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RESEARCH ARTICLE

Investigation of the chemical-induced selective type II (T_H2) allergic response in mice: Effect of the length of the sensitizing phase

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Abstract

Allergies are immune system disorders characterized by abnormal, acquired sensitivity to various environmental chemicals. We investigated the mechanism of chemical-induced selective type II (T_H2) allergy by using three different sensitization protocols and the well-known respiratory sensitizer trimellitic anhydride (TMA). Mice were sensitized for either 1, 2, or 3 weeks. For each sensitization schedule, the mice were allocated into 3 or 4 groups: -/- group, both sensitized and challenged with vehicle; +/- group, sensitized with vehicle and challenged with 0.1% TMA; +/+ group, sensitized with 1% TMA and challenged with vehicle; and +/+ group, both sensitized and challenged with 0.1% TMA. After challenge, we assayed the auricular lymph nodes of all mice for number of lymphocytes, surface antigen expression of B-cells, and local cytokine production, and we measured TMA-specific serum IgE levels. Some parameters in mice sensitized for 1 or 2 wk showed, at most, mild changes. In contrast, all parameters in animals receiving 3-wk sensitization showed marked increases, as well as marked increases in the IgE/major histocompatibility complex (MHC) Class II-positive B-cell population and T_H2 cell production of IL-10 and IL-13. These results indicate that 3 wk of sensitization according to our protocol led to overt respiratory allergic reactions. While these studies showed that using the approach here, positive reactions were elicited using a typical allergen; whether the same events occur after sensitization by other chemicals that are found in the environment remains uncertain. These findings here should be regarded moreover as preliminary in scope and that additional studies with irritants, dermal sensitizers and other respiratory sensitizers are needed to further evaluate the overall sensitivity and selectivity of this novel protocol.

Keywords: Chemical-induced selective type II (T_H2) allergy; BALB/c mouse; trimellitic anhydride

Introduction

Allergies are immune system disorders characterized by abnormal, acquired sensitivity to a given substance, such as pollen and numerous environmental chemicals (Hopkin, 1997). Environmental and lifestyle factors and increasing use of chemicals have often been suggested as contributors to allergies (Peden, 2000; Ban and Hettich, 2005). Multiple chemical sensitivities (MCS) are a representative chemically-induced allergy that has emerged as a public health problem

worldwide (Howarth, 1998; Kimber and Dearman, 2002; Ban and Hettich, 2005).

Allergic diseases take many forms, but in all instances, they can be defined as adverse health effects resulting from the stimulation of a specific immune response (Basketter et al., 1996). For example, a selective type II (T_H2) allergic response such as respiratory hypersensitivity can be induced in susceptible persons after airway exposure to a chemical allergen. A variety of chemicals (including various

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diisocyanates, acid anhydrides, and reactive dyes) can cause allergic sensitization of the respiratory tract associated with occupational asthma and other symptoms (Kimber and Dearman, 1997, 2002; Dearman et al., 2002; Arts et al., 2003; Pauluhn et al., 2003; Ban et al., 2006).

In guinea pigs, respiratory sensitization (induction) to numerous individual chemicals has been achieved through single/repeated inhalation exposures, and by intradermal or subcutaneous administration of the test agent (Dearman et al., 1990; Karol, 1994; Arts et al., 2003). Use of the guinea pig models have an additional advantage of allowing for more *global* functional endpoints to be tested, i.e., changes in respiratory functionality whose symptoms might then be studied/analyzed in the context of any changes in individual immunologic/histologic parameters being assessed in hosts treated in parallel. Despite these advantages, the respiratory sensitization tests are time-consuming, costly, and may require the use of hapten-protein conjugates, which hamper comparisons with the human situation (Sarlo et al., 1997; Arts et al., 2003).

In contrast, mouse model-associated approaches, such as the IgE test (Karol, 1994; Satoh et al., 1995; Dearman et al., 2000, 2003; Arts et al., 2003; Kimber et al., 2003), are based on the finding that chemicals with a potential to cause respiratory allergy in man, such as trimellitic anhydride (TMA), can provoke significantly elevated serum levels of both total and TMA-specific IgE in the exposed mice. Unfortunately, while this test is more cost- and time-effective than those associated with the studies using guinea pig models, it is not a good predictor of potential changes in host/organ-specific functionality. In addition, although environmental chemical allergens tend to be only weakly immunogenic, methods developed previously with either of these model systems have focused on the detection of strong allergic reactions (Arts et al., 2003). Thus, protocols are needed for the detection (to thereby facilitate treatment) of weakly immunogenic and low-dose allergic reactions. To achieve this goal, these studies here reflect out attempts to build upon these previous models by incorporating both new sensitization protocols and detection methods.

