 $\pm$ 5.95	1.00	349.11 $\pm$ 161.76 *	2.45
10.00% Benzocaine	20.92 $\pm$ 4.18	1.24	57.89 $\pm$ 10.55	1.67	74.85 $\pm$ 3.92	1.00	211.57 $\pm$ 48.41	1.49

Each value represents the mean $\pm$ S.D.. % represents the cell percentages. $\times 10^4$ represents multiplication of the number of cells by 10000. Index is the ratio of the mean of actual measurements for each test substance treatment group versus that of the AOO treatment control group. Each test substance treatment group and the AOO treatment control group were compared by Dunnett's multiple comparison. *: $p < 0.05$, **: $p < 0.01$.

dent standard LLNA, using the strong sensitizer DNCB and the weak sensitizer benzocaine.

In our study, non-radioisotope methods (BrdU *ex vivo* and *in vivo* incorporation, IL-2 production and PCNA expression) detected statistically significant increases in the 0.25% DNCB groups, as did the standard radioisotope method. Sensitization rate of DNCB was 100% in GPMT and it was classified as an extreme sensitizer (Basketter and Scholes, 1992). Some authors have stated that DNCB at doses of 0.1% and 0.25% is positive in the standard LLNA (Kimber *et al.*, 1989, 1991; Loveless *et al.*, 1996; Gerberick *et al.*, 1999a).

The BrdU *ex vivo* method detected statistically significant increases in the 5% and 10% benzocaine groups in one laboratory. The BrdU *in vivo* method detected statistically significant increases in the 2.5%, 5% and 10% benzocaine groups in one laboratory, but dose-dependent increases were not evident. Measuring IL-2 production detected statistically significant increases in the 5% benzocaine group. However, a dose-dependent response was not observed with any method used. In some experiments, there was an indication toward a bell-shaped dose response curve for benzocaine (Kimber *et al.*, 1991; Basketter *et al.*, 1994, 1995; Warbrick *et al.*, 2000; Van Och *et al.*, 2000).

LLNA with non-radioisotope alternative endpoints.

Table 3. Flow cytometric analysis for BrdU incorporation in lymph node cells-2.

Substance	BrdU+/B220+			BrdU+/CD4+			BrdU+/CD8+		
	(%)	Index	($\times 10^4$)	(%)	Index	($\times 10^4$)	(%)	Index	($\times 10^4$)
AOO	2.73 $\pm$ 0.35	1.00	19.2 $\pm$ 4.4	4.46 $\pm$ 0.41	1.00	31.6 $\pm$ 8.1	2.11 $\pm$ 0.19	1.00	14.9 $\pm$ 3.7
0.01% DNCB	2.61 $\pm$ 0.58	0.96	19.5 $\pm$ 3.7	4.37 $\pm$ 0.30	0.98	35.7 $\pm$ 5.2	2.95 $\pm$ 0.63	1.40	24.4 $\pm$ 6.7
0.05% DNCB	2.72 $\pm$ 0.49	1.00	25.2 $\pm$ 9.3	5.60 $\pm$ 0.60	1.26	50.7 $\pm$ 13.0	2.89 $\pm$ 0.53	1.37	25.9 $\pm$ 6.7
0.25% DNCB	4.38 $\pm$ 0.51 **	1.60	128.9 $\pm$ 23.1 **	8.71 $\pm$ 1.57 **	1.95	262.0 $\pm$ 85.4 **	4.43 $\pm$ 1.20 ***	2.10	131.8 $\pm$ 42.8 **

Substance	BrdU+/CD4+ & BrdU+/CD8+		
	(%)	Index	($\times 10^4$)
AOO	6.57 $\pm$ 0.59	1.00	46.5 $\pm$ 11.8
0.01% DNCB	7.32 $\pm$ 1.05	1.11	60.1 $\pm$ 10.3
0.05% DNCB	8.49 $\pm$ 0.89	1.29	76.5 $\pm$ 18.7
0.25% DNCB	13.14 $\pm$ 2.41 ***	2.00	393.8 $\pm$ 119.5 ***

Each value represents the mean $\pm$ S.D.. % represents the cell percentages. $\times 10^4$ represents multiplication of the number of cells by 10000. Index is the ratio of the mean of actual measurements for each test substance treatment group versus that of the AOO treatment control group. Each test substance treatment group and the AOO treatment control group were compared by Dunnett's multiple comparison. **: $p < 0.01$, ***: $p < 0.001$.

