



Allergic reaction induced by dermal and/or respiratory exposure to low-dose phenoxyacetic acid, organophosphorus, and carbamate pesticides

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ABSTRACT

Several types of pesticides, such as organophosphates, phenoxyacetic acid, and carbamate have a high risk of affecting human health, causing allergic rhinitis and bronchial asthma-like diseases. We used our long-term sensitization method and a local lymph node assay to examine the allergic reactions caused by several types of pesticides. BALB/c mice were topically sensitized (9 times in 3 weeks), then challenged dermally or intratracheally with 2,4-D, BRP, or furathiocarb. One day post-challenge, the mice were processed to obtain biologic materials for use in assays of total IgE levels in serum and bronchoalveolar lavage fluid (BALF); differential cell counts and chemokine levels in BALF; lymphocyte counts and surface antigen expression on B-cells within regional lymph nodes (LNs); and, ex situ cytokine production by cells from these LNs. 2,4-D-induced immune responses characteristic of immediate-type respiratory reactions, as evidenced by increased total IgE levels in both serum and BALF; an influx of eosinophils, neutrophils, and chemokines (MCP-1, eotaxin, and MIP-1 β) in BALF; increased surface antigen expression on B-cells IgE and MHC class II production) in both auricular and the lung-associated LNs; and increased Th2 cytokine production (IL-4, IL-5, IL-10, and IL-13) in both auricular and the lung-associated LN cells. In contrast, BRP and furathiocarb treatment yielded, at most, non-significant increases in all respiratory allergic parameters. BRP and furathiocarb induced marked proliferation of MHC Class II-positive B-cells and Th1 cytokines (IL-2, TNF- α , and IFN- γ) in only auricular LN cells. These results suggest that 2,4-D is a respiratory allergen and BRP and furathiocarb are contact allergens. As our protocol detected classified allergic responses to low-molecular-weight chemicals, it thus may be useful for detecting environmental chemical-related allergy.

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1. Introduction

Several types of pesticides, including organophosphates, phenoxyacetic acid, and carbamate, are widely used throughout the world in agriculture and/or eradicating termites around homes (Ellenhorn and Barceloux, 1988; Lopez et al., 2007; Nakadai et al., 2006; OECD, 2008; Wessels et al., 2003). These pesticides are sold in large quantities in many countries and are applied at yearly rates of 0.21 tonnes (t)/km² in the USA, 1.06 t/km² in the Netherlands, 1.29 t/km² in Korea, and 1.5 t/km² – the highest rate – in Japan (OECD, 2008). Many studies have reported deleterious effects of these pesticides on the nervous, immune, urinary, and reproductive systems (Blaylock et al., 1992; Jones and Miller, 2008; Li et al., 2007; Lopez et al., 2007; Nakadai et al., 2006; Takeuchi et al., 2002).

In particular, pesticides that have diffused into ambient air have a high risk of causing allergic rhinitis and bronchial asthma-like diseases in humans (Boers et al., 2008; Hernandez et al., 2004a,b, 2006, 2008; Hoppin et al., 2002, 2007, 2008; Proskocil et al., 2008; Stejskal and Hubert, 2008). Aerial spraying is a major source of exposure (Hoppin et al., 2007, 2008; Proskocil et al., 2008), and aerial application of pesticides has been restricted in Europe and the USA in recent years (European Commission, 2006b; US EPA, 2007). However, the ease of aerial spraying has still led to its wide use on many farm products in many countries (Hoppin et al., 2002, 2007, 2008; OECD, 2008; Proskocil et al., 2008). Therefore, there is a need for protocols for the treatment and detection of respiratory allergic diseases triggered by pesticides that have diffused into the environment.

Recently, several detection methods have been developed to identify chemicals that trigger allergic diseases (Arts et al., 2003; Ban et al., 2006). However, these methods have focused on the detection of strong allergic reactions, whereas environmental chemical allergens (such as diffused pesticides) tend to have weak or minimal immunogenicity. In addition, experimental allergic

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responses to allergens are dependent on many factors, including sensitizing route, application method, and animal species. Therefore, new protocols are needed to detect (as well as eventually treat) and treat respiratory allergies caused by weakly immunogenic and low-dose allergens. Previously, in our first stage of studies, we developed a new method of detecting environmental chemical-related dermal or respiratory allergic diseases. We used typical chemical sensitizers (2,4-dinitrochlorobenzene [DNCB], trimellitic anhydride [TMA], and toluene diisocyanate [TDI]) in a long-term dermal sensitization protocol followed by dermal or respiratory challenge (Fukuyama et al., 2008a,b). DNCB is a contact allergen, whereas the others (TMA and TDI) are respiratory allergens. Indeed, TMA-induced prominent increases in several parameters indicative of induced allergic responses, including IgE levels (antigen-specific IgE level and IgE-positive B-cell population), eosinophil proliferation, local (lung airway) chemokine levels (MCP-1, MIP-1 β , and eotaxin) and cytokine levels (interleukin [IL]-4, -10, and -13). TDI induced significant increases in IgE-positive B-cell numbers and local (lung airway) cytokine production. In contrast, DNCB sensitization only yielded non-significant increases in each of these parameters. These results demonstrated that our method is able to detect and classify allergic reactions caused by chemicals present in the environment at weakly immunogenic and low doses.

In this second stage study, we examined the allergic reactions caused by several types of pesticides using our long-term sensitization method (Fukuyama et al., 2008a,b) in conjunction with a local lymph node assay (LLNA) (National Institute of Environmental Health Sciences, 1999; Basketter et al., 2002). The LLNA was developed initially for hazard identification (Basketter et al., 1999; Dearman et al., 1999) and has now been evaluated extensively and validated formally (Dearman et al., 1999; Gerberick et al., 2000; Basketter et al., 2002). The chemicals used in the study reported here were the phenoxyacetic acid pesticide 2,4-D, the organophosphorus pesticide BRP, and the carbamate pesticide furathiocarb. These pesticides were specifically selected for study in that: Cushman and Street (1982) demonstrated the ability of 2,4-D to elicit respiratory allergy-specific IgE antibodies in Balb/c mice (although Hoppin et al. (2002) showed that 2,4-D was not associated with wheezing.); and, a material data sheet stated that furathiocarb and BRP induced a positive response in a guinea pig skin sensitization study (ACGIH, 2002).

2. Materials and methods

2.1. Chemicals

Standard 2,4-D ($C_{12}H_6H_3OCH_2COOH$), BRP ($C_4H_7Br_2C_{12}O_4P$), and furathiocarb ($C_{18}H_{26}N_2O_5S$), acetone, and olive oil were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). For the dermal sensitization and challenge, each test chemical was dissolved in 4:1 acetone:olive oil (AOO). For the intratracheal challenge, each test chemical was dissolved in acetone and diluted in sterile physiological saline solution. The concentrations of 2,4-D, BRP, and furathiocarb used are presented in Table 1. These concentrations were selected to avoid systemic toxicity and/or excessive local sensitization (particularly in the preliminary test) while still permitting comparisons of the sensitizing potencies of the chemicals. In the LLNA we focused on determining the EC3 value (the concentration of a chemical required to elicit a threshold positive response), which was used to select suitable concentrations of 2,4-D, BRP, and furathiocarb for the main study. To confirm the

allergenicity of 2,4-D, BRP, and furathiocarb, we adopted a sensitization dose that was less than the EC3 values and a challenge dose that was 10 or 100 times lower than the sensitization dose (see Table 1).

2.2. Animals

Female CBA/Jn mice (age, 7 weeks) (for the LLNA study) and female BALB/c mice (age, 7 weeks) (for the main study) were purchased from Charles River Japan Laboratories (Atsugi, Kanagawa, Japan) and housed individually under controlled lighting (lights on from 07:00 to 19:00 h), temperature ($22 \pm 3^\circ C$), 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).