In a series of previous studies, our laboratories developed a novel method for detection of environmental chemical-related hypersensitivity using typical T_H2 sensitizers (trimellitic anhydride [TMA] and toluene diisocyanate [TDI]) in a long-term dermal sensitization phase followed by dermal or respiratory challenge with the chemical (Fukuyama et al., 2008a, 2008b). Those studies showed that low-dose TMA induced a typical T_H2 response, i.e., an increase in antigen-specific serum IgE levels and interleukin (IL)-4 production in local lymph nodes (LN), and that TDI induced marked increases in IL-4, IL-10, IL-13, and interferon (IFN)- γ production by re-stimulated LN cells. These results demonstrated that our method was able to detect allergic reactions caused by chemicals occurring at weakly immunogenic and low doses in the environment. Building upon those findings, a major purpose of the study reported here was to further refine our methods to detect environmental chemical-

induced hypersensitivity (moreover) using shorter length-of-sensitization phases. To that end, in this study, three different (relatively short) sensitization schedules were employed and several highly sensitive endpoints utilized to evaluate these immunologic outcomes.

Materials and methods

Chemicals

The test chemical was dissolved in 4:1 acetone/olive oil (AOO). Trimellitic anhydride (TMA) was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Acetone and olive oil were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

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 0700 to 1900 h), temperature ($22 \pm 2^\circ\text{C}$), humidity ($55\% \pm 15\%$), and ventilation (at least 10 100% fresh air changes hourly). Food (Certified Pellet Diet MF, Oriental Yeast Co., Tokyo) 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).

Treatment protocol

After a 1-wk acclimatization period, mice were allocated randomly to dose and control groups ($n=8$ per group). For these studies, three different schedules for dermal sensitization and then dermal challenge were tested (Figure 1); a 1-wk (Days 1–3) sensitization and challenge on Day 17; 2-wk (Days 1–3, 8–10) sensitization and challenge on Day 24; and, 3-wk sensitization (Days 1–3, 8–10, 15–17) and challenge on Day 31. For each sensitization, a 25 μl aliquot of 1% TMA solution (or solvent only) was applied to the dorsum of both ears of each mouse. Two weeks after the final sensitization, a 25 μl aliquot of 0.1% TMA solution (or solvent only) was applied as the dermal challenge. One day after the challenge, mice were anesthetized (diethyl ether) and euthanized. Blood samples were taken from the inferior vena cava and plasma samples then isolated for assay of specific serum IgE. Each animal's auricular lymph nodes (LN) on both sides were removed, weighed, and pooled in RPMI 1640 medium (Gibco, Tokyo).

To determine the dosage used in this study, a local lymph node assay (LLNA) was conducted as a preliminary test (data not shown). Based on those results, 0.1% TMA was adopted for a challenge dose that was $< \text{EC}_{50}$, and 1% TMA for a sensitization dose that was 10 times the challenge dose.

Test and control groups

Table 1 depicts the experimental groups used in the main study. For each schedule, the mice were divided into three or four groups: Group -/- (both sensitization and challenge

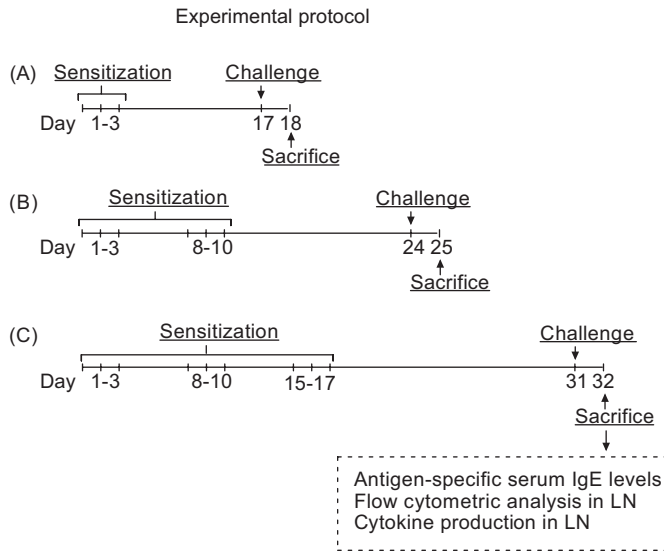


Figure 1. Experimental protocol used to sensitize and challenge BALB/c mice with TMA in acetone/olive oil solvent. For the sensitizing protocol, three different schedules were tested as follows; 1-wk sensitization (A) (Days 1-3 dermal sensitization and Day 17 dermal challenge), 2-wk sensitization (B) (Days 1-3, 8-10 dermal sensitization and Day 24 dermal challenge) and 3-wk sensitization (C) (Days 1-3, 8-10, 15-17 dermal sensitization and Day 31 dermal challenge). A 25 μ l aliquot of TMA solution or solvent only was applied to the dorsum of each ear of each mouse for dermal sensitization. Mice were then challenged 2 wk after last sensitization. The same volume of TMA solution or solvent only was applied to the dorsum of each ear of each mouse for dermal challenge.

Table 1. Experimental groups used in this study.

