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## Detection of low-level environmental chemical allergy by a long-term sensitization method

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### ABSTRACT

Multiple chemical sensitivity (MCS) is characterized by various signs, including neurological disorders and allergy. Exposure may occur through a major event, such as a chemical spill, or from long-term contact with chemicals at low levels. We are interested in the allergenicity of MCS and the detection of low-level chemical-related hypersensitivity. We used long-term sensitization followed by low-dose challenge to evaluate sensitization by well-known Th2 type sensitizers (trimellitic anhydride (TMA) and toluene diisocyanate (TDI)) and a Th1 type sensitizer (2,4-dinitrochlorobenzene (DNCB)). After topically sensitizing BALB/c mice (9 times in 3 weeks) and challenging them with TMA, TDI or DNCB, we assayed their auricular lymph nodes (LNs) for number of lymphocytes, surface antigen expression of B cells, and local cytokine production, and measured antigen-specific serum IgE levels. TMA and TDI induced marked increases in levels of antigen-specific serum IgE and of Th2 cytokines (IL-4, IL-5, IL-10, and IL-13) produced by ex vivo restimulated lymph node cells. DNCB induced a marked increase in Th1 cytokine (IL-2, IFN- $\gamma$ , and TNF- $\alpha$ ) levels, but antigen-specific serum IgE levels were not elevated. All chemicals induced significant increases in number of lymphocytes and surface antigen expression of B cells. Our mouse model enabled the identification and characterization of chemical-related allergic reactions at low levels. This long-term sensitization method would be useful for detecting environmental chemical-related hypersensitivity.

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### 1. Introduction

Multiple chemical sensitivity (MCS) has emerged as a public health problem worldwide. Its incidence is increasing, especially in infants and children (Ban and Hettich, 2005; Hopkin, 1997; Howarth, 1998; Kimber and Dearman, 2002). Environmental and lifestyle factors and increasing use of chemicals have often been suggested as contributors (Ban and Hettich, 2005; Peden, 2000). MCS is characterized by a wide range of signs and symptoms triggered when affected patients come into contact with a number of chemical products (Bartha et al., 1999; Brown and Jason, 2007). Common triggers include pesticides and perfumes (Brown and Jason, 2007; Kreutzer et al., 1999). However, it is still uncertain how environmental chemicals induce this sensitivity.

Several animal models have been tested to identify chemical allergy (Arts et al., 2003; Ban et al., 2006). However, although environmental chemical allergens tend to have weak, minimal immunogenicity, these methods have focused on the detection of strong allergic reactions. Therefore, there is a need for protocols for the treatment and detection of weakly immunogenic and low doses allergic reactions. Recently, the local lymph node assay (LLNA) was developed initially as an alternative approach to hazard identification (National Institute of Environmental Health Sciences, 1999). It has now been evaluated extensively and validated formally (Basketter et al., 2002; Dearman et al., 1999; Gerberick et al., 2000) because of having high sensitivity and convenience of use. This assay measures proliferation of the draining lymph node cells and it comprises a sensitizing phase only. Induction of a three-fold or higher proliferative response compared with that in solvent-treated controls identifies a test chemical as a sensitizer. In this issue, to clear up detection of weakly immunogenic and low doses allergic reactions, we develop an improved model of the LLNA that uses long-term dermal sensitization followed by dermal challenge.

Many newly developed allergy models have focused on the local lymph node (LN) (Manetz and Meade, 1999; Plitnick et al., 2003;

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Sailstad et al., 2003). The LN is a key site in the priming of cytokine production by T lymphocytes and IgE production by B lymphocytes (Manetz and Meade, 1999; Plitnick et al., 2003). In human and animal studies, it has become clear that the differentiation of T helper (Th) lymphocytes into Th1 and Th2 cells is a key factor in promoting or suppressing IgE antibody production (Mosmann and Coffman, 1989). From this point of view, it may be possible to identify chemicals capable of eliciting IgE-mediated (Th2) or T-cell-mediated (Th1) allergy response by measuring the levels of cytokine production in the LN. Generally speaking, respiratory hypersensitivity is considered an IgE-mediated (Th2) disease. To identify and classify the allergenicity of environmental chemicals, we analyzed antigen-specific serum IgE levels, surface antigen expression on B cells and local cytokine production in the LN.

Our primary objective was to develop a new test schedule and methods for detecting environmental chemical-related hypersensitivity at low and weakly immunogenic doses. To that end, we used a long-term sensitization method and the Th1 type sensitizers 2,4-dinitrochlorobenzene (DNCB) and the Th2 type sensitizers trimellitic anhydride (TMA) and toluene diisocyanate (TDI). Exposure to TMA and TDI is recognized as a leading cause of occupational asthma worldwide. Several reports (Ban et al., 2006; Vanoirbeek et al., 2003) showed that topical TMA and TDI exposure leads to increased total serum IgE levels and a Th2 cytokine secretion pattern in mice. In contrast, identical exposure to DNCB, that causes delayed-type hypersensitivity reactions but lack the potential to cause respiratory hypersensitivity, elicits the Th1 type cytokine secretion profile. These low-molecular weight chemicals are well-known, typical human allergens (Larsen et al., 2001; Matheson et al., 2005a,b) that have been used extensively in experimental research examining chemical hypersensitivity.

## 2. Materials and methods

### 2.1. Chemicals

Each test chemical was dissolved in 4:1 acetone/olive oil (AOO). TMA and DNCB were purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). TDI (95% 2,4 isomer), acetone, and olive oil were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). DNCB is used as a Th1 type allergen, whereas the others are Th2 type allergens. The concentrations used in the pilot study and the main study are presented in Table 1. The maximum doses used in the pilot study were selected to avoid systemic toxicity and/or excessive local sensitization (particularly in the preliminary test) while still permitting comparisons of the sensitizing potencies among the chemicals to be made.

