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Oral Administration of Chitin Down-Regulates Serum IgE Levels and Lung Eosinophilia in the Allergic Mouse

Yoshimi Shibata, L. Ann Foster, John F. Bradfield, and Quentin N. Myrvik

Previous studies showed that local macrophages phagocytose nonantigenic chitin particles (1–10 μm polymers of N-acetyl-D-glucosamine) through mannose receptors and produce IL-12, IL-18, and TNF-α. These cytokines lead to the production of IFN-γ by NK cells. To determine whether chitin could down-regulate Th2 responses, chitin was given orally (8 mg/day for 3 days before and 13 days during ragweed allergen immunization) in BALB/c and C57BL/6 mice. These ragweed-immunized mice were given ragweed intratracheally on day 11. Three days after the challenge, the immunized mice with saline (controls) showed increases in serum IgE levels and lung eosinophil numbers. The chitin treatment resulted in decreases of these events in both strains. To dissect the inhibitory mechanisms of Th2 responses, spleen cells (4 × 10^6 cells/ml) isolated from the ragweed-immunized mice (controls) were cultured in the presence of ragweed and/or chitin for 3 days (recall responses). Ragweed alone stimulated the production of IL-4 (0.6 ng/ml), IL-5 (20 U/ml), and IL-10 (3.2 ng/ml), but not IFN-γ. Ragweed/chitin stimulation resulted in significant decreases of IL-4, IL-5, and IL-10 levels and the production of IFN-γ (48 U/ml). Moreover, spleen cells isolated from the chitin-treated mice showed ragweed-stimulated IFN-γ production (15 U/ml) and significantly lower levels of the Th2 cytokines, suggesting that the immune responses were redirected toward a Th1 response. Collectively, these results indicate that chitin-induced innate immune responses down-regulate Th2-facilitated IgE production and lung eosinophilia in the allergic mouse. The Journal of Immunology, 2000, 164: 1314–1321.

 序列 prevalence studies have shown that childhood allergic diseases including asthma are becoming more common. A probable explanation for the increase is due to the effects of life in the industrialized world, resulting in an increase in exposure to exogenous factors (outdoor and indoor pollutants, cigarette smoke, and allergen exposure) that may induce asthma, a reduction in host resistance, or a combination of both mechanisms. Factors altering host resistance have received less attention (1). A recent study by Shirakawa et al. (2) on children in Wakayama, Japan, suggested that tuberculosis was instrumental in reducing atopic disease prevalence. This study demonstrated a marked decline in the incidence of positive tuberculin responses (95% in 1965 and 58% in 1995) and tuberculosis (154.4 per 100,000 in 1974 and 52.1 per 100,000 in 1994). This information, therefore, suggested that the enhancement of Th1 immune responses by appropriate vaccines may be therapeutically beneficial against asthma and allergic diseases.

Inflammation in allergic asthma is generated and activated by endogenous proinflammatory cytokines produced by Th2-type lymphocytes: IL-4 facilitates IgE production and humoral immunity; IL-5 facilitates replication and activation of eosinophils. These allergen-induced Th2 responses enhance airway hyperreactivity in mouse models, although the mechanism(s) appears to be multifactorial depending on mouse strains (3–5). In contrast, the development of Th1/Th2-mediated immune responses induced by intracellular bacterial infection is characterized by the initial production of IL-12, TNF-α, and IL-18 by macrophages (MΦ) phagocytosing bacteria. These MΦ cytokines subsequently induce IFN-γ production by NK cells and Th1 lymphocytes. It is of importance that Th1 cytokines reverse Th2 cell-facilitating allergic inflammation. Particularly, the administration of exogenous IFN-γ or IL-12 appears to inhibit such IL-4/IL-5-associated allergic asthma responses in patients and allergic animal models (6–8).