2.3. Local lymph node assay

We conducted an LLNA as a preliminary test to select the concentrations of 2,4-D, BRP, and furathiocarb for use in the main studies. The assay was performed as described by Kimber and Weisenberger (1989), with minor modifications. After a 1-week acclimatization period, CBA/Jn mice were allocated randomly to dose and control groups ($n=5$ per group). A 25- μ 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, ^3H -methyl thymidine (^3H -TdR, 20 μ Ci/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 the auricular LNs on both sides of each mouse were removed, weighed, and pooled by mouse in phosphate-buffered saline (PBS, Gibco, Tokyo, Japan). Single-cell suspensions of LNs in 5 ml PBS were prepared by passage through sterile 70- μ m nylon cell strainers (Falcon, Tokyo, Japan). The LN cell suspension was washed twice with an excess of PBS, and the cell pellet was incubated in 3 ml 5% trichloroacetic acid (TCA, Wako Pure Chemical Industries, Ltd.) at $4^\circ C$ for approximately 18 h. Each cell pellet was resuspended in 1 ml TCA and transferred to 9 ml of scintillation fluid (AtomLight, PerkinElmer Japan, Tokyo, Japan). Incorporation of ^3H -TdR was measured with a β -scintillation counter (LC-5100, Aloka, Tokyo, Japan) as disintegrations per minute (DPM) for each mouse.

Stimulation indexes (SIs) and EC3 values were calculated from the ^3H -TdR incorporation data. The SI was calculated by dividing the mean ^3H -TdR incorporation value for each treatment group by that of the solvent control group. The EC3 value is an estimate of the amount of 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).

2.4. Experimental design (main study: long-term sensitization)

For the main study, two different protocols (dermal challenge and respiratory challenge) were used. In both the dermal and respiratory challenge protocols, the BALB/c mice were divided into five groups for each chemical (2,4-D, BRP, and furathiocarb): Group–/– (both sensitized and challenged with solvent only), Group–/+ (sensitized with solvent only and challenged with high-dose test solution), Group+/- (sensitized with test solution and challenged with solvent only), Group+/-^{low} (sensitized with test solution and challenged with low-dose test solution), and Group+/-^{high} (sensitized with test solution and challenged with high-dose test solution). In this study, we define –/–, –/+ and +/- groups as the control groups.

After a 1-week acclimatization period, mice were allocated randomly to dose and control groups ($n=6$ or 7 per group). In both the dermal and respiratory challenge protocols, a 25- μ l aliquot of the test solution or solvent alone was applied to the dorsum of each ear of each mouse on days 1–3, 8–10, and 15–17 for dermal sensitization. In the dermal challenge protocol, 2 weeks after the last sensitization (day 31), a 25- μ l aliquot of test solution or solvent only was applied as previously for the challenge. In the respiratory challenge protocol, 2 weeks after the last sensitization (day 31), mice were anesthetized by intraperitoneal injection of pentobarbital sodium (50 mg/kg), and then challenged with a 50- μ l aliquot of test solution or solvent alone injected intratracheally via a 29 G needle. On the day after challenge (day 32), all mice were anesthetized with pentobarbital sodium (75 mg/kg) and sacrificed. Blood samples were taken from the inferior vena cava, and serum samples were assayed for total IgE. Each animal's auricular (dermal challenge protocol) or lung-associated (respiratory challenge protocol) LNs were removed and pooled by mouse in RPMI 1640 medium (Gibco, Tokyo, Japan). In the respiratory challenge protocol, bronchoalveolar lavage fluid (BALF) was collected by cannulating the trachea and lavaging the lungs three times with 1 ml phosphate-buffered saline (PBS, Gibco, Tokyo, Japan) supplemented with 1% heat-inactivated fetal calf serum (FCS, Gibco) warmed to $37^\circ C$. The first BALF fraction from each animal was then centrifuged at $350 \times g$ for 5 min, and then all the supernatants from all mice in a group were pooled. The supernatant was assayed for total IgE, chemokine (eotaxin, MCP-1, MIP-1 β , and RANTES), and cytokine (IL-6 and TNF- α) levels. The cell pellets of the first fraction and the other two fractions were pooled and centrifuged at $350 \times g$ for 5 min. The supernatant was removed, and the cell pellet was used for cell counts and differentials.

Table 1
Chemical concentrations used.

Chemical	Local lymph node assay (LLNA)	Main study	
		Sensitization	Challenge
2,4-D	0%/1%/3%/10%/30%	5%	Low: 0.05%, high: 0.5%
BRP	0%/0.1%/0.3%/1%/3%	0.3%	Low: 0.003%, high: 0.03%
Furathiocarb	0%/0.1%/0.3%/1%/3%	1%	Low: 0.01%, high: 0.1%

2.5. Total IgE

Total IgE in serum and BALF was measured by ELISA (Mouse IgE Set, Becton Dickinson [BD] Pharmingen, San Diego, CA, USA) in accordance with the manufacturer's protocol. Optical density (OD) was read at a wavelength of 450 nm with an ImmunoMini reader (NJ-2300, Nippon Intermed, Tokyo, Japan).

2.6. Bronchoalveolar lavage fluid analysis

The number of cells was determined with a Coulter counter Z2 (Beckman Coulter Co., Tokyo, Japan). For differential cell counts, Cytospin (Shandon Inc., Pittsburgh, PA) preparations and flow cytometric analysis (de Haar et al., 2005) were used. The Cytospin preparations were stained with Giemsa stain (Wako Pure Chemical Industries, Ltd.), and 200 cells in each sample were examined to determine the numbers of macrophages, eosinophils, and neutrophils present (according to standard leukocyte typing).

2.7. Lymph node analyses

For cell counts, flow cytometry, and cell culture, single-cell suspensions of LNs in 1 ml RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum were prepared by passage through sterile 70- μ m nylon cell strainers. The number of LN cells was determined with a Coulter Z2 counter (Beckman Coulter Co.).

To stimulate T-cell-receptor signaling, we cultured single-cell suspensions of LN (1×10^6 cells/well) with anti-CD3 (2 μ g/ml) and/or anti-CD28 (2 μ g/ml) (BD Pharmingen) T-cell antibodies for 24 or 96 h in 24-well plates (Multidish, Nalge Nunc International K.K., Tokyo, Japan) at 37 °C in a 5% carbon dioxide atmosphere. IL-2, TNF- α , and IFN- γ in the supernatants were quantified following culture for 24 h in the presence of anti-CD3 antibody. Production of IL-13 was analyzed in the supernatants prepared following culture for 96 h in the presence of anti-CD3. Production of IL-4, IL-10, and IL-5 in the supernatants was analyzed following culture for 24 h in the presence of anti-CD3 and anti-CD28.

2.8. Antibodies and flow cytometry

All antibodies for flow cytometric analysis – fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse IgE (clone R35-72), PE-Cy5-conjugated rat anti-mouse CD45R/B220 (clone RA3-6B2), FITC-conjugated rat anti-mouse Gr-1 (clone RB6-8C5), and phycoerythrin (PE)-conjugated hamster anti-mouse CD11c (HL3) – were purchased from BD Pharmingen. To avoid non-specific binding, 1×10^6 cells were incubated with 20% normal goat serum (Sigma, St. Louis, MO, USA) for 10 min at 4 °C; this was followed by incubation with FITC-, PE-, and PE-Cy5-conjugated mAbs for 30 min at 4 °C in the dark. The cells were washed twice with a wash buffer, resuspended at 1×10^6 cells per tube in 1 ml PBS, and then analyzed with a FACSCaliber flow cytometer (BD Pharmingen) by using the Cell Quest program (BD Pharmingen). For each sample, 10,000 events were collected and analyzed for the expression of antigens.