### 2.2. Animals

Female BALB/c mice (age, 7 weeks) purchased from Charles River Japan Laboratories (Atugi, Kanagawa, Japan) were housed individually under controlled lighting (lights on from 07:00 to 19:00 h), temperature ( $22 \pm 2^\circ\text{C}$ ), humidity ( $55 \pm 15\%$ ), and ventilation (at least ten 100% fresh air changes hourly). Food (Certified Pellet Diet MF, Oriental Yeast Co., Tokyo, Japan) and water were available ad libitum.

This study was conducted in accordance with the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science (Japanese Association for Laboratory Animal Science, 1987).

**Table 1**  
Chemical concentrations used in pilot study (LLNA) and main study

| Chemical | Pilot study           | Main study        |               |
|----------|-----------------------|-------------------|---------------|
|          |                       | Sensitization (%) | Challenge (%) |
| TMA      | 0%/0.1%/0.3%/1%/3%    | 1                 | 0.1           |
| TDI      | 0%/0.03%/0.1%/0.3%/1% | 0.1               | 0.01          |
| DNCB     | 0%/0.03%/0.1%/0.3%/1% | 0.3               | 0.03          |

TMA: trimellitic anhydride; TDI: toluene diisocyanate; DNCB: 2,4-dinitrochlorobenzene.

### 2.3. Pilot study (local lymph node assay)

#### 2.3.1. Experimental design

The assay was performed as described by Kimber and Weisenberger (1989) with minor modifications. After a 1-week acclimatization period, mice were allocated randomly to dose and control groups ( $n=5$  per group). A 25- $\mu\text{L}$  aliquot of test solution or solvent only was applied daily to the dorsum of each ear of each mouse for 3 consecutive days (days 1–3). On day 6,  $^3\text{H}$ -methyl thymidine ( $^3\text{H}$ -TdR, 20  $\mu\text{Ci}/\text{animal}$ ; GE Healthcare Bioscience, Tokyo, Japan) was injected via the tail vein into all test and control mice; 5 h after injection, the mice were euthanized, and each animal's auricular LNs on both sides were removed, weighed, and pooled per mouse in phosphate-buffered saline (PBS, Gibco, Tokyo, Japan).

#### 2.3.2. Incorporation of $^3\text{H}$ -TdR

The remaining LN cell suspension was washed twice with an excess PBS, and the cell pellet was incubated in 3 ml 5% trichloroacetic acid (TCA, Wako Pure Chemical Industries, Ltd., Osaka, Japan) at  $4^\circ\text{C}$  for approximately 18 h. Each cell pellet was resuspended in 1 ml TCA and transferred to 9 ml of scintillation fluid (Atom-Light, PerkinElmer Japan, Tokyo, Japan). Incorporation of  $^3\text{H}$ -TdR was measured by  $\beta$ -scintillation counter (LC-5100, Aloka, Tokyo, Japan) as disintegrations per minute (dpm) for each mouse.

#### 2.3.3. Statistical analysis

Stimulation indexes (SIs) and EC3 values were calculated from the  $^3\text{H}$ -TdR incorporation data. The SI was calculated by dividing the mean  $^3\text{H}$ -TdR incorporation value for each treatment group by that of the solvent control group. The EC3 value is an estimate of the amount of the test solution required to induce an SI of 3 (Basketter et al., 1999). In the standard LLNA, the criterion for a positive response is an SI of 3 or greater (Dearman et al., 1999). Data on LN weights and incorporation of  $^3\text{H}$ -TdR were compared by Dunnett's multiple comparison test, and differences were considered significant at  $P < 0.05$ .

### 2.4. Main study: a long-term sensitization method of detecting chemical allergy

#### 2.4.1. Test and control groups

The mice were divided into four groups for each chemical (TMA, TDI and DNCB): Group  $-/-$  (both sensitization and challenge with solvent only); Group  $-/+$  (sensitized with solvent only and challenged with test solution); Group  $+/-$  (sensitized with test solution and challenged with solvent only); and Group  $+/+$  (sensitized and challenged with test solution).

#### 2.4.2. Treatment protocol

After a 1-week acclimatization period, mice were allocated randomly to dose and control groups ( $n=8$  per group). Fig. 1 depicts the experimental protocol used in the main study. On days 1–3, 8–10, and 15–17, a 25- $\mu\text{L}$  aliquot of test solution or solvent only was applied to the dorsum of each ear of each mouse for dermal sensitization. Two weeks after the last sensitization (day 31), a 25- $\mu\text{L}$  aliquot of test solution or solvent only was applied as previously for dermal challenge. On the day after the challenge, mice were anesthetized with diethyl ether and euthanized. Blood samples were taken from the inferior vena cava and serum samples were assayed for specific serum IgE levels. Each animal's auricular LNs on both sides were removed, weighed, and pooled per mouse in RPMI 1640 medium (Gibco, Tokyo, Japan).

#### 2.4.3. Antigen-specific serum IgE

TMA-ovalbumin (OVA) conjugate was prepared by Imject Immunogen EDC Kit in accordance with the manufacturer's protocol (Pierce Biotechnology, Inc., Rockford, MD, USA). TDI-MSA conjugate (mouse serum albumin, Sigma-Aldrich Chemical

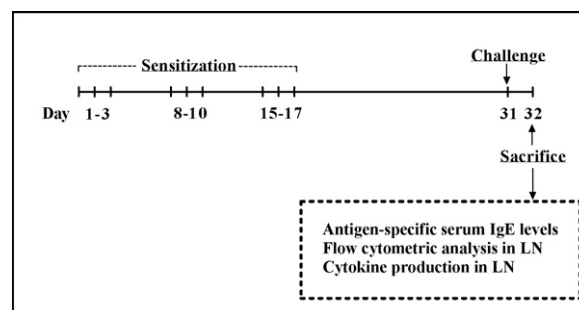


**Fig. 1.** Experimental protocol used to sensitize and challenge BALB/c mice with TMA, TDI, or DNCB in acetone/olive oil solvent. On days 1–3, 8–10, and 15–17, a 25- $\mu\text{L}$  aliquot of test solution or solvent only was applied to the dorsum of each ear of each mouse for dermal sensitization. Mice were then challenged 2 weeks after last sensitization (day 31). The same volume of test solution or solvent only was applied to the dorsum of each ear of each mouse for dermal challenge.