Recently, we have studied the mechanisms of innate immunity induced by heat-killed (HK) Mycobacterium bovis BCG or Propionibacterium parvum. We have found that when MΦ phagocytose bacteria or their products through the MΦ mannose receptors, they initiate production of IL-12, IL-18, and TNF-α, all of which are extracellular signaling cytokines for IFN-γ production (9–11). To imitate this response, we have created nonantigenic mimetic microbes consisting of 1–10 μm chitin (polymers of N-acetyl-D-glucosamine) particles, which are recognized and ingested by MΦ through the mannose receptor (11). Our previous study (10) clearly indicated that 1–10 μm chitin particles, but not soluble chitin or nonphagocytosable size particles (>50 μm), induce the production of Th1 cytokines including IFN-γ in vitro and in vivo in mouse models. The capacity to induce Th1 cytokines by chitin is comparable with that induced by HK-BCG or HK-Propionibacterium acnes suspensions (9, 11). In contrast, other phagocytosable particles, such as chitosan, β-glucan, latex beads, IgG-opsonized SRBC, and complement (C³b)-opsonized SRBC, do not induce Th1 cytokines (11, 12).

To evaluate the potential for chitin to down-regulate allergic inflammation in vivo, we utilized a mouse model of allergic airway inflammation elicited by ragweed allergen clinically relevant to...
Table I. Schedule of immunization and challenge of ragweed and oral administration of chitin

<table>
<thead>
<tr>
<th>Schedule</th>
<th>Chitin or saline administration (once/day)</th>
<th>Naive I</th>
<th>Naive II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RW immunization</strong></td>
<td>1. Chitin (≤3 to 13)</td>
<td>Days ≤3 to 13</td>
<td></td>
</tr>
<tr>
<td>Experimental groups 1–3</td>
<td>2. Chitin (12, 13)</td>
<td>Days 12 and 13</td>
<td></td>
</tr>
<tr>
<td>Day 0 i.p., RW/Alum</td>
<td>3. Saline</td>
<td>Days ≤3 to 13</td>
<td></td>
</tr>
<tr>
<td>Day 4 i.p., RW/Alum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 11 i.t., RW</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 14 BAL, blood, lung, spleen</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* At each chitin injection interval, six to seven allergic mice in each group received chitin orally at 8 mg/0.5 ml/day. Seven other allergic mice in group 3 received saline (0.5 ml/day). Three days after the ragweed challenge i.t., mice were sacrificed and BAL, blood, and lungs were harvested. Nonsensitized control groups (naive I and naive II) were challenged i.t. with saline and ragweed, respectively, on day 11 and harvested on day 14.

**Materials and Methods**

**Mice**

BALB/c and C57BL/6 female mice (6–7 wk old) were obtained from Harbor Laboratory (Indianapolis, IN) and The Jackson Laboratory (Bar Harbor, ME), respectively, maintained in barrier-filtered cages, and fed Purina Laboratory Chow and tap water ad libitum.

**Chitin particle preparation**

Chitin particles (1–10 µm diameter) were prepared from purified chitin powders (Sigma, St. Louis, MO), as described previously (10, 13), suspended in saline (10–16 mg/ml), autoclaved, and stored at 4°C until use. The chitin preparations contained undetectable levels of endotoxin (<0.03 EU/ml), as determined by the Limulus amebocyte lysate assay (Sigma).

**Immunization of mice with ragweed**

Mice were sensitized with ragweed allergen, as described previously (6). The schedule of the sensitization is shown in Table I. Briefly, mice were immunized with two i.p. injections of endotoxin-free ragweed allergen (<0.03 EU/ml; Geer Laboratories, Lenoir, NC), 100 µg/dose (5000 protein nitrogen units/dose) suspended in 1:4 aluminum hydroxide adjuvant (Inject Alum; Pierce, Rockford, IL) on days 0 and 4. Eleven days after the initial sensitization, an i.t. challenge was performed with 100 µg of ragweed allergen. Control groups included unimmunized mice receiving i.t. challenge of 0.1 ml of saline (naive I) or ragweed 100 µg (naive II) on day 11. After 3 days, unless indicated otherwise, mice were humanely sacrificed, and the blood, spleens, BAL cells, and lungs were harvested.

BAL eosinophils and other inflammatory cells were enumerated by differential counts following cytoospin preparations and staining with Diff-Quik. Following BAL, the lungs were removed, Formalin fixed, and used for histological analysis, as described below. Spleen cells were utilized for recall responses, as described below. Serum IgE levels were determined by ELISA, as described below.