Table 4. Flow cytometric analysis for lymphocyte subsets in lymph nodes.

Substance	CD3+				CD4+CD8-				CD4-CD8+				B220+				CD25+			
	(%)	Index	($\times 10^5$)	Index	(%)	Index	($\times 10^5$)	Index	(%)	Index	($\times 10^5$)	Index	(%)	Index	($\times 10^5$)	Index	(%)	Index	($\times 10^5$)	Index
AOO	87.78	1.00	202.1	1.00	63.39	1.00	146.0	1.00	24.53	1.00	56.5	1.00	9.17	1.00	21.1	1.00	3.58	1.00	8.2	1.00
0.01% DNCB	88.67	1.01	149.0	0.74	63.19	1.00	106.2	0.73	24.71	1.01	41.5	0.73	9.01	0.98	15.1	0.72	3.34	0.93	5.6	0.68
0.05% DNCB	89.61	1.02	246.0	1.22	60.17	0.95	165.2	1.13	26.26	1.07	72.1	1.28	10.77	1.17	29.6	1.40	3.96	1.11	10.9	1.32
0.25% DNCB	75.90	0.86	835.8	4.14	52.50	0.83	575.2	3.95	24.10	0.98	256.7	4.54	20.60	2.25	206.1	9.76	4.61	1.29	51.2	6.21
	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
AOO	91.52	1.00	173.9	1.00	62.93	1.00	119.6	1.00	30.91	1.00	58.7	1.00	6.23	1.00	11.8	1.00	3.95	1.00	7.5	1.00
2.50% Benzocaine	91.59	1.00	201.5	1.16	60.02	0.95	132.0	1.10	31.26	1.01	68.8	1.17	6.37	1.02	14.0	1.18	3.73	0.94	8.2	1.09
5.00% Benzocaine	90.99	0.99	196.5	1.13	60.73	0.97	131.1	1.10	30.28	0.98	65.4	1.11	6.71	1.08	14.5	1.22	4.19	1.06	9.1	1.21
10.00% Benzocaine	91.34	1.00	169.9	0.98	60.76	0.97	113.0	0.94	29.91	0.97	55.6	0.95	6.68	1.07	12.4	1.05	4.02	1.02	7.5	1.00

% represents the cell percentages. $\times 10^5$ represents multiplication of the number of cells by 100000. Index is the ratio of the mean of actual measurements for each test substance treatment group versus that of the AOO treatment control group.

Table 5. Flow cytometric analysis for B220+/I-A+ and CD69+/I-A+ cells in lymph nodes.

Substance	B220+/I-A+		CD69+/I-A+	
	(%)	Index	(%)	Index
AOO	8.54	1.00	1.34	1.00
0.13% DNCB	8.41	0.98	1.99	1.51
0.25% DNCB	16.16	1.85	3.40	2.50
0.50% DNCB	17.39	1.97	6.28	4.62

% represents the cell percentages. Index is the ratio of the mean of actual measurements for each test substance treatment group versus that of the AOO treatment control group.

This could be due to local toxicity or other inhibitory effects, dependent on the potential of benzocaine to act as a local anesthetic, or a direct effect on local blood vessels with dose-dependent changes in skin clearance of benzocaine (Basketter *et al.*, 1995). OECD Test Guideline 406 (1992) recommended a regular 6-month evaluation of 1 of 3 moderately-sensitizing positive control substances, hexylcinnamic aldehyde (HCA), mercaptobenzothiazole (MBT) and benzocaine. Sensitization rates of benzocaine are 19% and 50% in human maximization test and GPMT, respectively (Kligman, 1966; Basketter and Scholes, 1992). In previous reports, benzocaine has given highly variable and equivocal results in LLNA (Kimber *et al.*, 1989; Kimber *et al.*, 1991; Basketter *et al.*, 1994; Warbrick *et al.*, 2000; Van Och *et al.*, 2000). Therefore, Basketter *et al.* (1995) concluded that benzocaine is not a suitable positive control for use in predictive tests for skin sensitization. OECD Test Guideline 429 (2000) accepted LLNA as a stand-alone skin sensitization test, and recommended HCA and MBT as positive controls, but not benzocaine. We used benzocaine as a weak sensitizer to compare non-radioisotope methods and the radioisotope-dependent standard LLNA.