Company, Inc., Tokyo, Japan) and DNCB–MSA conjugate were prepared as described, by a modified version of the method of Ye et al. (2006). Briefly, TDI or DNCB was added to 1% HSA in PBS with constant stirring. Aliquots were taken 5, 10, 20, 30, and 40 min after the beginning of the reaction. Excess ammonium carbonate (2 M, Sigma–Aldrich Chemical Company, Inc., Tokyo, Japan) was added to each aliquot to terminate the reactions. All reactive samples were centrifuged at  $3000 \times g$  for 40 min to remove unreacted TDI and DNCB. The samples were then extensively dialyzed for 3 days against 0.1 mol/l ammonium carbonate and precipitated with an equal volume of 20% trichloroacetic acid (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Samples were then redissolved in 1 mol/l sodium hydroxide (Sigma Aldrich) and dialyzed with deionized water for 24 h. The protein content of each conjugated sample was determined by the Lowry method (Lowry et al., 1951).

Specific serum IgE was measured by ELISA. Briefly, each flat-bottomed microplate well (Nalge Nunc International K.K., Tokyo, Japan) was coated with chemical–protein conjugates (100  $\mu$ l, 0.1 mg/ml) in coating buffer (BD Pharmingen, San Diego, CA, USA) and incubated overnight at 4 °C. The content of each well was removed and the plate was washed fifth with wash buffer (BD Pharmingen) following each addition–incubation procedure. Non-specific binding was blocked by incubation with 200  $\mu$ l 10% heat-inactivated goat serum (Sigma–Aldrich Chemical Company, Inc., Tokyo, Japan) in PBS for 1 h at room temperature. Mouse serum was diluted in PBS (from 1:4 to 1:4096). The dilute serum (100  $\mu$ l) was added to each well and incubated for 2 h at room temperature. Biotin-conjugated rat anti-mouse IgE monoclonal antibody (BD Pharmingen, 2  $\mu$ g/ml, 100  $\mu$ l) was added to each well and incubated for 1 h at room temperature. Streptavidin–horseradish peroxidase (SAV-HRP) conjugate (BD Pharmingen, dilution 1:1000, 100  $\mu$ l) was added to each well and incubated for 30 min at room temperature. The plate was developed with tetramethylbenzidine (TMB) (100  $\mu$ l/well) in the dark, at room temperature, for 30 min. Optical density (OD) was read at a wavelength of 450 nm with an ImmunoMini reader (NJ-2300, Nippon Intermed, Tokyo, Japan).

#### 2.4.4. Tissue preparation

Single-cell suspensions of LNs in 1 ml RPMI 1640 (Gibco) supplemented with 5% heat-inactivated fetal calf serum (FCS; Gibco) were prepared by passage through sterile 70  $\mu$ m nylon cell strainers (Falcon). The number of LN cells was determined with a Coulter counter Z2 (Beckman Coulter Co., Tokyo, Japan).

#### 2.4.5. Cell staining and flow cytometric analysis

Flow cytometric analysis was confirmed by staining lymphocytes with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgE (clone R35-72), phycoerythrin (PE)-conjugated rat anti-mouse I-A/I-E (clone M5/114.15.2), and PE-Cy5-conjugated rat anti-mouse CD45R/B220 (clone RA3-6B2). All antibodies for flow cytometric analysis were purchased from BD Pharmingen (San Diego, CA, USA). To avoid non-specific binding,  $1 \times 10^6$  cells were incubated with 15% normal goat serum (Sigma) for 10 min at 4 °C, followed by FITC-, PE- and PE-Cy5-conjugated mAbs for 30 min at 4 °C in the dark. The cells were washed twice with wash buffer and resuspended at  $1 \times 10^6$  cells per tube in 1 ml PBS, and then analyzed by FACSCaliber flow cytometer (BD Pharmingen) using the Cell Quest program (BD Pharmingen). For each sample, 10,000 events were collected and analyzed for the expression of antigens to IgE and major histocompatibility complex (MHC) class II in B cells.

#### 2.4.6. Cytokine assay

To stimulate T-cell-receptor signaling, we cultured single-cell suspensions of LNs ( $1 \times 10^6$  cells/well) with T-cell antibodies to CD3 and/or CD28 (BD Pharmingen) for 24 or 96 h in Multidish 24-well plates (Nalge Nunc) at 37 °C in a controlled atmosphere of 5% carbon dioxide. Production of IL-2 was analyzed in the supernatants prepared following culture for 24 h in the presence of anti-CD3 antibody (2  $\mu$ g/ml). Production of IL-10, IL-13, IFN- $\gamma$ , and TNF- $\alpha$  was analyzed in the supernatants prepared following culture for 96 h in the presence of anti-CD3 (2  $\mu$ g/ml). Production of IL-4 was analyzed in the supernatants prepared following culture for 24 h in the presence of anti-CD3 (2  $\mu$ g/ml) and anti-CD28 (2  $\mu$ g/ml). Production of IL-5 was analyzed in the supernatants prepared following culture for 96 h in the presence of anti-CD3 (2  $\mu$ g/ml) and anti-CD28 (2  $\mu$ g/ml). Supernatants were assayed by BD Cytometric Bead Array (BD Pharmingen) in accordance with the manufacturer's protocol.

#### 2.4.7. Statistical analysis

For the statistical analysis, the data were transformed logarithmically to equal the variances. Then analysis of variance (ANOVA) was used to evaluate the results. When ANOVA was significant, the differences between groups were assessed by means of ANOVA followed by the Tukey–Kramer test. A probability value ( $P$ ) of <0.05 was considered to be significant.

### 3. Results

#### 3.1. Pilot study: local lymph node assay

To confirm the sensitizing potential of TMA, TDI, and DNCB in BALB/c mice, we performed the LLNA as preliminary test.