Groups	Sensitization	Challenge
-/-	Solvent	Solvent
-/+	Solvent	TMA (0.1%)
+/-	TMA (1%)	Solvent
+/+	TMA (1%)	TMA (0.1%)

Solvent: 4:1 acetone in olive oil.

using 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).

TMA-specific serum IgE

TMA-ovalbumin (OVA) conjugate was prepared by Imject Immunogen EDC Kit in accordance with the manufacturer's protocol (Pierce Biotechnology, Rockford, MD). The protein content of each conjugated sample was determined by the Lowry method (Lowry et al., 1951).

TMA-specific serum IgE was measured by ELISA. In studies by our laboratory (and by other Investigators), use of this protocol has been shown not to result in false positive data due to binding/detection of IgG species (Fukuyama et al., 2008a, 2008b). This is because the rat anti-mouse IgE antibody (clone R35-72; BD Pharmingen [San Diego, CA])—more precisely its biotin-conjugated form (clone R35-118)—used in the assays then and now: reacts specifically with mouse IgE of the Igh-C [a] and Igh-C [b] haplotypes; has been reported not to react with other Ig isotypes; and, has been used in

the detection of surface immunoglobulin on IgE-secreting hybridoma cells (Pharmingen Technical Data Sheet, product 553419 and 553413). In addition, parallel studies (data not shown) testing the serum of these hosts in the current studies against wells coated with OVA only or other types of chemical-OVA conjugates, as well as tests using biotin-conjugated rat anti-mouse IgG in place of biotin-conjugated rat anti-mouse IgE, confirmed that the assay here (as in our two cited earlier studies) was in fact measuring TMA-specific serum IgE only.

Briefly, flat-bottom microplate wells (Nalge Nunc International K.K., Tokyo, Japan) were coated with TMA-OVA conjugate (100 μ l, 0.1 mg/ml) in coating buffer (Pharmingen) and incubated overnight at 4°C. The content of each well was removed and the plate was rinsed with wash buffer (Pharmingen). Non-specific binding was blocked by incubation with 200 μ l 10% heat-inactivated goat serum (Sigma Aldrich, Tokyo) in PBS for 1 hr at room temperature. Mouse serum was diluted in PBS (1:4 to 1:4096), aliquots (100 μ l) added to each well, and the plates incubated 2 hr at room temperature. After rinsing each well with buffer, monoclonal biotin-conjugated rat anti-mouse IgE (2 μ g/ml, 100 μ l) was added to each well and incubated for 1 hr at room temperature. After each well was rinsed of non-adherent secondary antibody, streptavidin-horseradish peroxidase (SAV-HRP) conjugate (Pharmingen, dilution 1:1000, 100 μ l) was added to each well and the plates incubated for 30 min at room temperature. The presence of bound secondary antibody was then detected by addition of tetramethylbenzidine (TMB) (100 μ l/well) and incubation in the dark (at room temperature) for 30 min. Optical density (OD) values for each well were read at 450 nm in an ImmunoMini reader (NJ-2300, Nippon Intermed, Tokyo).

Tissue preparation

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

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 Pharmingen. To avoid nonspecific binding, 1×10^6 cells were incubated with 15% normal goat serum (Sigma) for 10 min at 4°C, followed by incubation with FITC-, PE-, and PE-Cy5-conjugated monoclonal antibodies for 30 min at 4°C in the dark. The cells were then washed twice with wash buffer and resuspended at 1×10^6 cells/tube (in 1 ml PBS) and then analyzed in a FACSCaliber flow cytometer (Pharmingen) using the Cell Quest program. For

each sample, 10,000 events were collected and analyzed for expression of antigens to IgE and major histocompatibility complex (MHC) Class II antigen in B-cells.

Cytokine assay

To stimulate T-cell-receptor signaling, we cultured single-cell suspensions of LN (1×10^6 cells/well) with T-cell antibodies to CD3 and/or CD28 (Pharmingen) for 24 or 96 hr in Multidish 24-well plates (Nalge Nunc) at 37°C in a controlled atmosphere of 5% CO₂. Production of IL-3 was analyzed in supernatants isolated following a 24-hr culture in the presence of anti-CD3 antibody (2 µg/ml). Production of IL-10 and IL-13 was analyzed in supernatants isolated after 96 hr of culture in the presence of anti-CD3 (2 µg/ml). Production of IL-4 was analyzed in supernatants isolated following culture for 24 hr in the presence of anti-CD3 and anti-CD28 (each 2 µg/ml). Production of IL-5 and IL-6 was analyzed in supernatants isolated after 96 hr of culture in the presence of anti-CD3 and anti-CD28 (each 2 µg/ml). Each supernatant was assayed using a BD Cytometric Bead Array (Pharmingen) in accordance with manufacturer's protocols.

Statistical analysis

Mean and standard deviation were calculated for all parameters. All data were evaluated by the Tukey–Kramer multiple comparison test, and differences were considered significant at $p < 0.05$.