2.9. Cytokine and chemokine assays

Cytokines and chemokines (IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN γ , TNF α MCP-1, MIP1- β , and RANTES) in the supernatants of LN or BALF were assayed by BD

Cytometric Bead Array (BD Pharmingen) in accordance with the manufacturer's protocol. Eotaxin in the supernatants of BALF was measured by ELISA (Quantikine, R&D Systems, Inc., Minneapolis, MN, USA) in accordance with the manufacturer's protocol.

2.10. Statistical analysis

The data were transformed logarithmically to equalize the variances, and analysis of variance (ANOVA) was used to evaluate the results. When the ANOVA was significant, the differences between groups were assessed by Dunnett's multiple comparison test (LLNA) or the Tukey–Kramer test (main study). A probability value (P) of <0.05 was considered to indicate significance.

3. Results

3.1. Local lymph node assay

To confirm the sensitizing potential of 2,4-D, BRP, and furathiocarb in CBA/Jn mice, we performed an LLNA as a preliminary test. The LLNA results are presented in Table 2. When 2,4-D dissolved in AOO was used, both LN weight and incorporation of ^3H -TdR increased. The difference between test and control values achieved statistical significance in the 10% group ($P < 0.01$) in the case of LN weight, and 2,4-D induced a positive response in [^3H]-TdR incorporation (defined as $\text{SI} \geq 3$) in the 10% ($P < 0.01$) and 30% ($P < 0.05$) treatment groups. When BRP dissolved in AOO was used, LN weight increased and incorporation of ^3H -TdR also increased in a dose-dependent manner, and the differences between test and control values achieved statistical significance in the 1% and 3% ($P < 0.01$) treatment groups. When furathiocarb was dissolved in AOO, LN weight and incorporation of ^3H -TdR increased in a dose-dependent manner, and differences between test and control values achieved statistical significance in the 0.3% ($P < 0.05$), 1% ($P < 0.05$), and 3% ($P < 0.01$) treatment groups in the case of LN weight and in the 3% ($P < 0.01$) group in the case of incorporation of ^3H -TdR. Furathiocarb induced a positive [^3H]-TdR incorporation response, defined as $\text{SI} \geq 3$, in the 3% treatment group.

In the standard LLNA, the criterion for a positive response is $\text{SI} \geq 3$ (Dearman et al., 1999). According to the LLNA results, the EC3 values of 2,4-D, BRP, and furathiocarb were 5.91%, 0.46%, and 1.08%, respectively. For the studies herein, levels of 5.0%, 0.3%, and 1.0%, respectively of 2,4-D, BRP, and furathiocarb were used for all sensitization protocols.

Table 2
Results of local lymph node assay (LLNA).

	Dose (%)	Lymph node weight (mg)	^3H -TdR incorporation (DPM)	
		Mean $\pm$ S.D.	Mean $\pm$ S.D.	SI
2,4-D	0	5.00 $\pm$ 0.42	567 $\pm$ 237	1.00
	1	5.38 $\pm$ 1.76	609 $\pm$ 569	1.07
	3	6.68 $\pm$ 1.76	1109 $\pm$ 329	1.96
	10	9.90 $\pm$ 3.16**	2526 $\pm$ 1171**	4.46
	30	8.10 $\pm$ 0.42	2101 $\pm$ 771*	3.71
BRP	0	3.58 $\pm$ 0.67	334 $\pm$ 178	1.00
	0.1	5.32 $\pm$ 0.99	555 $\pm$ 398	1.66
	0.3	5.16 $\pm$ 1.50	704 $\pm$ 465	2.11
	1	7.46 $\pm$ 1.05**	2012 $\pm$ 672**	6.02
	3	11.46 $\pm$ 1.66**	5277 $\pm$ 1524**	15.80
Furathiocarb	0	4.14 $\pm$ 0.15	179 $\pm$ 61	1.00
	0.1	5.74 $\pm$ 0.56	310 $\pm$ 190	1.73
	0.3	6.96 $\pm$ 1.97*	397 $\pm$ 294	2.22
	1	6.88 $\pm$ 0.95*	468 $\pm$ 215	2.61
	3	8.22 $\pm$ 2.60**	2264 $\pm$ 762**	12.64

SI is the ratio of the mean measurement 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 $\text{SI} \geq 3$. Values for treatment and solvent control groups were compared by Dunnett's multiple comparison test. DPM: Disintegrations per minute.

* $P < 0.05$.

** $P < 0.01$.

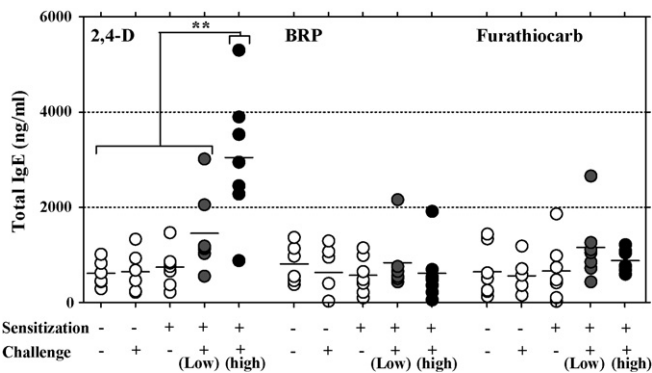


Fig. 1. Total serum IgE levels in dermal challenge protocol 1 day after challenge with test solution or solvent alone. Figure shows individual values of the IgE level (ng/ml) and bars are expressed as mean (ng/ml). Statistical significance is marked by asterisks: ** for $P < 0.01$ (Tukey's t -test). $n = 6$ –7 per group.

3.2. Main study (dermal challenge protocol)

In the dermal challenge protocol, we examined the total IgE levels in serum (Fig. 1), performed flow cytometric analysis of B-cell activation that had occurred in the LNs (Fig. 2), and examined cytokine production by the LN cells (Fig. 3).

The total serum IgE levels (Fig. 1) of the 2,4-D $+/+^{high}$ treatment group showed a significant increase ($P < 0.01$) relative to those in the control groups ($-/-$, $-/+$ and $+/-$ group) and the $+/+^{low}$ treatment group. The 2,4-D $+/+^{low}$ treatment group showed an increasing trend, but the reaction was mild, and no statistically significant differences compared with control groups were observed. On the other hand, there was no increase in total serum IgE levels in the BRP and furathiocarb $+/-$ treatment groups, and no statistically significant differences compared with control groups were observed.

To evaluate the activation of B-cells in auricular LNs following treatment with 2,4-D, BRP, or furathiocarb treatment, flow cytometric analysis was performed (Fig. 2A–C). In the 2,4-D-treated groups, the total numbers of lymphocytes and IgE- and MHC-Class II-positive B-cells were also significantly higher in the $+/+^{high}$ group than in control and $+/+^{low}$ treatment groups ($P < 0.01$). In addition, the total numbers of IgE+ B-cells in all sensitized hosts were significantly greater than in their non-sensitized counterparts. In the BRP-treated mice, the total number of lymphocytes was significantly higher in the $+/+^{high}$ group than in the control groups ($P < 0.01$); levels in the $+/+^{low}$ hosts were only significantly greater than those in the non-sensitized mice. The numbers of MHC Class II+ B-cells were significantly higher in the $+/+^{high}$ group than in the controls ($P < 0.01$). However, no elevation in the number of IgE+ B-cells due to any BRP exposures was observed. In the furathiocarb-treated groups, the total numbers of lymphocytes and MHC-Class II-positive B-cells were significantly higher in the $+/+^{high}$ group than in all other treatment groups ($P < 0.01$). The numbers of IgE-positive B-cells were significantly higher in the $+/+^{high}$ group than in the control groups ($P < 0.05$), but not versus that in the $+/+^{low}$ hosts. With all three pesticides, the total number of B-cells (i.e., B220+ cells) were increased in parallel with the numbers of IgE+ and/or MHC Class II+ B-cells (data not shown).