**Table 2**

Results of pilot study (local lymph node assay)

| Dose (%) | Lymph node weight (mg) | <sup>3</sup> H-TdR incorporation (dpm) |       |
|----------|------------------------|----------------------------------------|-------|
|          |                        | Mean ± S.D.                            | SI    |
| TMA      |                        |                                        |       |
| 0        | 6.50 ± 1.87            | 300 ± 99                               | 1.00  |
| 0.1      | 8.98 ± 2.00            | 707 ± 374                              | 2.36  |
| 0.3      | 9.50 ± 1.35            | 1138 ± 556                             | 3.79  |
| 1        | 13.83 ± 1.89**         | 4217 ± 554**                           | 14.06 |
| 3        | 24.35 ± 4.00**         | 9806 ± 2016**                          | 32.69 |
| TDI      |                        |                                        |       |
| 0        | 9.08 ± 2.22            | 1724 ± 755                             | 1.00  |
| 0.03     | 11.80 ± 2.05           | 5761 ± 927                             | 3.34  |
| 0.1      | 23.08 ± 1.72**         | 18,821 ± 3312**                        | 10.92 |
| 0.3      | 33.60 ± 4.51**         | 31,816 ± 8932**                        | 18.45 |
| 1        | 42.32 ± 3.34**         | 44,586 ± 2669**                        | 25.87 |
| DNCB     |                        |                                        |       |
| 0        | 9.23 ± 4.68            | 1391 ± 636                             | 1.00  |
| 0.03     | 8.37 ± 1.63            | 2219 ± 1297                            | 1.59  |
| 0.1      | 10.60 ± 1.48           | 6910 ± 923**                           | 4.97  |
| 0.3      | 21.93 ± 3.20**         | 22,005 ± 2010**                        | 15.81 |
| 1        | 28.57 ± 1.43**         | 34,264 ± 2806**                        | 24.63 |

\*\* $P$  < 0.01. SI is the ratio of the mean of the measurements of the treatment group to that of the solvent control group. The criterion for a positive response in the standard local lymph node assay was  $SI \geq 3$ . Values for treatment and solvent control groups were compared by Dunnett's multiple comparison test. dpm: disintegrations per minute.

The LLNA results are presented in Table 2. When TMA was dissolved in AOO, LN weight and incorporation of <sup>3</sup>H-TdR increased in a dose-dependent manner, and differences between test and control values achieved statistical significance in the 1% ( $P$  < 0.01) and 3% ( $P$  < 0.01) treatment groups. In the context of [<sup>3</sup>H]-TdR incorporation, TMA induced a positive response, defined as  $SI \geq 3$ , in the 0.3–3% treatment groups. When TDI was dissolved in AOO, LN weight and incorporation of <sup>3</sup>H-TdR also increased in a dose-dependent manner, and differences between test and control values achieved statistical significance in the 0.1–1% ( $P$  < 0.01) treatment groups. TDI induced a positive response, defined as  $SI \geq 3$ , in the 0.03–1% treatment groups. When DNCB was dissolved in AOO, LN weight and incorporation of <sup>3</sup>H-TdR increased in a dose-dependent manner, and differences between test and control values achieved statistical significance in the 0.3–1% in the case of LN weight treatment groups ( $P$  < 0.01) and 0.1–1% ( $P$  < 0.01) in the case of incorporation of <sup>3</sup>H-TdR increased. DNCB induced a positive response, defined as  $SI \geq 3$ , in the 0.1–1% treatment groups.

In the standard LLNA, the criterion for a positive response was  $SI \geq 3$  (Dearman et al., 1999). According to the LLNA results, the EC3 values of TMA, TDI, and DNCB were 0.20%, <0.03%, and 0.06%, respectively.

#### 3.2. Main study: long-term sensitization for detection of chemical allergy

##### 3.2.1. Antigen-specific serum IgE

Antigen-specific serum IgE levels are shown in Fig. 2. The TMA +/+ treatment group (sensitized and challenged with test solution; relative unit value:  $182 \pm 110$ ) showed a significant increase in specific serum IgE levels relative to the –/– group (sensitized and challenged with solvent) (1) and the –/+ group (only chemical challenge) (1). The TDI +/+ treatment group ( $324 \pm 140$ ) had a significant increase in specific serum IgE levels relative to the –/– group (1) and the –/+ group (1). The DNCB +/+ treatment group ( $12 \pm 20$ ) showed an increasing trend, but the reaction was mild and no significant difference was observed.

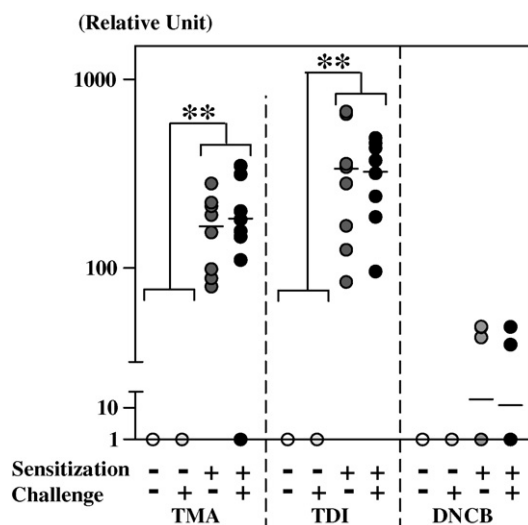


**Fig. 2.** Antigen-specific IgE levels in mouse serum isolated 1 day after challenge with test solution or solvent alone. Results are expressed as mean (titer)  $\pm$  S.D. Statistical significance is marked by asterisks: \*\* $P < 0.01$  (Tukey's  $t$ -test).

### 3.2.2. Numbers of lymphocytes and flow cytometric analysis

To evaluate the state of activation of B cells following TMA, TDI and DNCB treatment, the flow cytometric analysis was confirmed by staining the lymphocytes with anti-B220, -IgE, and -MHC class II (I-A/I-E) antibodies. Total numbers of lymphocytes are shown in Fig. 3A. Numbers of IgE-positive B cells (B220<sup>+</sup>IgE<sup>+</sup>) and MHC class II-positive B cells (B220<sup>+</sup>I-A/I-E<sup>+</sup>) are shown in Fig. 3B and C.