**Oral administration of chitin**

As shown in Table I, allergic mice (6–7 mice/group) were given chitin (5–8 mg/mouse/day) orally on days 3 to 13 (group 1). Chitin was given 4 h before i.t. challenge of ragweed on day 11. Control mice received saline (0.5 ml/day) (group 3). In some experiments, allergic mice received chitin on days 12 and 13 (group 2).

**Endotoxin removal**

It was noticed that the presence of endotoxin in some allergen preparations caused neutrophilia (rather than eosinophilia) labeled anti-inflammatory allergen challenge in patients with asthma (11). Endotoxin induces the production of inflammatory mediators including IL-12, IL-18, and TNF-α (9), major targets in this study. To remove possibly contaminating endotoxin, all soluble materials including ragweed preparations were passed through 0.22-µm Zetapor membrane filters (Cuno, Meriden, CT) manufactured for this purpose (15). The effectiveness of endotoxin removal was monitored by the Limulus amebocyte lysate assay (Sigma).

**Cytokine production in recall responses: spleen cell cultures stimulated with ragweed allergen in the presence or absence of chitin**

Spleens were isolated from ragweed-sensitized/challenged mice (3 days after the challenge) and naive I control mice. Spleens in each group were pooled. Spleen cells (4 × 10⁶ cells/ml) suspended in RPMI 1640 plus 10% heat-inactivated FBS were incubated with ragweed at 10 µg/ml and/or chitin at 100 µg/ml at 37°C for 3 to 4 days in a CO₂ incubator. After the incubation, the culture supernatants were collected and their selected cytokine levels (IL-4, IL-5, IL-10, IFN-γ) were determined by their respective ELISA assays (9).

In some experiments, CD4⁺ and asialo GM1⁺ NK cells were eliminated with Abs against CD4 (clone GK1.5; American Type Culture Collection, Manassas, VA) and asialo GM1⁺ rabbit polyclonal; Accurate Chemical, Westbury, NY), respectively, followed by treatment with guinea pig serum (1:10; Accurate) as a source of complement. The expression of CD4 and DX5 (pan NK cell marker) was determined by indirect immunofluorescence by the staining with anti-CD4 mAb and anti-DX5 mAb (PharMingen, San Diego, CA) in the presence of 5% heat-inactivated newborn calf serum (Life Technologies, Grand Island, NY), pH 7.2, as described previously (9).

**Detection of total IgE and ragweed-specific IgE and IgG2a Abs in sera**

Ragweed-immunized allergic responses were monitored by measuring total IgE levels. Blood (5 µl/mouse/interval) was collected from the tail vein, and serum IgE levels were detected by ELISA using purified mouse IgE κ isotype (PharMingen) as a standard. Ragweed-specific IgE and IgG2a levels were measured by ELISA with ragweed Ag-coated 96-well plates; 5 µg ragweed/0.1 ml/well in 0.1 M sodium acetate buffer, pH 6.5, overnight at 4°C (16). For the detection, alkaline phosphatase-labeled anti-IgE and anti-IgG2a were used. The enzyme substrate was p-nitrophenylphosphate: optical absorbance at 405 nm was read.

**Lung histology**

Lungs were removed on day 14 and immediately fixed with 10% neutral-buffered Formalin. The fixed lungs were embedded in paraffin, sectioned at a thickness of 4 µm, and stained with hematoxylin and eosin. The slides were evaluated for any abnormalities by a pathologist blinded to the treatment groups. The degree of allergen-induced peribronchial and perivascular inflammation was graded on a subjective scale of 0, 1, 2, 3, and 4 corresponding to severity nomenclature of absent, mild, moderate, marked, or severe, respectively (17).

**Statistics**

Differences between mean values were analyzed by unpaired Student’s t test. p values less than 0.05, which were calculated as two-tailed p values, were considered statistically significant.