To determine the effects of the chemicals on the production of allergy-associated cytokines, we cultured auricular 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- γ , and TNF- α) (Fig. 3A–C) induced by 2,4-D, BRP, or furathiocarb treatment revealed significant differ-

ences between the $+/+^{high}$ group and the other treatment groups ($P < 0.01$).

On the other hand, marked increases in Th2 cytokine production (IL-4, IL-5, IL-10, and IL-13) (Fig. 3D–F) were seen in the 2,4-D $+/+^{high}$ treatment groups. For clarity purposes, IL-10 has, until recently, been deemed a “Th2” cytokine; in fact, a subclass of CD4+ T-cells (i.e., the T-regulatory [Treg] cells) is the likely major source of the IL-10 being measured in these studies. Analysis of Th2 cytokine production induced by 2,4-D treatment revealed significant differences between the $+/+^{high}$ and other treatment groups ($P < 0.01$). In particular, marked increases in IL-5 and IL-13 levels were observed in the 2,4-D $+/+^{high}$ treatment group relative to the BRP and furathiocarb $+/+^{high}$ treatment groups. Regarding the production of Th2 cytokines induced by BRP and furathiocarb treatment, significant differences were observed in the $+/-$ groups compared with the control groups (Fig. 3E and F), but the reactions were milder than those observed in mice in the 2,4-D treatment groups. The only instance where there was no significant difference between the $+/-$ group and the control groups was with respect to IL-4 production among the BRP-treated mice.

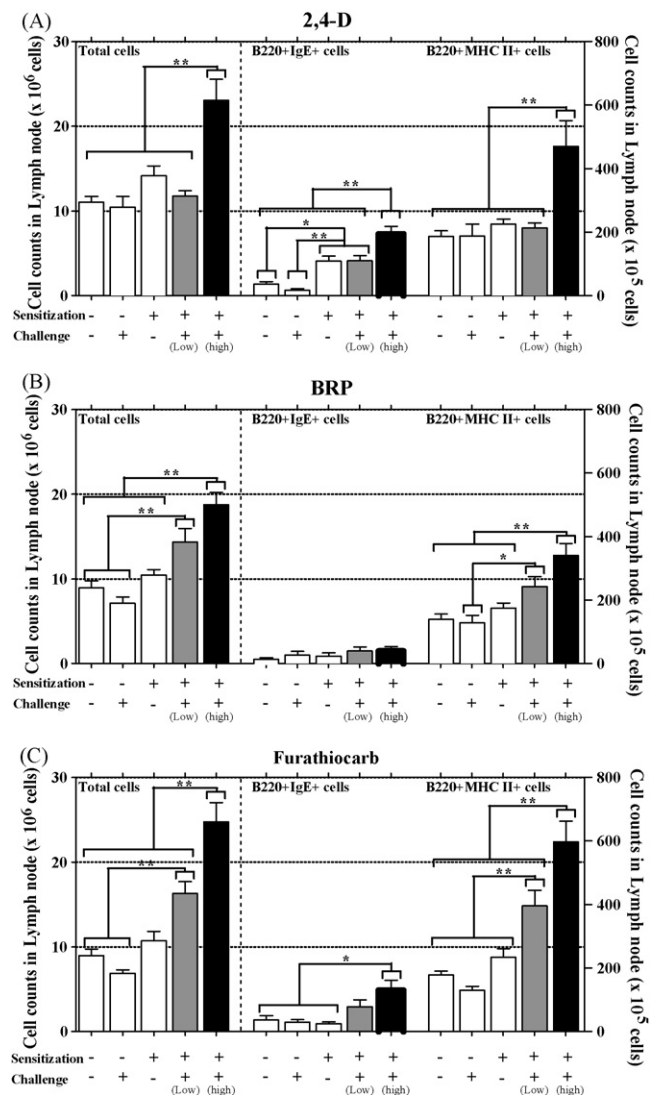


Fig. 2. Cell counts in auricular lymph node, B220⁺ IgE⁺ cells, and B220⁺ MHC class II⁺ cells (A: 2,4-D; B: BRP; C: furathiocarb) in auricular lymph nodes from mice treated with test solutions or solvent alone in dermal challenge protocol. Results are expressed as mean (no. of cells) $\pm$ S.D. Statistical significance is marked by asterisks: * for $P < 0.05$, ** for $P < 0.01$ (Tukey's t -test). $n = 6$ –7 per group.