TMA-treated groups, the total number of lymphocytes and numbers of IgE- and MHC class II-positive B cells were significantly higher in the +/+ group than in all other treatment groups (-/-, -/+, and +/-) ( $P < 0.01$ ) (Fig. 3A–C). In the +/+ group, the populations of IgE- and MHC class II-positive B cells were  $66.00 \pm 19.2$  ( $\times 10^6$  cells, mean  $\pm$  S.D.) and  $66.05 \pm 19.7$ , respectively—an increase of approximately 900% over the -/- group and 300% over the +/- group (Fig. 3B and C). In the TDI-treated group, the total number of lymphocytes and the numbers of IgE- and MHC class II-positive B cells were significantly higher in the +/+ group than in all other treatment groups ( $P < 0.01$ ) (Fig. 3A–C). In the +/+ group, the populations of IgE- and MHC class II-positive B cells were  $69.57 \pm 23.0$  and  $69.86 \pm 22.5$ , respectively—approximately 800% increase over the -/- group and the -/+ group, and a 200% increase over the +/- group (Fig. 3B and C). In the DNCB-treated group, the total number of lymphocytes and the numbers of IgE- and MHC class II-positive B cells were significantly greater in the +/+ group than in all other treatment groups ( $P < 0.01$ ) (Fig. 3A–C). In the +/+ group, the populations of IgE- and MHC class II-positive B cells were  $86.22 \pm 21.5$  and  $90.44 \pm 19.42$ —approximately 800% increase over the -/- group and a 200% increase over the +/- group (Fig. 3B and C).

### 3.2.3. Cytokine production

To determine the effects of the chemicals on the level of allergy-associated cytokine production, we cultured LN cell suspensions with T-cell antibodies (CD3 and/or CD28) for 24 or 96 h and assayed the supernatant by cytometric bead array.

Analysis of Th1 cytokine production (IL-2, IFN- $\gamma$ , and TNF- $\alpha$ ) induced by TMA, TDI, and DNCB treatment revealed significant differences between the +/+ group and the control groups (-/-, -/+, and +/-) ( $P < 0.01$ ) (Fig. 4A–C). Marked increases in IL-2, IFN- $\gamma$  and TNF- $\alpha$  levels were observed in the DNCB treatment group. In the TMA and TDI +/+ groups, production of IL-2 was  $802 \pm 207$  pg/animal (mean  $\pm$  S.D.) and  $1522 \pm 841$  pg/animal,

respectively. In the DNCB treatment group, production of IL-2 was  $3640 \pm 1766$  pg/animal—approximately 400% increase over the TMA treatment group and a 200% increase over the TDI treatment group (Fig. 4A). IFN- $\gamma$  production induced by TMA and TDI treatment was  $12,114 \pm 8147$  pg/animal and  $12,182 \pm 5051$  pg/animal, respectively (Fig. 4B). In the DNCB treatment group, production of IFN- $\gamma$  was  $17,603 \pm 5970$  pg/animal—approximately 150% increase over the TMA and TDI treatment groups (Fig. 4C). TNF- $\alpha$  production induced by TMA and TDI treatment was  $3294 \pm 964$  pg/animal and  $5892 \pm 1698$  pg/animal, respectively (Fig. 4C). In the DNCB treatment group, production of TNF- $\alpha$  was  $8828 \pm 2575$  pg/animal—approximately 300% increase over the TMA treatment group and a 150% increase over the TDI treatment group (Fig. 4C).

On the other hand, marked increases in Th2 cytokine production (IL-4, IL-5, IL-10, and IL-13) were seen in the TMA and TDI treatment groups (Fig. 5). Analysis of the Th2 cytokine production induced by TMA and TDI treatment revealed significant differences between the +/+ group and the control groups (-/-, -/+, and +/-) (Fig. 5A–D). When mice were treated with TMA or TDI, production of IL-4 in the +/+ group was  $359 \pm 158$  pg/animal (a 500% increase over the +/- group) and  $211 \pm 79$  pg/animal (a 150% increase over the +/- group), respectively (Fig. 5A). IL-5 production induced by TMA and TDI +/+ group was  $658 \pm 303$  pg/animal (a 700% increase over the +/- group) and  $599 \pm 232$  pg/animal (a 250% increase over the +/- group), respectively (Fig. 5B). However, there was no increase in IL-4 production in the DNCB treatment group (Fig. 5A) and there was no significant difference between the +/+ group and the +/- group in IL-5 production in the DNCB (Fig. 5B). In the production of IL-10 and IL-13 induced by DNCB treatment, significant differences were observed in the +/+ groups compared with the control groups (-/-, -/+, and +/-).

## 4. Discussion

Our initial goal was to develop a new method for detecting environmental chemical-related hypersensitivity at weakly immunogenic and low doses. To that end, we developed a new long-term sensitizing protocol in a mouse model treated with typical Th1 (DNCB) and Th2 (TMA and TDI) type sensitizers, and we then looked for sensitive methods for detecting low-level allergic reactions.

We first evaluated the response to TMA, TDI, and DNCB in the LLNA. Both Th1 (DNCB) and Th2 (TMA and TDI) type sensitizers gave positive reactions in the LLNA (see Table 2). This test assesses, in mice, the potential of chemicals to cause a primary T-lymphocyte proliferative response after topical application of the chemicals to the skin (Dearman et al., 1999). Because the LLNA involves dermal application, it is, strictly speaking, appropriate only for assessing the risk of allergic contact dermatitis. However, Vanoirbeek et al. (2003) demonstrated that this test could detect the “generic” ability of chemicals to cause sensitization. If this is the case, then the LLNA could be relevant not only for predicting the risk of allergic dermatitis but also the risk of airway hypersensitivity from inhaled chemical sensitizers. In practice, the technical ease of dermal application makes it a more appealing route of exposure for model development than other routes of sensitization. However, environmental chemical allergens tend to have weak, minimal immunogenicity; this methods have not focused on the detection of such a weak allergic reactions. To clear up these problems, we next set up a new method that uses long-term dermal sensitization followed by dermal challenge. To confirm the availability of this method, we adopted a challenge dose (TMA: 0.1%; TDI: 0.01%; DNCB: 0.03%) that was less than the EC3 value and a sensitization dose (TMA: 1%; TDI: 0.1%; DNCB: 0.3%) that was 10 times the chal-