Results

TMA-specific serum IgE

TMA-specific serum IgE levels are shown in Figure 2. The +/+ treatment group (both sensitized and challenged with TMA) that underwent 1 wk of sensitization showed no significant increase in TMA-specific serum IgE (relative unit value, 12 ± 14) compared with that of the -/- group (sensitized and challenged with vehicle; relative unit value set at 1). The +/+ treatment group that underwent 2 wk of sensitization showed a significant ($p < 0.05$) increase in TMA-specific serum IgE (70 ± 43) compared with that of the -/- group (sensitized and challenged with vehicle; relative unit value set at 1). The +/+ treatment group receiving a 3-wk sensitization had a significant increase in TMA-specific serum IgE levels (182 ± 110) relative to those of the -/- group (1; $p < 0.01$) and the -/+ group (sensitized with vehicle and challenged with TMA; 1; $p < 0.01$). Among the +/+ treatment groups, the TMA-specific IgE levels in mice sensitized for 3 wk were $\approx 1500\%$ greater than those of mice with 1-wk sensitization and 250% greater than those with 2-wk sensitization.

Interestingly, among the animals that received only the sensitization regimens (i.e., the +/- hosts) for 1, 2, or 3 wk, the changes (increases) in IgE levels relative to the -/- controls were highly significant ($p < 0.01$) for animals in the 2- and 3-wk groups. As with their +/+ counterparts, these animals also showed time-related trends in these increases (albeit that the change from 2-wk to 3-wk was not dramatic).

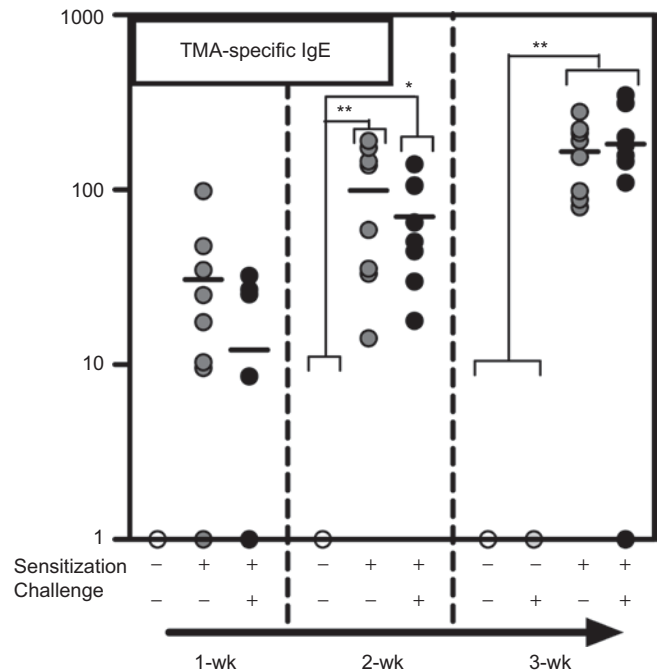


Figure 2. TMA-specific IgE levels in mouse serum isolated 1 d after challenge with TMA solution or solvent alone. Experimental groups are as in Table 1. Results (circles) are values for (titer); bar indicates mean titer value. Statistical significance is marked by asterisks: * $p < 0.05$, ** $p < 0.01$ (Tukey's *t*-test).

Numbers of lymphocytes and flow cytometric analysis

The total numbers of lymphocytes are shown in Figure 3A; data for IgE-positive B-cells (B220⁺IgE⁺) and MHC-class-II-positive B-cells (B220⁺I-A/I-E⁺) are shown in Figures 3B and 3C, respectively. The flow cytometric identification of IgE-positive B-cells after TMA treatment is illustrated as dot plots in Figure 3D.

The total number of lymphocytes from the +/+ treatment group sensitized for 1 wk showed tended to increase, but the reaction was mild, and no significant difference was present (Figure 3A). However, among the groups sensitized for 1 wk, significantly more IgE- and MHC Class-II-positive B-cells were present in the +/+ group ($20.18 \pm 5.20 \times 10^5$ cells [$p < 0.01$] and $20.50 \pm 4.30 \times 10^5$ cells [$p < 0.05$], respectively) than in the -/- group ($9.73 \pm 4.20 \times 10^5$ cells and $10.54 \pm 3.90 \times 10^5$ cells, respectively) (Figures 3B and 3C). After 2 wk of sensitization, the total numbers of lymphocytes and the numbers of IgE- and MHC Class-II-positive B-cells were significantly higher in the +/+ group ($19.09 \pm 5.50 \times 10^6$ cells [$p < 0.01$], $34.01 \pm 10.20 \times 10^5$ cells [$p < 0.05$] and $35.01 \pm 10.20 \times 10^5$ cells [$p < 0.01$], respectively) than in the -/- group ($9.95 \pm 2.10 \times 10^6$ cells, $7.15 \pm 2.40 \times 10^5$ cells and $8.83 \pm 2.40 \times 10^5$ cells, respectively; Figures 3A–3C).