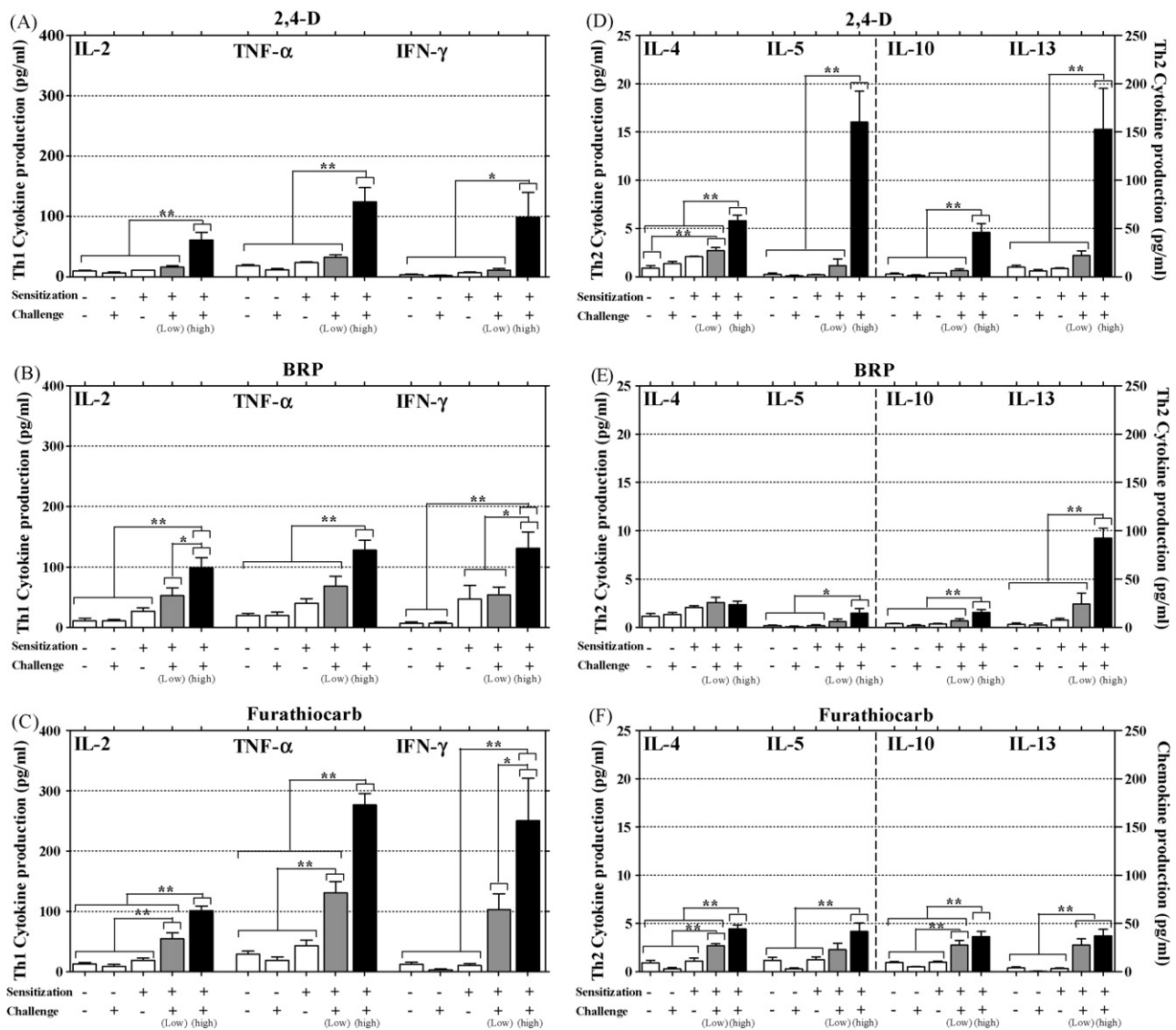


Fig. 3. Ex situ Th1 (IL-2, TNF- α , and IFN- γ) (A: 2,4-D; B: BRP; C: furathiocarb) and Th2 (IL-4, IL-5, IL-10, and IL-13) (D: 2,4-D; E: BRP; F: furathiocarb) cytokine production by auricular lymph node cells of mice treated with test solutions or solvent alone in dermal challenge protocol. To determine 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: * for $P < 0.05$, ** for $P < 0.01$ (Tukey's t -test). $n = 6-7$ per group.

3.3. Main study (respiratory challenge protocol)

In the respiratory challenge protocol, we examined the total IgE levels in serum and BALF (Fig. 4), total cell and differential cell counts in BALF (Fig. 5), chemokine/cytokine levels in BALF (Fig. 6), B-cell activation in lung-associated LNs (as determined by flow cytometric analysis) (Fig. 7), and cytokine production in lung-associated LNs (Fig. 8).

Total IgE levels in serum (Fig. 4A) in the 2,4-D $+/+^{high}$ treatment group showed a significant increase relative to those in the control groups. Total IgE levels in BALF (Fig. 4B) in the 2,4-D $+/+$ treatment groups (both low and high) showed a significant increase ($P < 0.01$) relative to those in the control groups. However, in the BRP- and furathiocarb-treated groups, no elevation of total IgE levels in serum and BALF were observed.

We examined the total numbers of macrophages, eosinophils, and neutrophils in BALF cells (Fig. 5A–C). To discriminate the different cell types in BALF, we used a combination of anti-Gr-1 and -CD11c antibodies. We also examined production of MCP-1, IL-6, eotaxin, and MIP-1 β in the BALF response to each pesti-

cide (Fig. 6A–F). The 2,4-D $+/+^{high}$ group showed greatly increased numbers of eosinophils and neutrophils and high levels of MCP-1, MIP1- β , eotaxin, and IL-6. The number of eosinophils was significantly higher in the $+/+^{high}$ group than in the control groups ($P < 0.01$). The number of neutrophils was significantly higher in the $+/+^{high}$ group than in all the other groups ($P < 0.01$). Production of MCP-1 was significantly higher in the $+/+^{high}$ group than in the control groups ($-/-$, $-/+$, $P < 0.05$; $+/-$, $P < 0.01$). Production of MIP1- β was significantly higher in the $+/+^{high}$ group than in the control groups ($P < 0.05$). Production of eotaxin was significantly higher in the $+/+^{high}$ group than in the other groups ($-/-$, $-/+$, $+/-$, $P < 0.01$; $+/+^{low}$, $P < 0.01$). Production of IL-6 was significantly higher in the $+/+^{high}$ group than in the $+/-$ group ($P < 0.05$).

Elevations in total and differential cell counts and levels of RANTES, eotaxin, IL-6, and TNF- α were not observed in mice treated with BRP or furathiocarb. Significant increases in MCP-1 and MIP1- β were observed in the high-dose BRP- and furathiocarb-treated groups, respectively, but these up-regulations were mild compared with those in the 2,4-D-treated groups. Interestingly, in none of the treatment groups was an effect on RANTES expression noted. In

contrast, significant increases in $+/\text{high}$ 2,4-D-treated groups. The differential cell counts from the Cytospin preparations showed very similar patterns as those of the flow cytometric analysis (data not shown).

We examined the total numbers of lymphocytes, IgE-positive B-cells ($\text{B220}^+\text{IgE}^+$), and MHC-Class II-positive B-cells ($\text{B220}^+\text{MHC II}^+$) in lung-associated LNs (Fig. 7A–C). With 2,4-D treatment, the numbers of IgE-positive B-cells were significantly higher in the $+/\text{high}$ group than in the control groups ($P < 0.01$) and significantly higher in the $+/\text{low}$ group than in the $-/-$ group ($P < 0.05$). As with the IgE-positive B-cells, the numbers of MHC-Class II-positive B-cells were significantly higher in the 2,4-D $+/\text{high}$ group than in the $-/+$ ($P < 0.05$) and $-/-$ groups ($P < 0.01$). However, in the BRP- and furathiocarb-treated groups, no elevations in the number of IgE-positive B-cells or MHC-Class II-positive B-cells were observed. As in the LN analyses, with all three pesticides studies, the total number of B-cells (i.e., B220^+ cells) were increased in tandem with the numbers of IgE+ and/or MHC Class II+ B-cells (data not shown).

We examined cytokine production (IL-4, IL-10, IL-13, and IFN- γ) in the lung-associated-LN supernatants of mice exposed to 2,4-D, BRP, or furathiocarb (Fig. 8A–C). Marked increases in IL-4, IL-10, and IL-13 production were seen in the 2,4-D $+/\text{high}$ treatment groups. Analysis of IL-4 and IL-10 production induced by 2,4-D treatment revealed significant differences between the $+/\text{high}$ and all other groups ($P < 0.05$ or $P < 0.01$). Analysis of the IL-13 production induced by 2,4-D treatment revealed significant differences between the $+/\text{high}$ and control groups ($P < 0.01$). There was no increase in IL-4, IL-10, or IL-13 production in any of the BRP groups (relative to controls or one another); this was also the case with the furathiocarb treatment groups.

In contrast, significant differences in IFN- γ production were observed only in the BRP treatment groups and only between either

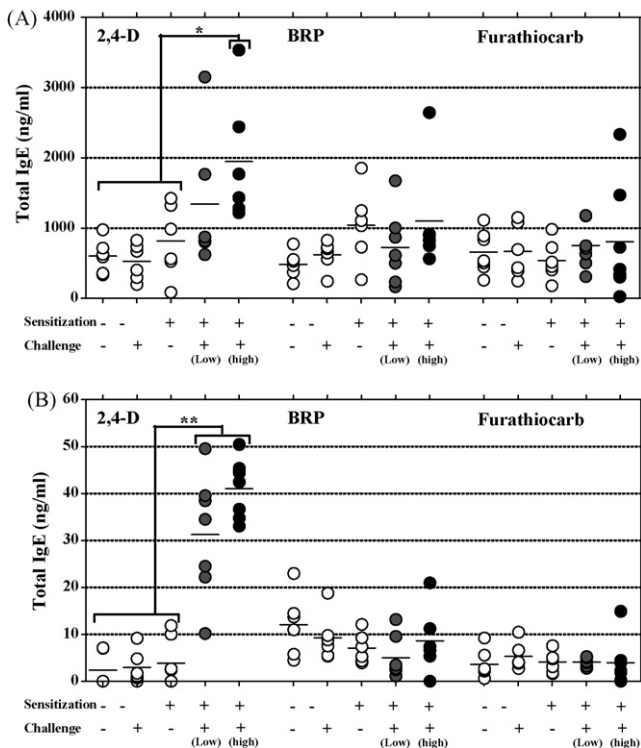


Fig. 4. Total IgE levels in mouse serum (A) and BALF (B) isolated 1 day after challenge with test solution or solvent alone in respiratory challenge protocol. Figure shows individual values of the IgE level (ng/ml) and bars are expressed as mean (ng/ml). Statistical significance is marked by asterisks: ** for $P < 0.01$ (Tukey's t -test). $n = 6$ –7 per group.