**Results**

The release of IL-4, IL-5, and IL-10 production in ragweed-induced recall responses ex vivo is inhibited by chitin

To assess whether chitin modulates the production of selected Th2 cytokines, spleen cells were isolated from the spleens of ragweed-sensitized BALB/c mice and incubated with ragweed allergen and/or chitin. When spleen cells from ragweed-sensitized mice were incubated with the optimal endotoxin concentration (10–16 mg/ml), as determined by the amebocyte lysate assay (Sigma) (Table I), chitin treatment resulted in inhibition of Th2 cytokine production (IL-4, IL-5, IL-10, IFN-γ) compared to saline-treated controls (Figure 1). This inhibitory effect was dose-dependent, as shown by the decrease in cytokine production in response to increasing concentrations of chitin (Figure 2). The inhibition of Th2 cytokine production was accompanied by a decrease in the number of BAL eosinophils and other inflammatory cells (Figure 3). These results suggest that chitin treatment may be an effective therapeutic strategy for IgE-mediated allergic diseases.
NK cell-eliminated spleen cells contained 0.5% CD4 or chitin, either CD4 cells or NK cells were eliminated by specific reduced compared with those stimulated by chitin alone.

The data shown are representative of three independent experiments.

were rechallenged with ragweed allergen ex vivo (recall responses), they released significant levels of IL-4, IL-5, and IL-10, but not IFN-γ (Fig. 1). Control spleen cells from unimmunized mice (naïve I control). Spleen cells (4 x 10^6 cells/ml) were cultured in the presence of ragweed (RW, 10 μg/ml), chitin (100 μg/ml), or ragweed mixed with chitin (RW/chitin) for 3 days. The levels of indicated cytokines in the supernatants were measured by ELISA. Mean ± SD in triplicate cultures. Naïve control and ragweed-immunized mice, respectively. *p < 0.05, and #p < 0.0005 compared with the cytokine levels induced by ragweed (RW) alone and chitin alone, respectively. The data shown are representative of three independent experiments.

To characterize cell populations responding to ragweed allergen or chitin, either CD4 cells or NK cells were eliminated by specific Abs, followed by complement-dependent cell lysis. CD4 cell- and NK cell-eliminated spleen cells contained 0.5% CD4^+ cells and 0.3% DX5^+ cells, respectively (data not shown). As shown in Fig. 2, when CD4 cells were eliminated, ragweed-stimulated IL-4, IL-5, and IL-10 production was significantly impaired, whereas chitin-induced IFN-γ production was still intact. In contrast, when asialo GM1^+ NK cells were eliminated, ragweed-induced IL-4, IL-5, and IL-10 production was intact, but chitin-induced IFN-γ production was diminished (Fig. 2). Interestingly, when NK cell-eliminated spleen cells were stimulated simultaneously by ragweed and chitin, the down-regulation of IL-4/IL-5/IL-10 production by chitin was not observed (Fig. 2).

Taken together, these results suggest that IL-4 and IL-5 are produced by ragweed Ag-specific CD4^+ Th2 spleen cells. When co-stimulated with chitin, Th1 cytokines including IFN-γ produced by NK cells appear to inhibit the Th2 cytokine production. It is likely, therefore, that the administration of chitin in the allergic mouse will down-regulate the allergen-induced Th2 immune responses.

The prophylactic effects of chitin administration in the allergic BALB/c mouse

Mice immunized with ragweed at the schedule shown in Table I had been established by Sur et al. (6), and showed high levels of total serum IgE and lung inflammation including eosinophilia. To assess whether chitin administration down-regulates these inflammatory parameters, ragweed-sensitized mice were given chitin orally on days 3 to 13 (group 1 in Table I).

As shown in Fig. 3A, a significant elevation of serum IgE levels was observed after day 8. The levels increased to 4607 ± 1086 ng/ml on day 14. When mice were treated with chitin, the levels were significantly reduced (2014 ± 939 ng/ml, p < 0.0005). Ragweed-specific IgE levels were also reduced significantly by the chitin treatment, whereas ragweed-specific IgG2a levels were enhanced by the chitin treatment (Fig. 3B). The inhibition of total IgE levels was also observed on days 8, 10, and 12. Less than 50 ng/ml was detected on day 14 in naïve I and naïve II controls. BAL cells were harvested on day 14. Control groups (naïve I, naïve II) showed less than 3 x 10^5 total nucleated cells that contained >95% Mφ, 3% of lymphocytes, and <2% neutrophils (data not shown). As shown in Fig. 4, i.t. challenge of ragweed allergen in ragweed-immunized mice resulted in the migration of lymphocytes, eosinophils, and neutrophils in BAL fluid. The eosinophil and lymphocyte levels were reduced significantly by the chitin treatment (Fig. 4).