After the 3-wk sensitization protocol, the total number of lymphocytes and numbers of IgE- and MHC Class-II-positive B-cells were significantly greater in the +/+ group ($27.73 \pm 5.82 \times 10^6$ cells, $65.99 \pm 19.20 \times 10^5$ cells and $66.05 \pm 19.70 \times 10^5$ cells, respectively) than in the control groups (-/-, -/+, and +/-; $p < 0.01$ for all comparisons; Figures 3A–3C). Among the +/+ treatment groups, the IgE- and MHC Class-II-positive

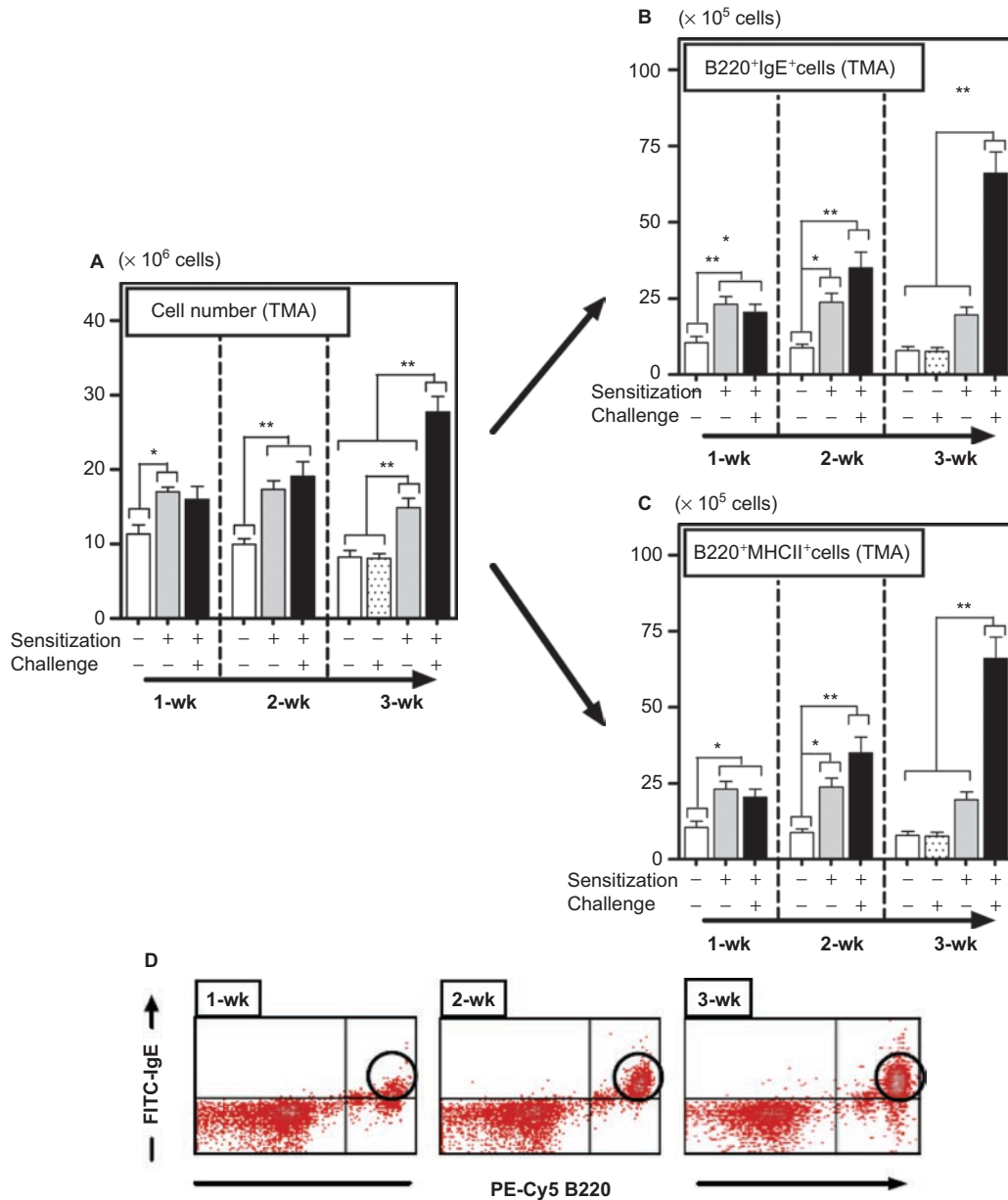


Figure 3. Total numbers of lymphocytes (A), numbers of B220⁺ IgE⁺ cells (B), and numbers of B220⁺ MHC Class II⁺ cells (C) in auricular lymph nodes from mice treated with TMA solution or solvent alone. Dot-plots of B220⁺ IgE⁺ cells in the +/+ treatment group receiving a 1-wk, 2-wk or 3-wk sensitization (D) are also shown. Circles show the staining pattern of B220⁺ IgE⁺ cells. Experimental groups are as in Table 1. Results are expressed as number of cells (mean $\pm$ SD). Statistical significance is marked by asterisks: * p < 0.05, ** p < 0.01 (Tukey's t -test).