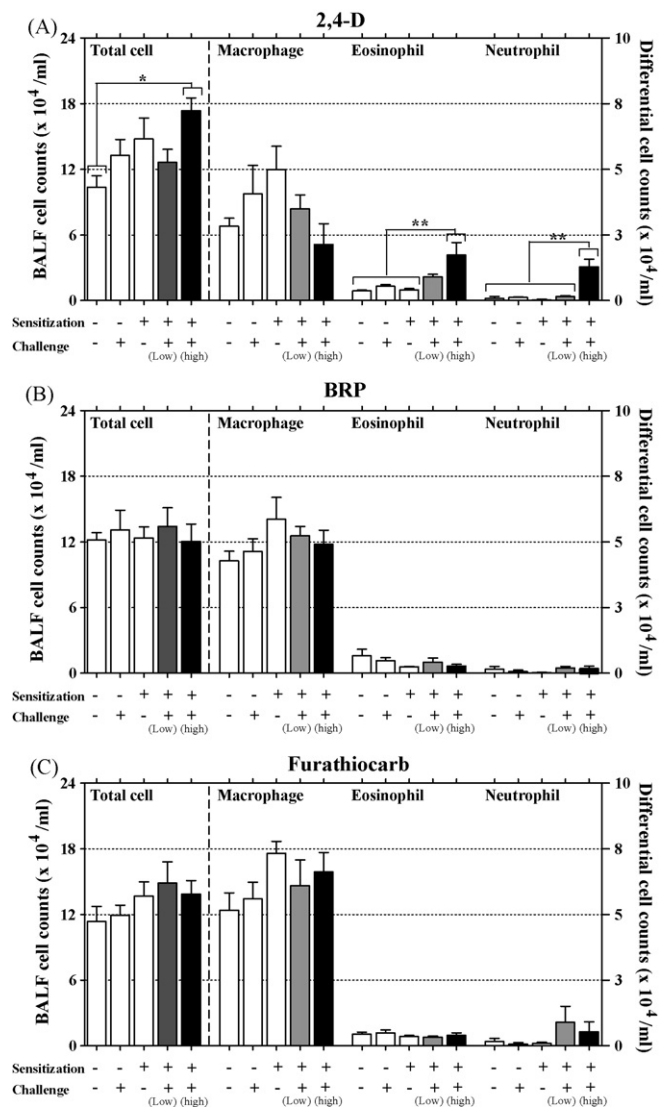


Fig. 5. Total and differential cells counts (A: 2,4-D; B: BRP; C: furathiocarb) in BALF of mice treated with test solution in respiratory challenge protocol. Total and differential counts are expressed as mean (no. of cells) $\pm$ S.D. BALF cells were differentiated by Gr-1 and CD11c staining and flow cytometry. The combination of these markers was used to discriminate the different cell types in BALF. Statistical significance is marked by asterisks: * for $P < 0.05$, ** for $P < 0.01$ (Tukey's t -test). $n = 6$ –7 per group.

$+/\text{low}$ and $+/\text{high}$ groups and the control groups ($+/\text{low}$, $P < 0.05$; $+/\text{high}$, $P < 0.01$).

4. Discussion

Our primary objective was to clarify the allergenicity of an environmental chemical-related hypersensitivity at weakly immunogenic and low doses. To that end, we use several types of pesticides (2,4-D, BRP, and furathiocarb), our original long-term sensitization method and LLNA. In conclusion, weakly immunogenic pesticides can be detected using our protocol. The current need for methods of detecting allergy has been further highlighted by the implementation of the European Union (EU) regulatory framework for registration, evaluation, and authorization of chemicals (Boverhof et al., 2008; REACH). In addition, under the EU authorization directive 91/414/EEC, the contact-sensitization potential of active substances in pesticides has been linked to their potential for respiratory allergy without a distinction being made between the two conditions (Boverhof et al., 2008; European