Total lung inflammation on day 14 was determined histologically and defined as the sum of peribronchial and perivascular inflammation scores. Fig. 5. A (naïve II control), B (saline, group 3), and C (chitin, group 1), show representative hematoxylin and
eosin-stained peribronchial and perivascular areas of the lungs (×40). Naive animals had no detectable lung inflammation, whereas the allergic mice treated with saline (group 3) had significant peribronchial and perivascular inflammation. Compared with

FIGURE 3. The effects of chitin treatment on serum IgE levels in ragweed-sensitized BALB/c mice. Groups of BALB/c mice (seven mice/group) were given chitin (8 mg/mouse/day) orally 3 days before ragweed immunization and continued to receive chitin during the immunization periods (group 1 in Table I). Immunized mice receiving saline (0.5 ml/mouse/day) served as controls (group 3). Blood samples were collected from tail veins at indicated days. A, Total serum IgE levels in the chitin-treated group (■) and in the saline-treated control group (□) were measured by ELISA. Mean ± SD, n = 7, *, p < 0.05; **, p < 0.01; #, p < 0.0005 compared with the saline-treated group. B, Ragweed-specific IgE (■) and IgG2a levels (□) in sera isolated from unimmunized groups (naive I controls, naive II controls) and ragweed-immunized groups (saline treated, chitin treated) on day 14 were quantified as described in Materials and Methods. The sera were diluted 1/5 and 1/20 with saline before they were assayed for ragweed-specific IgE and IgG2a levels, respectively. Mean ± SD, n = 7, *, p < 0.01; #, p < 0.0005 compared with the saline-treated group.

FIGURE 4. The effect of chitin treatment on ragweed-induced eosinophilia in BAL (BALB/c mice). BAL was collected from the saline-treated control group (■), chitin-treated group (days 12 and 13, □) of immunized BALB/c mice on day 14. The numbers of lymphocytes (Lymph), eosinophils (Eo), neutrophils (Neut), and macrophages (Mac) were determined as described in Materials and Methods. Mean ± SD, n = 7, *, p < 0.05; #, p < 0.005; ##, p < 0.0005 compared with the saline-treated group.

FIGURE 5. The effects of chitin on peribronchial and perivascular lung inflammation. Shown are photographs of lung sections from representative naive mice (A, naive II in Table I), saline-treated allergic mice (B, group 3), and chitin-treated allergic mice (C, group 1) at ×40 magnification. The lung of a naive mouse (A) showed no peribronchial or perivascular inflammation. In contrast, the lung of a mouse from the saline-treated group (B) showed grade 4 peribronchial inflammation, grade 4 perivascular inflammation, and grade 8 total lung inflammation. The lung from a mouse in the chitin-treated group (C) showed grade 1 peribronchial inflammation, grade 1 perivascular inflammation, and grade 2 total lung inflammation.
the saline group, the peribronchial, the perivascular, and total lung inflammation were inhibited in the chitin-treated group (Table II).

Although BAL fluid on day 14 did not have detectable levels of IL-4 or IFN-γ in the chitin-treated group (group 1) and saline-treated control (group 3) (data not shown), we monitored IL-4 and IFN-γ levels in BAL fluid on day 12 (Fig. 6). BAL contained IL-4 at 613 pg/ml and IFN-γ at <5 U/ml in saline-treated group (group 3). The chitin treatment (group 1) showed significant inhibition of IL-4 levels (163 pg/ml) and enhancement of IFN-γ levels (19.5 U/ml) (Fig. 6). IL-5 and IL-10 levels were not detected in BAL fluid of these two groups obtained on either day 12 or 14 (data not shown).

It should be noted that mice that received chitin orally exhibited a slight weight gain (Table III) and showed no distress or systemic toxicity. The activity of chitin to induce IL-12, IL-18, TNF-α, and IFN-γ was determined whether the chitin treatment at the effector phase of allergic inflammation is still effective. Chitin was given only on days 12 and 13 (group 2 in Table I) when total serum IgE levels were relatively high (1000–2800 ng/ml). As shown in Fig. 7, the chitin treatment resulted in significant decrease of IgE levels on day 14. There were significant decreases of lymphocyte and eosinophil numbers in BAL on day 14 (Fig. 4). These results suggest that chitin appears to have therapeutic effects.