B-cell counts of mice that underwent 3 wk of sensitization were $\approx$ 300% greater than those of mice sensitized for 1 wk and 200% greater than those sensitized for 2 wk.

As occurred with the TMA-specific serum IgE levels, among the +/- hosts that underwent the 1-, 2-, or 3-wk sensitization regimens, the levels of total lymphocyte numbers and in numbers of IgE- and MHC Class-II-positive B-cells (relative to those associated with the -/- controls) were consistently found to be significantly higher. However, unlike what was seen with their +/+ counterparts, the effect of an increasing length of sensitization period on each parameter's final average value was not strong. In fact, by the end

of the 3-wk regimen, the values for the +/+ hosts (which displayed very clear time-associated increases over the span from 1- to 2- to 3-wk) were significantly greater than those of +/- hosts whose values generally appeared somewhat static.

Cytokine production

To determine the effects of the TMA on the level of allergy-related cytokine production, LN cell suspensions were cultured with T-cell antibodies (CD3 or CD28 or both) for 24 or 96 hrs, and the culture supernatant were then assayed using cytometric bead arrays.

Among mice sensitized for 3 wk, those in the $+/+$ group showed significant ($p < 0.01$) increases in cytokine production (IL-3, IL-4, IL-5, IL-6, IL-10, and IL-13) compared with those in the $-/-$, $-/+$, and $+/-$ groups (Figure 4). Among the $+/+$ groups, mice sensitized for 3 wk produced 3041 ± 891 pg IL-3/animal - an $\approx 400\%$ increase over levels from the 1-wk and 2-wk sensitization groups (Figure 4A). The production of IL-4 in $+/+$ mice sensitized for 3 wk was 359 ± 158 pg/animal - an $\approx 1100\%$ increase over that in the 1-wk sensitization group and a 500% increase over that in mice sensitized for 2 wk (Figure 4B). The IL-5 level after 3 wk of sensitization was 658 ± 303 pg/animal - $\approx 200\%$ greater than that of the 1-wk $+/+$ group and 300% greater than that of the 2-wk $+/+$ group (Figure 4C). IL-6 production in the 3-wk $+/+$ group was 1028 ± 241 pg/animal - 500% increased over that of mice sensitized for 1 wk and 250% increased over that after 2 wk (Figure 4D). The production of IL-10 was 3586 ± 1360 pg/animal - $\approx 1200\%$ more than that from the 1-wk $+/+$ group and 1800% more than that from the 2-wk $+/+$ group (Figure 4E). The IL-13 yield in $+/+$ mice sensitized for 3 wk was 2720 ± 1193

pg/animal - $\approx 10000\%$ more than after 1 wk of sensitization and 1000% increase more than after 2 wk (Figure 4F).

In general, as observed with the other endpoints examined here, among the $+/-$ hosts that underwent the 1-, 2-, or 3-wk sensitization regimens, the changes (increases) in the production of each of the cytokines (relative to values associated with the $-/-$ controls) were significant only among the animals in the 1- and 2-wk groups (with the exception being the 2-wk IL-3 level). However, unlike what was seen with their $+/+$ counterparts, the ultimate effect (i.e., by after 3 wk) of the increasing length of sensitization period on each parameter's final average value was not strong. In fact, for some of the cytokines (i.e., IL-5 and IL-6), the values actually dropped to their lowest/near-lowest levels by the end of the 3-wk regimen. With IL-3 and IL-4, the values again generally appeared somewhat static over the three time frames examined. As with the cell population studies, the results for each of the four cytokines indicated that by the end of the longest sensitization regimen, body levels of each in the $+/+$ hosts were uniformly significantly greater than those in their $+/-$ counterparts.