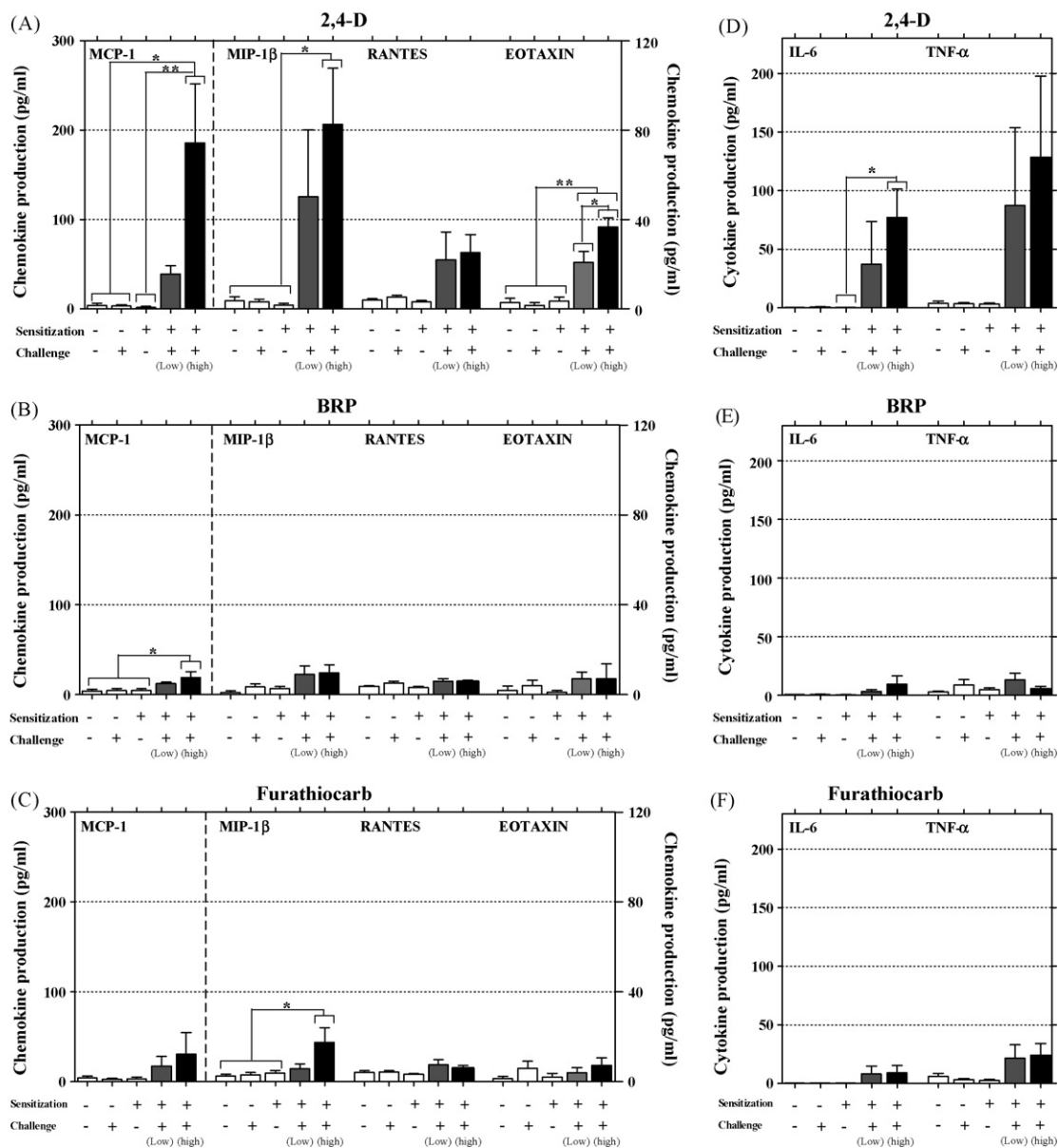


Fig. 6. Chemokine (A: 2,4-D; B: BRP; C: furathiocarb) and cytokine (D: 2,4-D; E: BRP; F: furathiocarb) levels in BALF of mice treated with test solution in respiratory challenge protocol. Chemokine and cytokine levels are expressed as mean (pg/mouse) $\pm$ S.D. To determine the chemokine and cytokine levels, BALF were assayed by cytometric bead array. Statistical significance is marked by asterisks: * for $P < 0.05$, ** for $P < 0.01$ (Tukey's t -test). $n = 6-7$ per group.

Commission, 2006a,b). Within the Globally Harmonized System for the Classification and Labeling of Chemicals, classification as a respiratory allergen relies heavily on human evidence and proposed animal studies, which include the mouse IgE test and pulmonary responses in guinea pigs, despite the lack of consensus and guidance for these assays (Boverhof et al., 2008; United Nations Economic Commission for Europe, 2007). The drafting and impending implementation of these directives further highlights the need to develop assays that are able to identify compounds that are respiratory allergens and differentiate them from contact allergens.

We first evaluated the response to 2,4-D, BRP, and furathiocarb in the LLNA as a preliminary test to assess the potential of each agent to cause a primary T-lymphocyte proliferative response after application to the skin (Dearman et al., 1999). Because we were particularly interested in low-dose reactions, we focused on establishing the agents' respective EC3 values (the concentration of a chemical required to elicit a threshold positive response) based on ^3H -TdR incorporation data in the LLNA (Basketter et al., 1999). The

EC3 values of 2,4-D, BRP, and furathiocarb in CBA/J mice were 5.91%, 0.46%, and 1.08%, respectively (Table 2); the actual levels of each used throughout the long-term dermal sensitization portions of our study were 5.0%, 0.3%, and 1.0%, respectively.

Allergy, in general, is defined as an adverse condition that is manifested following an immune response to an otherwise innocuous antigen. It is member of a class of outcomes termed 'hypersensitivity reactions,' which are defined as immune responses that result in tissue injury (Boverhof et al., 2008; Janeway et al., 2004). Therefore, to confirm the low-level allergic reactions to 2,4-D, BRP, and furathiocarb, we adopted a sensitization dose for each that was less than their respective EC3 value, and a challenge dose that was 10 or 100 times lower than the sensitization dose used (Table 1).

The conditions that are of particular concern to allergy researchers include both respiratory allergy and allergic contact dermatitis (ACD). A respiratory allergy is a hypersensitivity reaction of the respiratory tract to a xenobiotic protein or chemical

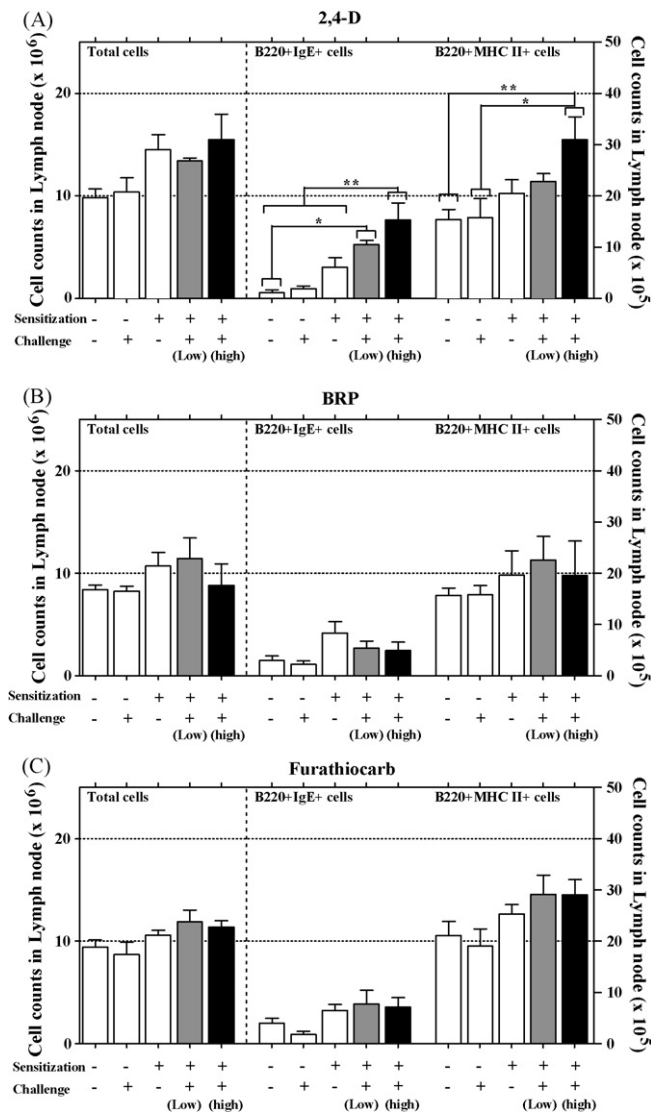


Fig. 7. Cell counts in lung-associated lymph node, of B220⁺ IgE⁺ cells, and of B220⁺ MHC class II⁺ cells (A: 2,4-D; B: BRP; C: furathiocarb) in lung-associated lymph nodes of mice treated with test solution or solvent alone in respiratory challenge protocol. Numbers of lymphocytes are expressed as mean (no. of cells) $\pm$ S.D. Statistical significance is marked by asterisks: * for $P < 0.05$, ** for $P < 0.01$ (Tukey's *t*-test). ND = None detected. $n = 6-7$ per group.

(Boverhof et al., 2008). This hypersensitivity reaction is immediate, with clinical signs occurring within minutes to hours after chemical exposure (Boverhof et al., 2008). On the other hand, a larger number of compounds are associated with skin hypersensitivity and the development of ACD and are believed to have no sensitizing effect on the immediate type hypersensitivity responses in the respiratory tract (Boverhof et al., 2008; Kimber and Dearman, 2005). Unlike respiratory allergy, ACD is an example of a delayed hypersensitivity reaction resulting from a cell-mediated immune response (Janeway et al., 2004). In this case, to detect and classify the low-level allergic reactions to pesticides (2,4-D, BRP, and furathiocarb) here, we used two different protocols and assessed several immune endpoints: IgE antibody levels, MHC Class II molecule levels, eosinophil proliferation, and local chemokine and cytokine levels (Fukuyama et al., 2008a,b).