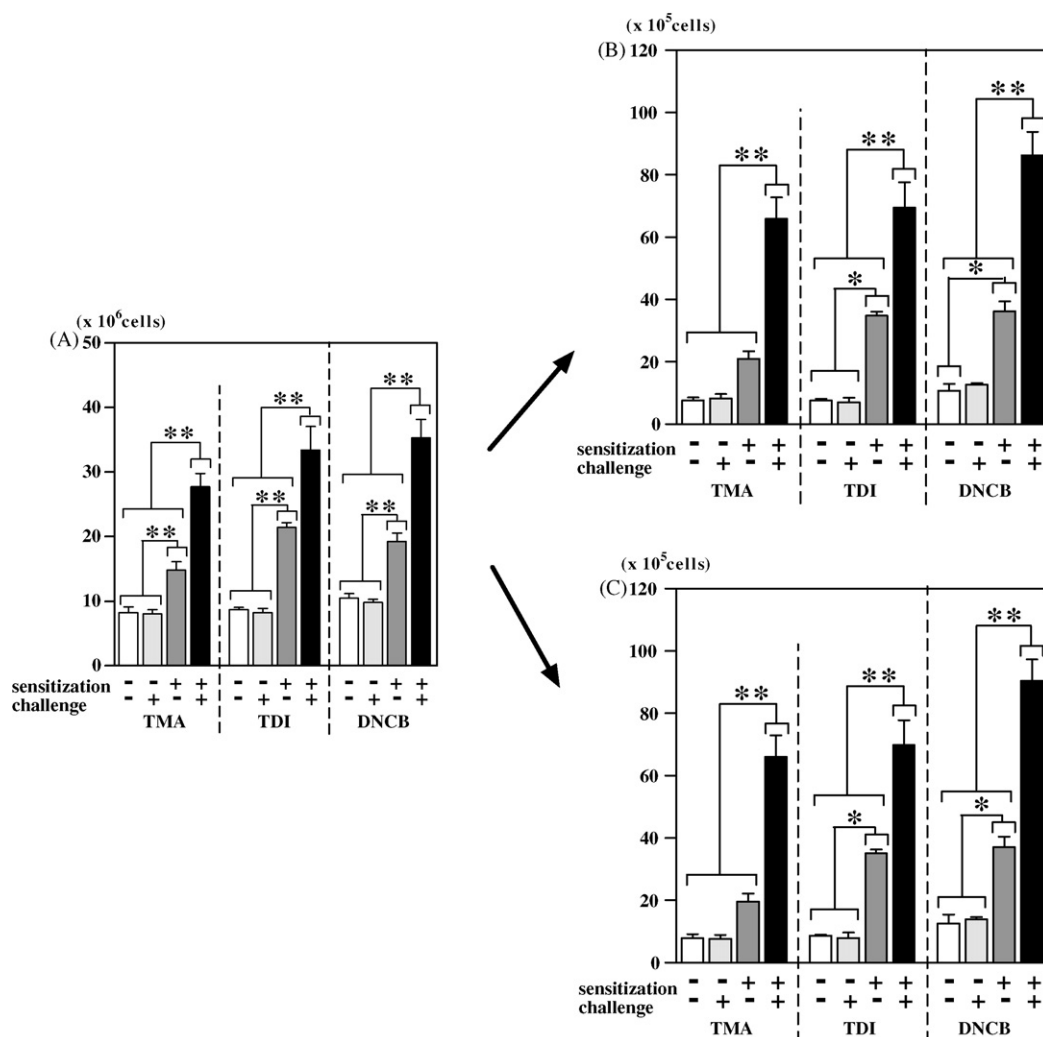


**Fig. 3.** Total number of lymphocytes (A), B220<sup>+</sup>IgE<sup>+</sup> cells (B), and B220<sup>+</sup>MHC class II<sup>+</sup> cells (C) in auricular lymph nodes from mice treated with test solutions or solvent alone. Results are expressed as mean (cells)  $\pm$  S.D. Statistical significance is marked by asterisks: \**P* < 0.05, \*\**P* < 0.01 (Tukey's *t*-test).

challenge dose. These challenge doses of TMA, TDI and DNCB that were not capable of inducing a proliferative response at a single dosing could, after multiple doses, induce an increase in the population of LN cells.

To detect the environmental chemical-related hypersensitivity at weakly immunogenic and low doses, we focused on several immune endpoints in main study. Among them, we used IgE as a central marker of chemical-induced allergy. Especially, there is no doubt that specific IgE antibody plays a key role in allergy to inhaled chemicals (Kimber and Dearman, 2002). In our study, TMA and TDI induced a prominent increase in antigen-specific serum IgE levels compared to DNCB-treated groups. Generally speaking, DNCB is Th1 type allergens and TMA and TDI are Th2 type allergens. This upregulation would be a useful endpoint for identifying and characterizing chemical-related sensitizers.

Along with the serum IgE levels, we analyzed the IgE-expressing B-cell population in the LN by flow cytometry. IgE is produced by plasma cells located in the lymph nodes draining the site of antigen entry, and locally, at the sites of allergic reactions, by plasma cells derived from germinal centers developing within the inflamed tissue (Janeway et al., 2004). Surface IgE-expressing B cells have recently been shown to be necessary for IgE production. Following stimulation with known IgE-inducing allergens, animals lacking B cells with the ability to express surface IgE produced negligi-

ble amounts of total and antigen-specific IgE (Achatz et al., 1997; Manetz and Meade, 1999). According to our results, significant increases in the IgE-expressing B-cell population were observed in both TMA- and TDI-treated groups (see Fig. 3). In this model schedule for the detection of sensitizers, challenge doses of TMA and TDI that were not capable of inducing a proliferative response at a single dosing could, after multiple doses, induce an increase in the population of IgE-expressing B cells. This upregulation of IgE-expressing B cells would be a useful endpoint for identifying chemical-related respiratory sensitizers. However, significant increases in the numbers of these cells were observed not only in the TMA- and TDI-treated groups (Th2 type) but also in the DNCB-treated groups (Th1 type) (see Fig. 3).