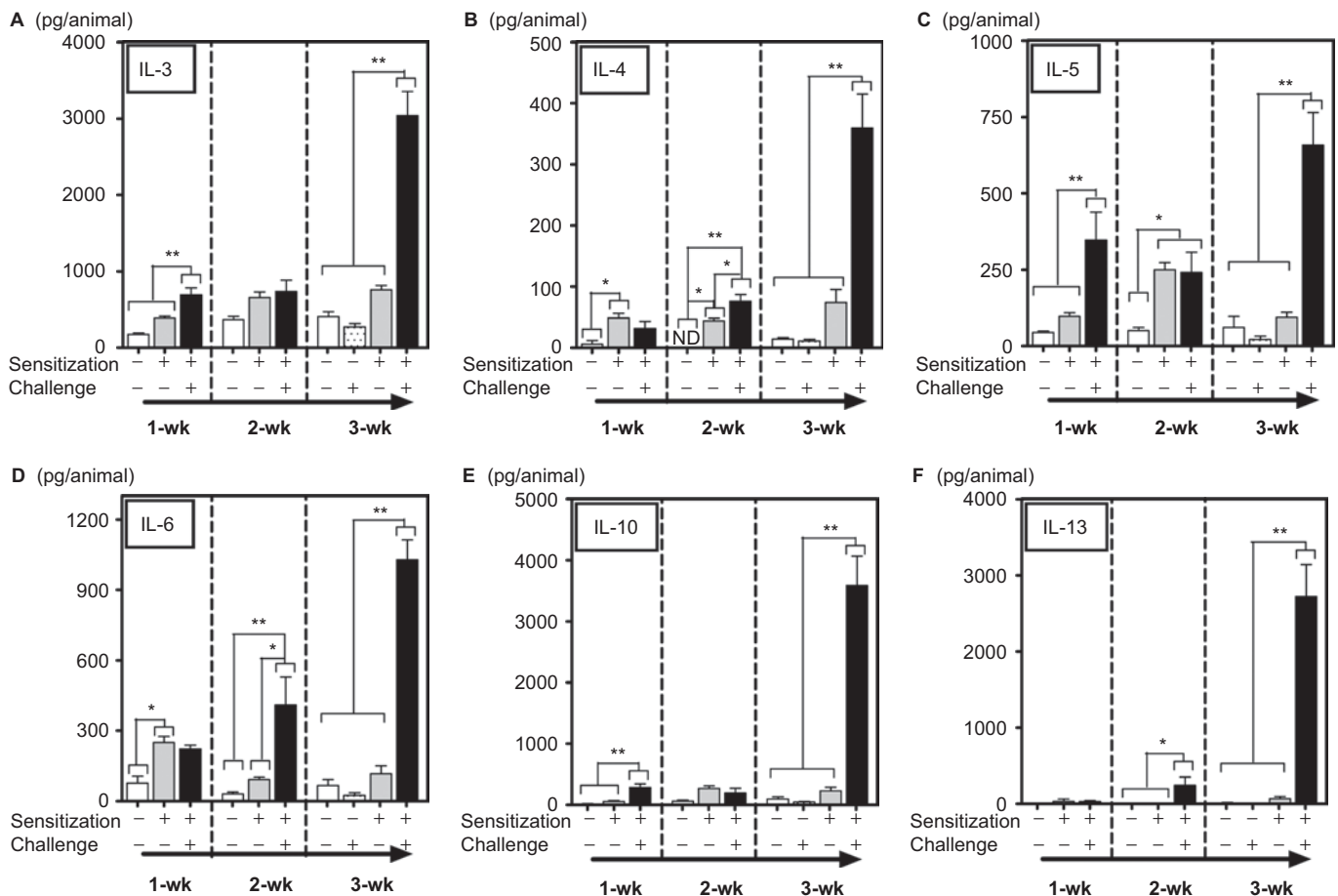


Figure 4. Cytokine production (A: IL-3, B: IL-4, C: IL-5, D: IL-6, E: IL-10, F: IL-13) by auricular lymph node calls of mice treated with TMA solution or solvent. To determine the effects on cytokine production, lymph node cell suspensions were cultured with T-cell antibodies (CD3 and/or CD28) for 24 or 96 hr (see details of culturing conditions for specific cytokines in Methods), and the supernatant was assayed by cytometric bead array. Experimental groups are as in Table 1. Results are expressed as mean (pg/animal) levels $\pm$ SD. Statistical significance is marked with asterisks: * $p < 0.05$, ** $p < 0.01$ (Tukey's t -test).

Discussion

According to our results, the mechanisms of chemical-induced selective type II (T_H2) allergy are heavily dependent on the length of the sensitization phase. Indeed, 3-wk dermal TMA sensitization followed by low-dose TMA challenge induced prominent allergic responses in parameters including TMA-specific serum IgE levels, the numbers of IgE- and MHC-Class-II-positive B-cells, and local cytokine production. In contrast, 1- and 2-wk TMA sensitization protocols yielded, at most, small non-significant increases in some parameters.

In this study, we used IgE as a central marker of chemical-induced T_H2 type allergy. Antigen-specific serum IgE levels and the IgE-positive B-cells from most draining LN are effective for the detection of several types of allergy (Manetz and Meade, 1999; Kimber and Dearman, 2002; Goutet et al., 2005; Ban et al., 2006). After stimulation with known IgE-inducing allergens, animals lacking B-cells with the ability to express surface IgE produced negligible amounts of total and antigen-specific serum IgE (Achatz et al., 1997; Manetz and Meade, 1999). In addition, exposure to known chemical irritants likely results in increased numbers of lymphocyte cells without markedly altering the IgE-positive B-cell populations (Manetz and Meade, 1999). In the present study, serum IgE levels and IgE-specific B-cells were significantly increased in the +/+ group after 3 wk of TMA sensitization (Figures 2 and 3).