Above all, IgE antibody is one of the major markers of chemically induced respiratory allergy. Changes in levels of total IgE and of IgE⁺ B-cells within draining LNs are especially useful for the detection of respiratory allergies (Ban et al., 2006; Goutet et al., 2005; Kimber

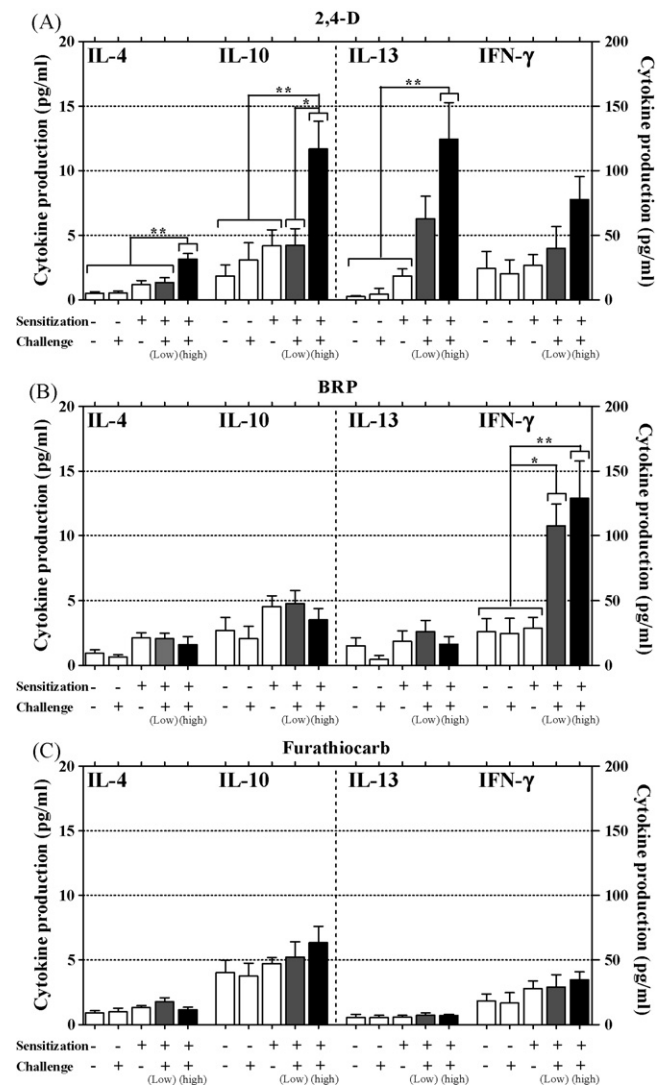


Fig. 8. Ex situ cytokine (IL-4, IL-10, IL-13, and IFN- γ) (A: 2,4-D; B: BRP; C: furathiocarb) production by lung-associated lymph node cells of mice treated with test solution or solvent alone in respiratory challenge protocol. To determine the effects of cytokine production, lymph node cell suspensions 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: * for $P < 0.05$, ** for $P < 0.01$ (Tukey's *t*-test). $n = 6-7$ per group.

and Dearman, 2002; Manetz and Meade, 1999). Here, we analyzed the total IgE levels in serum and/or BALF by ELISA and the IgE-expressing B-cell population in LNs (auricular or lung-associated) by flow cytometry. According to our data, a 2,4-D challenge after sensitization induced significant increases in total IgE levels and in IgE-expressing B-cell populations. In contrast, there was no significant increase in total IgE levels in the BRP and furathiocarb treatment groups. In terms of IgE-expressing B-cell populations, significant increases were observed only in the furathiocarb dermal challenge groups, but the reaction was less than with that in the 2,4-D-treated groups; there was no significant effect of this agent when it was used as a respiratory challenge.

In the development of immune responses, activated local B lymphocytes can act as antigen-presenting cells for CD4⁺ and CD8⁺ T-cells (Goutet et al., 2005). The CD4⁺ 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 2,4-D, BRP, or furathiocarb sensitization and then challenge, we assessed the membrane expression of MHC Class II molecules important for antigen presentation and regulation of the immune response (Murray, 1998). According to our results, significant increases in MHC-Class II-expressing B-cell populations were observed in 2,4-D, BRP, or furathiocarb dermally challenged groups (Fig. 2). In contrast, among those mice assessed in the respiratory challenge protocols, a significant increase was observed only in the 2,4-D-treated hosts (Fig. 7).

In general, an airway allergen challenge causes cellular inflammation that is dominated by eosinophils in the airways. It has been suggested that eosinophils contribute to several of the clinical features of respiratory allergy, including tissue damage and airway hyperresponsiveness (Lotvall et al., 1998). Furthermore, eosinophils have the capacity to release substantial amounts of allergy-related chemical mediators, such as MCP-1, MIP-1 β , RANTES, eotaxin, IL-6, and TNF- α (Janeway et al., 2004). Therefore, we analyzed eosinophil numbers and chemokine/cytokine levels in BALF by flow cytometry. Eosinophil levels and the levels of various chemokines/cytokines released (primarily) by cells likely to also be present in the BALF of 2,4-D-treated mice were enhanced; however, no such increases were noted in these endpoints assessed in the BALF from BRP- or furathiocarb-treated mice, except for the lone case of MIP-1 β that had been released by cells of the furathiocarb-treated hosts.

The mechanism of chemical-induced allergy is dependent on Th1 and Th2 cytokines (Andersson et al., 2000). Th2 cells produce IL-4, IL-10, and IL-13 (Mosmann and Coffman, 1989; Yssel and Groux, 2000), and these responses are down-regulated by Th1 cytokines (IFN- γ) (Gavett et al., 1995; Lack et al., 1996). In contrast, the Th1 cytokine secretion profile is stimulated by treatment with Th1-type allergen, with the production of high levels of IFN- γ , TNF- α , and IL-2, but is not stimulated by treatment with Th2-type allergens (Janeway et al., 2004). In our study, all chemicals induced significant increases in the levels of Th1 cytokines (IL-2, TNF- α , IFN- γ) produced by restimulated ex vivo LN cells in the dermal challenge protocol (Fig. 3). However, in the respiratory challenge protocol, only BRP induced a significant increase, and only in the levels of IFN- γ (Fig. 8). In terms of Th2 cytokines, each agent induced a significant increase in the levels of IL-4, -5, -10, and -13 (albeit that the increase in IL-4 due to BRP just missed achieving significance) in the dermal challenge studies (see Fig. 3). In contrast to the Th1 products, there was no increase in local Th2 cytokine production in BRP or furathiocarb respiratory-challenged groups, but levels of IL-4, -10, and -13 were all significantly increased by the 2,4-D treatment.

Overall, these results show that LLNA and our long-term sensitization method can be used to detect allergic responses to low-levels of pesticides. Up-regulation of eosinophil proliferation, chemokine levels, and Th2 cytokine production in 2,4-D-treated groups was associated with a subsequent increase in IgE production in the serum and BALF. On the other hand, BRP or furathiocarb treatment-induced MHC-class-II-molecule expression and Th1 cytokine production, but there was almost no eosinophil or Th2 cytokine production in the respiratory challenge protocol, and this lack of reaction was associated with the lack of increase in IgE production observed in the serum and BALF analyses. These results suggest that 2,4-D is a respiratory allergen and the others are contact allergens. Furthermore, our original protocol demonstrated that analysis of BALF and lung-associated LNs made it possible to detect and classify a chemical-induced allergy by its type for further validation.

The results of sensitization assays with other types of pesticides might be greatly influenced by the sensitizing route, dose of chemical, and animal species used. Several investigators have shown that dermal sensitization, followed by a dermal or airway challenge,

results in disparate immediate and late airway reactions that do not correspond to elevated levels of IgE antibodies and levels of other mediators (Ban et al., 2006; Dearman et al., 2002; Farraj et al., 2007; Fukuyama et al., 2008a,b; Goutet et al., 2005; Manetz and Meade, 1999; Vandebriel et al., 2003). It is our belief that the important thing is not to overlook the minute signs and symptoms triggered when affected patients come into contact with any of a number of environmental chemical products at low doses. For that purpose, our ongoing studies continue to focus on the detection of weak allergic reactions and attempt to develop methods for detecting environmental chemical allergens, including pesticides. We elicited positive reactions to several types of pesticides using our protocol.

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