**Table II. Effects of chitin on peribronchial and perivascular lung inflammation**

<table>
<thead>
<tr>
<th>Group</th>
<th>Peribronchial</th>
<th>Perivascular</th>
<th>Total</th>
<th>% Inhibition</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>2.64 ± 0.85</td>
<td>2.93 ± 0.73</td>
<td>5.57 ± 1.51</td>
<td>1.36 ± 0.75</td>
<td>1.64 ± 0.69</td>
</tr>
<tr>
<td>Chitin</td>
<td>1.38 ± 0.98</td>
<td>2.28 ± 0.46</td>
<td>3.56 ± 0.94</td>
<td>46</td>
<td>0.006</td>
</tr>
</tbody>
</table>

*Inflammation score was determined by a pathologist blinded to the treatment groups. Peribronchial and perivascular inflammation were graded on a subjective scale of 0, 1, 3, and 4 corresponding to mild, moderate, marked, or severe inflammation, respectively, with an increment of 0.5 if the inflammation fell between two integers. The total lung inflammation was defined as the sum of peribronchial and perivascular inflammation scores. The values are expressed as mean ± SD for 7 animals.

**Table III. Growth of mice given chitin particles orally**

<table>
<thead>
<tr>
<th>Group</th>
<th>Mouse Number</th>
<th>Day 0</th>
<th>Day 30</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline (control)</td>
<td>20</td>
<td>17.07 ± 0.93</td>
<td>19.37 ± 1.24</td>
</tr>
<tr>
<td>Chitin</td>
<td>22</td>
<td>17.28 ± 0.98</td>
<td>20.16 ± 0.98*</td>
</tr>
</tbody>
</table>

*Group of mice were given chitin (5 mg/mouse/day) orally for 30 days, (mean ± SD). *p < 0.03 compared with saline (day 30).

**Prophylactic treatment with chitin.** To determine whether ragweed allergen-specific Th2 cell functions were modulated by the prophylactic treatment with chitin (group 1 in Table I), recall responses of spleen cells were conducted. As shown in Fig. 8, ragweed-stimulated IL-4, IL-5, and IL-10 production was significantly reduced by the chitin treatment, whereas slight but significant amounts of IFN-γ were detected. The production of either IFN-γ or IL-4/IL-5/IL-10 was not observed when spleen cells (group 1) were depleted of CD4+ cells by anti-CD4+ plus guinea pig serum before the recall responses (data not shown).

IL-4/IL-10 and IFN-γ are known to down-regulate Th1 and Th2 responses, respectively (9, 10, 18–20). IL-10 also inhibits allergen-stimulated IL-4 and IL-5 production (21). To assess regulatory roles of these endogenous cytokines (IL-4, IL-10, IFN-γ), neutralizing Abs for these cytokines at 20 μg/ml were added to the ragweed-stimulated spleen cell cultures. When rat Ig at 20 μg/ml was added to the cultures as a control, the cytokine profiles were identical to those untreated (data not shown). Fig. 8 shows that ragweed-stimulated IL-4/IL-5 production by spleen cells was significantly enhanced by either anti-IL-10 or anti-IFN-γ treatments in the chitin groups, suggesting that endogenous IL-10 and IFN-γ inhibit IL-4/IL-5 production. Ragweed-stimulated IL-4/IL-5/IL-10 production by spleen cells from chitin-treated mice was also enhanced by anti-IFN-γ treatments. However, the treatment with anti-IFN-γ did not enhance IL-4/IL-5/IL-10 production in allergic mice given saline (group 3). It is likely, therefore, that endogenous IFN-γ produced by spleen cells from chitin-treated mice inhibits...
production of these Th2 cytokines. Finally, the treatment with either anti-IL-4 or anti-IL-10 resulted in significant increases in ragweed-stimulated IFN-γ production. It is suggested that the immunized mice generate ragweed-specific Th1 cells when they were prophylactically treated with chitin.

**Therapeutic treatment with chitin.** Unlike the prophylactic treatment, the therapeutic treatment resulted in no change of the levels of IL-4, IL-5, and IL-10 in the recall responses. There was no detectable level of IFN-γ produced by the recall responses. Furthermore, the inhibition of endogenous IL-4 or IL-10 by the Ab treatments did not show the production of IFN-γ (data not shown).