In the development of immune responses, activated local B lymphocytes can act as antigen-presenting cells for CD4<sup>+</sup> and CD8<sup>+</sup> T-cells (Goutet et al., 2005). The CD4<sup>+</sup> T-cells that recognize MHC class II molecules are of two functional types: Th1 and Th2 (Santana and Rosenstein, 2003). Th1 cells are specialized to activate macrophages that are promoted by, or have ingested, pathogens. Th2 cells are specialized for B cell activation (Santana and Rosenstein, 2003). To evaluate the state of activation of Th1 and Th2 cells following DNCB, TMA and TDI treatment, we assessed the membrane expression of MHC class II molecules, which are important for antigen presentation and regulation of the immune

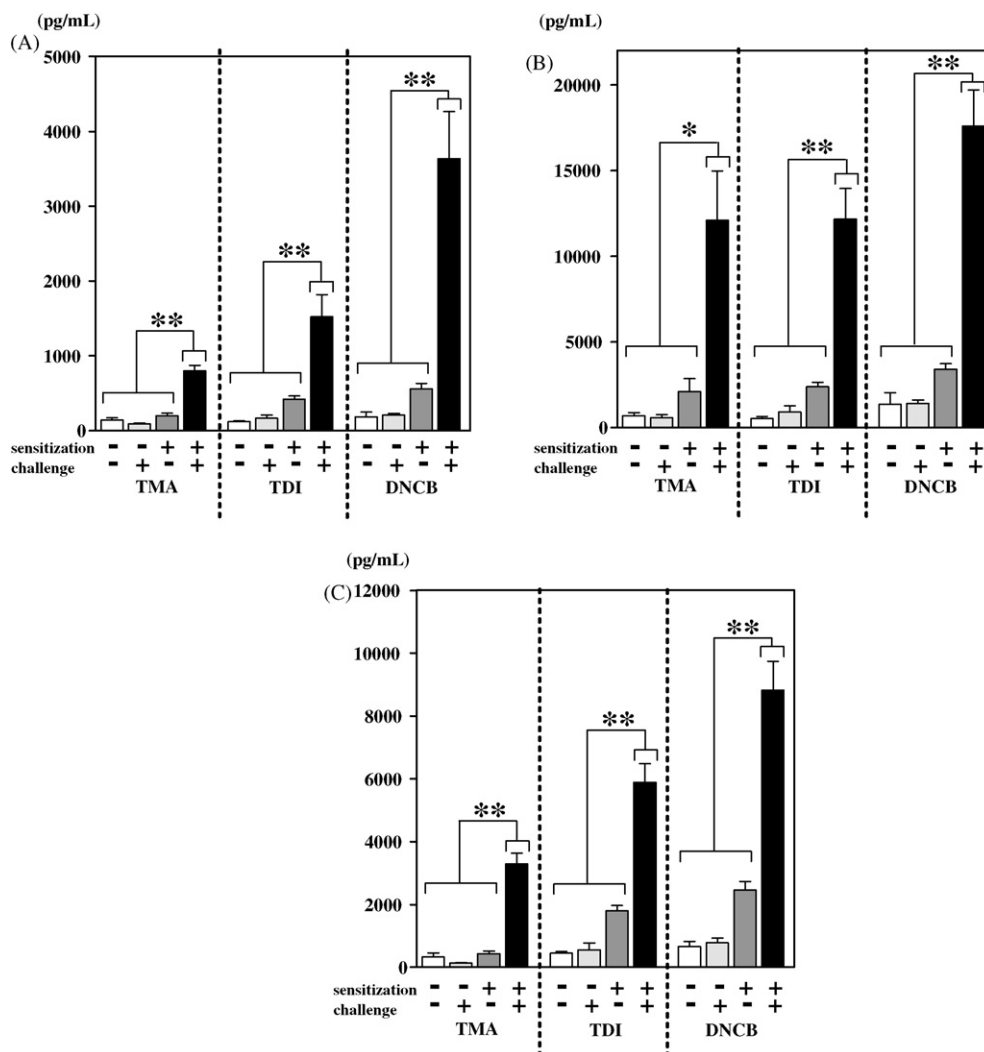


**Fig. 4.** Th1 cytokine production (A: IL-2; B: IFN- $\gamma$ ; C: TNF- $\alpha$ ) in auricular lymph nodes of mice treated with test solution or solvent alone. To determine the effects of cytokine production, lymph node cell suspensions were cultured with T-cell antibodies (CD3 and/or CD28) for 24 or 96 h, and the supernatant was assayed by cytometric bead array. Results are expressed as mean (pg/mouse)  $\pm$  S.D. Statistical significance is marked by asterisks: \*\* $P$  < 0.01 (Tukey's  $t$ -test).

response (Murray, 1998). Significant increases in the MHC class II-expressing B cell population were observed in TMA-, TDI- and DNCB-treated groups (see Fig. 3). This upregulation of MHC class II-expressing B cells would be a useful endpoint for demonstrating activation of the immune system. In addition, Goutet et al. (2005) and Gerberick et al. (2002) demonstrate that measuring the increase in the membrane expression of B220 cells and MHC class II molecules in the draining LN after dermal sensitization is useful in discriminating between allergen and irritant response. According to these reports, these factors were useful for identifying the weakly immunogenic sensitizers and discriminating between sensitizers and irritants.