The standard LLNA has the disadvantages of technical difficulties for 3H-TdR injection into the tail vein of the brown CBA mice and for disposal of the radioisotope-contaminated animals. Therefore, several methods without using any radioisotope are advantageous for the practical LLNA application.

Boussiquet-Levoux *et al.* (1995) histologically observed proliferative responses of LNC in LLNA using BrdU. However, this method may not be quantitatively so accurate as the original LLNA, since cell counting under microscopic observation is required. In

the BrdU *in vivo* method, BrdU positive cells are counted using a flow cytometer, which is more accurate. Furthermore, 2-color staining in FCM analysis can reveal a proliferating LNC subset.

The IL-2 assay is closely related to T cell proliferation which plays an important role in the induction of Type IV allergy. In contrast, *in vivo* 3H-TdR incorporation tends to reflect not only the proliferation of T cells but also an unidentified proliferation of other cells in lymph nodes. Hatao *et al.* (1995) reported that the amount of IL-2 was increased by strong sensitizers such as oxazolone, TNBS, hydroquinone, cinnamaldehyde, Sudan I, PPDA as well as the moderate sensitizer citral, but was not increased by moderate sensitizer eugenol. In the present study, 0.25% DNCB and 5% benzocaine significantly increased IL-2 production. These results suggest that this method can detect a wider range of skin sensitizers.

PCNA is constitutively expressed during the S phase of mitosis. Kuhn *et al.* (1995) described the good correlation between the incorporation of 3H-TdR and the nuclear expression of PCNA in LLNA. They stated that the total population of PCNA positive cells was significantly increased after application of contact sensitizers (DNFB, TNCB, oxazolone) in comparison to solvent-treated mice (Kuhn *et al.*, 1995). In addition, they stained LNC of mice treated with oxazolone for PCNA and the T cell markers CD4 and CD8. As a result, the majority of PCNA-positive cells belonged to the CD4-positive T cell subsets. These data show that skin sensitization potential can be accurately evaluated by PCNA expression without the technical limitations associated with the use of radioisotopes, and a more detailed view of the immune response induced by allergens can be obtained. In our study, although no combined staining was performed, the SI of the 0.25% DNCB group was high.

Many investigators have examined the FCM analysis of proliferating cells in LLNA. Sikorski *et al.* (1996) reported that the percentage of B220-positive cells increased dose-dependently in response to allergenic chemicals (TNCB, DNCB). The strong sensitizer DNCB induced significant increases in CD3 positive cells and CD25-positive cells compared with vehicle control and irritant SDS (De Silva *et al.*, 1993). In our study, FCM analysis data showed that SIs of the percentage of B220-positive cells and CD25-positive cells in the 0.25% DNCB group were 2.25 and 1.29, respectively.

The IMDS method can differentiate between irritative and allergic skin reactions based on cellularity index and ear swelling index. In our study, since DI

Table 6. Cellularity indices determined in each laboratory.