Among the +/+ treatment groups, the TMA-specific IgE levels in mice sensitized for 3 wk were much greater than those of mice with 1- and 2-wk sensitizations. Interestingly, while the effects of the sensitization alone appeared to induce significant increases in IgE levels, the time-related trend was not as clear cut after the first week. Finally, with the 2- and 3-wk treatments, the mean titers of the +/- hosts were approximately equivalent to that of their +/+ counterparts; this outcome might be more attributable to the excessive sensitivity of our method than to the issue of the added step of the challenge following the sensitization protocols. In addition, 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 regard to our model schedule for the detection of sensitizers, this suggests that repeat(ed) application to the same site could be part of the reason(s) for enhancement of IgE levels in the serum. Experiments are currently underway in our laboratories to examine if use of another route of challenge (along with an examination of additional endpoints) might help to resolve this issue.

Nevertheless, it is important for investigators to remember to not overlook minute signs and symptoms that may be triggered when sensitive patients come into contact with any one of a number of environmental chemical products at low doses. It is for that reason this study focused on enhancing the ability to detect weak allergic reactions and attempted to develop a method for detecting environmental chemical

allergens. As our earlier studies (Fukuyama et al., 2008a, 2008b) indicated that we elicited positive reactions using typical allergens, we concluded that antigen-specific IgE levels would be a useful endpoint for identifying (and then characterizing effects of) chemical-related sensitizers in hosts exposed to these agents at low doses.

In addition to IgE production, measurement of local cytokine production can be useful for identifying T_H2 type sensitizers. Studies in mice have demonstrated that known chemical respiratory allergens provoke T_H2 immune responses associated with antigen-specific serum IgE production and induced or elevated expression of cytokines that favor the elicitation of immediate-type allergic reactions (Dearman et al., 1999, 2000, 2001, 2005; Kimber and Dearman, 2002; Vanoirbeek et al., 2003; Farraj et al., 2004; Matheson et al., 2005a, 2005b; Ban et al., 2006). T_H2 cells produce IL-3, IL-4, IL-5, IL-6, IL-10, and IL-13 (Mosmann and Coffman, 1989; Yssel and Groux, 2000), and these responses are down-regulated by T_H1 cytokines (Gavett et al., 1995; Lack et al., 1996). IL-4 promotes T-cell activation and differentiation into the T_H2 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, a 3-wk TMA sensitization prominently increased the levels of T_H2 cytokines (IL-3, IL-4, IL-5, IL-6, IL-10, and IL-13) produced by *ex vivo* re-stimulated lymph node cells (Figure 4). Production of IL-10 and IL-13 was particularly dramatic, leading to ~1000% increases over levels in other sensitization groups.

We wondered whether antigen-presenting cells might be used to detect chemical-induced T_H2 type allergy. Antigen-presenting cells play an important role in the immune system by stimulating the production of IgE antibody and cytokine production. In the development of immune responses, activated local B-cells 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: T_H1 and T_H2 (Santana and Rosenstein, 2003). In particular, T_H2 cells are specialized for B-cell activation (Santana and Rosenstein, 2003). To assess whether TMA treatment activated allergy-related cytokine B-cells, we evaluated membrane expression of MHC Class II molecules, which are important for antigen presentation and regulation of the immune response (Murray, 1998). In this issue, +/- mice sensitized for 3 wk showed prominent increases in the MHC Class-II-expressing B-cell population (Figure 3).

The length of the sensitizing phase seems to be very important in the development of T_H2 type allergy models (Arts et al., 2003), and numerous sensitization protocols have been tested in animal models to identify T_H2 type chemical allergy (Arts et al., 2003; Plitnick et al., 2003; Ban et al., 2006; Vanoirbeek et al., 2006). However, the duration of sensitization that is optimal for detection of T_H2 type chemical allergy remains unknown.

The results of the present study, in which three different sensitizing protocols were tested, offer important information about the development of T_H2 -type allergy models.

Based upon helpful comments provided during the formulation of this manuscript, we are more aware that while our goal was to develop a protocol that was increasingly sensitive for the detection of respiratory allergens, we need to keep in mind whether or not the new protocol is overly specific/selective. That is, has increased sensitivity been won at the cost of decreased selectivity? For now, the answer as to whether the same events occur after sensitization by other chemicals that appear in many places in the environment remains uncertain. Even though we have previously demonstrated selectivity (to detect T_H2 sensitizers) using the same endpoints examined here—but with a longer length-of-sensitization phase—the findings here must ultimately be regarded as preliminary overall. Clearly, additional studies with irritants, dermal sensitizers and other respiratory sensitizers are needed to further evaluate assay sensitivity and selectivity. Such studies are already underway in our laboratories.

In summary, in mice, 3 wk of TMA sensitization followed by low-level TMA challenge in mice induced prominent T_H2 type allergic responses and overt T_H2 type allergic reactions such as class switching to IgE antibody. Our sensitizing method seems sufficiently sensitive for detecting T_H2 type allergic reactions caused by TMA at weakly immunogenic and low doses. In addition, this protocol may prove useful in further investigations of the mechanisms of chemical-induced T_H2 type allergy.

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