There are two main components of the immune response leading to IgE production. The first consists of the signals that favor the differentiation of naïve Th0 cells to the Th2 phenotype. The second comprises the action of cytokines and co-stimulatory signals from the Th2 cells that stimulate B cells to switch to producing IgE antibodies. Th2 cells produce IL-4, IL-5, IL-10, and IL-13 (Mosmann and Coffman, 1989; Yssel and Groux, 2000), and these responses are down-regulated by Th1 cytokines (IFN- $\gamma$ , TNF- $\alpha$ , and IL-2) (Gavett et al., 1995; Lack et al., 1996). Previous reports have shown that IL-4 promotes T-cell activation and differentiation into the Th2 subtype, whereas both IL-4 and IL-13 can switch the antibody isotype from

IgM to IgE (Kaplan et al., 1996; Oettgen, 2000; Shim et al., 2001). In our study, TMA and TDI induced a prominent increase in levels of IL-4 produced by ex vivo restimulated lymph node cells. The IL-4 was associated with a subsequent increase in antigen-specific serum IgE level (see Fig. 5). In contrast, the Th1 cytokine secretion profile was induced by identical treatment with the Th1 type allergen DNCB, with the production of high levels of IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 compared with TMA and TDI (see Fig. 4). However, our results showed an elevation in Th1 cytokine levels following TMA or TDI exposure and in Th2 cytokine levels following DNCB exposure.

In recent years, several investigators have shown that the dermal sensitization followed by dermal or airway challenge results in disparate immediate and late airway reactions that do not correspond with elevated IgE-expressing B-cell population and Th2 type cytokine production in LN draining the site of dermal application (Ban et al., 2006; Farraj et al., 2007; Goutet et al., 2005; Manetz and Meade, 1999; Vandebriel et al., 2003). In fact, elevation in IgE-expressing B-cell population and Th2 cytokine levels following DNCB exposure were observed in our study. However, it is possible that the divergence in these results might be due partly to differences in experimentation, e.g. sensitizing routes and doses of chemical used to sensitize and challenge (Ban et al., 2006). Especially, in our model schedule for the detection of sensitizers, it

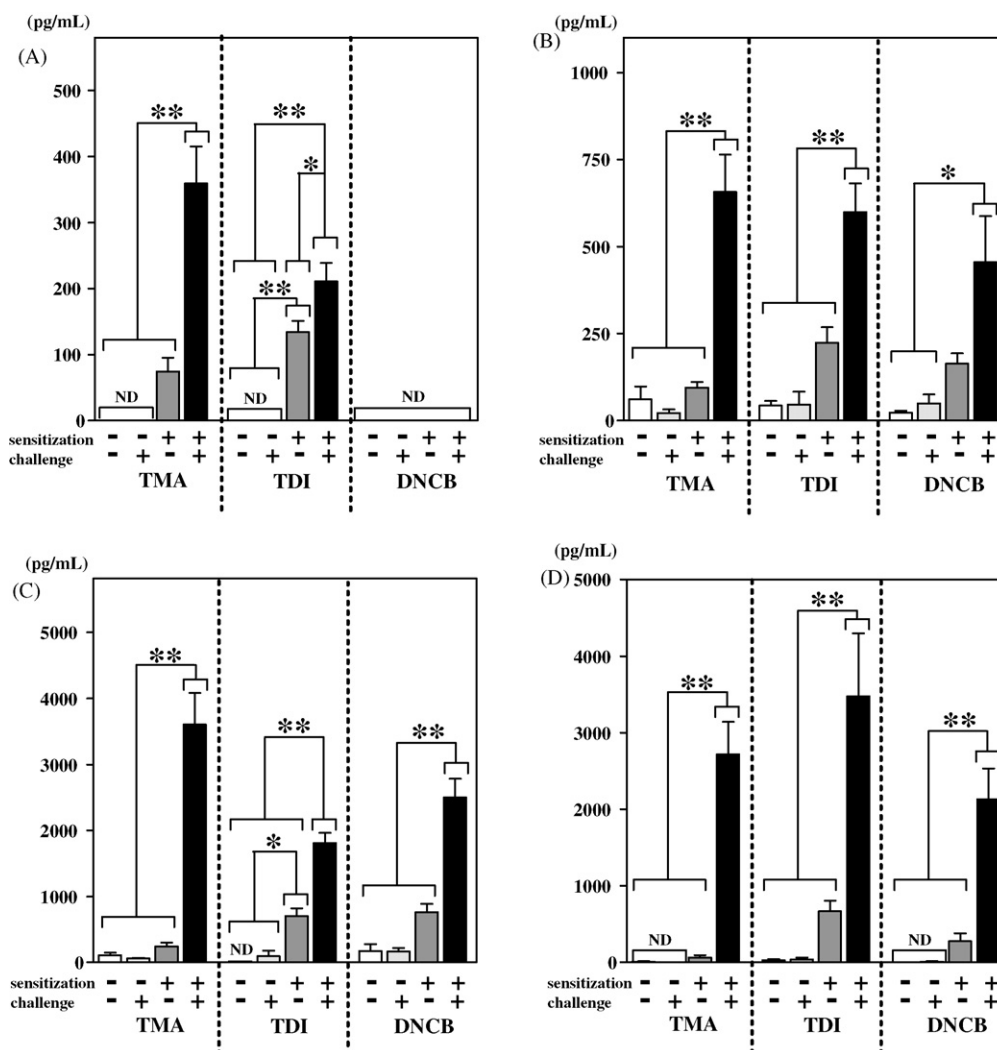


**Fig. 5.** Th2 cytokine production (A: IL-4; B: IL-5; C: IL-10; D: IL-13) in auricular lymph nodes of mice treated with test solution or solvent alone. To determine the effects of cytokine production, lymph node cell suspensions were cultured with T-cell antibodies (CD3 and/or CD28) for 24 or 96 h, and the supernatant was assayed by cytometric bead array. Results are expressed as mean (pg/mouse)  $\pm$  S.D. Statistical significance is marked with asterisks: \* $P < 0.05$ ; \*\* $P < 0.01$  (Tukey's  $t$ -test).

suggests that repeat application to same site is part of the reason for enhancement of IgE and cytokine production in LN. We thought that another route of challenge and other endpoints could clear up these problems. The experiments are currently under investigation in our laboratories.

Although allergic effects of environmental chemical exposure have been studied extensively, allergic effects at low doses remain unknown (Arts et al., 2003; Dearman et al., 2005; Larsen et al., 2001; Manetz and Meade, 1999; Satoh et al., 1995). Our long-term sensitizing method should be valuable for detecting allergic reactions caused by chemicals at weakly immunogenic and low doses in environment. It might be useful for further investigating the mechanisms of MCS.

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