Substance	Count with hemocytometer					
	Cellularity-1		Cellularity-2		Cellularity-3	
	($\times 10^6$)	Index	($\times 10^6$)	Index	($\times 10^6$)	Index
AOO	0.63 ± 0.53	1.00	1.04 ± 0.27	1.00	1.93 ± 0.59	1.00
0.01% DNCB	3.30 ± 0.85	5.24	0.89 ± 0.48	0.85	4.02 ± 2.69	2.08
0.05% DNCB	$3.90 \pm 0.88^{**}$	6.18	1.17 ± 0.61	1.12	6.60 ± 2.49	3.42
0.13% DNCB	—	—	—	—	—	—
0.25% DNCB	$6.31 \pm 2.04^{***}$	10.00	$6.32 \pm 1.73^{**}$	6.06	$27.7 \pm 16.5^{**}$	14.37
0.50% DNCB	—	—	—	—	—	—
AOO	0.63 ± 0.53	1.00	1.04 ± 0.27	1.00	1.93 ± 0.59	1.00
2.50% Benzocaine	0.62 ± 0.20	0.98	1.08 ± 0.61	1.04	3.70 ± 1.50	1.92
5.00% Benzocaine	0.27 ± 0.10	0.43	0.88 ± 0.56	0.84	$4.75 \pm 2.33^*$	2.46
10.00% Benzocaine	0.10 ± 0.04	0.16	1.01 ± 0.67	0.96	2.56 ± 0.96	1.32

Substance	Count with automatic cell counter					
	Cellularity-4		Cellularity-5		Cellularity-6	
	($\times 10^6$)	Index	($\times 10^6$)	Index	($\times 10^6$)	Index
AOO	7.0 ± 1.4	1.00	5.56 ± 2.04	1.00	3.77 ± 1.05	1.00
0.01% DNCB	7.8 ± 2.1	1.14	6.12 ± 1.90	1.10	4.13 ± 0.79	1.10
0.05% DNCB	9.0 ± 2.0	1.29	8.02 ± 2.58	1.44	4.32 ± 0.96	1.15
0.13% DNCB	—	—	—	—	—	—
0.25% DNCB	$29.5 \pm 4.8^{***}$	4.21	$23.32 \pm 1.99^{**}$	4.19	$14.70 \pm 6.27^{**}$	3.90
0.50% DNCB	—	—	—	—	—	—
AOO	5.6 ± 0.4	1.00	5.56 ± 2.04	1.00	3.77 ± 1.05	1.00
2.50% Benzocaine	$10.0 \pm 3.3^*$	1.78	6.19 ± 1.91	1.11	4.30 ± 1.15	1.07
5.00% Benzocaine	$13.3 \pm 4.0^{**}$	2.38	5.09 ± 0.60	0.91	4.27 ± 0.72	1.13
10.00% Benzocaine	$10.1 \pm 1.2^*$	1.86	6.65 ± 1.31	1.20	4.02 ± 1.08	1.07

$\times 10^6$ represents multiplication of the number of cells by 1000000. Each value represents the mean $\pm$ S.D. (—: Not examined). In Cellularity-7, (the mean $\pm$ S.D.) represents data obtained using the same method on another day. Index is the ratio of the mean of actual measurements for each test substance treatment group versus that of the AOO treatment control group. Each test substance treatment group and the AOO treatment control group are compared by Dunnett's multiple comparison. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. —: Not examined.

showed values > 1 for all concentrations tested, DNCB is an allergic compound, as supported by the results of FCM analysis, *i.e.*, increases in B220+/I-A+ cells (B cell) and CD69+/I-A+ cells (activated B cell) were observed (Table 5). Homey *et al.* (1998) reported that the most prominent differences between irritant and allergic reactions were seen for the expression of CD69+/I-A+ lymph node cells. Allergen (oxazolone) treatment dramatically upregulated CD69 on I-A+ lymph node cells. On the other hand, an irritant (croton oil) failed to induce significant changes in CD69 expression. Similar results were obtained for the expression of B220+/I-A+ lymph node cells. They concluded that if a test chemical produced a DI of 1, further studies such as FCM analysis of cell surface markers were needed.

In conclusion, our data suggest that BrdU data obtained *ex vivo* and *in vivo* and measuring IL-2 production are sensitive and useful methods. The decision process to identify a positive response in the LLNA standard method should include a $SI \geq 3$, statistical significance and dose response information (NIH Publication, 1999). On the other hand, non-radioisotope methods do not yet have the standard of a positive response. In our study, we considered the statistical significance when making a judgement. We believe that these non-radioisotope methods to assess allergic contact dermatitis caused by chemicals can be used even in a laboratory where it can be difficult to handle radioisotopes.

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