**The prophylactic effects of chitin administration in the allergic C57BL/6 mouse**

To further confirm the effects of chitin, we employed C57BL/6 mice, which are higher responders for cell-mediated immunity/Th1 responses, but lower responders for allergic responses compared with BALB/c mice. C57BL/6 mice were immunized with ragweed at the identical schedules (Table I). Serum IgE levels detected on day 14 (801 ± 357 ng/ml) were lower than those in ragweed-immunized BALB/c mice (Fig. 3A). After the treatment with chitin (5 mg/dose) on days −3 to 13, the levels decreased to 330 ± 158 ng/ml (p < 0.01, data not shown). Eosinophil levels were comparable with those found in BALB/c mice, and the chitin treatment resulted in a decrease in eosinophil numbers (Fig. 9).

**Discussion**

Previously, we showed that phagocytosable nonantigenic chitin, a seemingly inert molecule, induces endogenous Th1 cytokines (IL-12, IL-18, TNF-α, IFN-γ) (9–11). These are cytokines that are generally seen at early stages of infection (innate immunity) caused by intracellular bacteria such as *M. bovis* BCG (9–11). Innate immunity is important for protection against intracellular bacterial infections and to induce acquired immunity specific for bacteria (22). It is well established that Th1 cytokines, produced in innate immunity, down-regulate allergic immune (Th2) responses (18). The present study of recall responses clearly shows that allergen-stimulated IL-4, IL-5, and IL-10 production is significantly inhibited by the presence of chitin in the spleen cell cultures (Fig. 1). Our results suggest that IFN-γ produced by NK cells, at least in part, is responsible for the inhibition of the production of these Th2 cytokines.

The provocative findings are that chitin, given orally, down-regulates allergen-induced IgE production and lung inflammation including eosinophilia in our ragweed-immunized allergic model. We also found that an increase in IFN-γ levels and a reduction of IL-4 levels in BAL are associated with a reduction of allergen-specific IgE levels and an increase in allergen-specific IgG2a levels, indicating isotype switching by B cells. Interestingly, when chitin is given prophylactically, the chitin-treated mice appear to generate ragweed-specific Th1 cells, although the Th1 cell generation would significantly be reduced by endogenous IL-4 and IL-10, which are also produced during ragweed-stimulated recall responses. Our results strongly suggest that IFN-γ produced by these Th1 cells as well as NK cells down-regulates IL-4-facilitated IgE formation and IL-5-facilitated eosinophil migration. However, ragweed-specific Th1 cells were not detected when chitin was given only during the effector phase of allergic inflammation. In this case, IFN-γ produced by NK cells would play an important role for the down-regulation of IgE levels and lung eosinophilia.

Consistent with earlier observations (9, 23), our study clearly showed that IL-10 is produced by ragweed-stimulated CD4 cells and chitin-stimulated Moφ and NK cells. In addition, IL-10 is produced by many other diverse cell populations, including bronchial epithelial cells and B cells (24, 25). The immunoregulatory roles of IL-10 for allergic responses appear to be complex. It has been
suggested that IL-10 inhibits innate immunity as well as both Th1 and Th2 responses to Ags most probably by inhibiting APC (9, 21, 26). In our preliminary studies using IL-10-deficient C57BL/6 (KO) mice, sensitization with endotoxin-free ragweed in KO mice resulted in significantly higher levels of serum IgE levels and lung eosinophilia compared with those of allergic wild-type controls. The increases in serum IgE levels and lung eosinophil numbers in KO mice, both of which are facilitated by IL-4 and IL-5, respectively, were closely associated with the inhibitory effects of IL-10 on allergen-specific IL-4/IL-5 production in the recall responses. Oral administration of chitin at identical schedules employed in the present study significantly reduced serum IgE levels and lung eosinophilia in KO mice (manuscript in preparation). In sharp contrast to the anti-inflammatory effects, IL-10 may be highly relevant for proinflammatory responses involving mast cell activation: IL-10 significantly enhances mast cell proliferation, allergen-induced histamine release, and expression of chymases (27, 28). Therefore, exact regulatory mechanisms of endogenous IL-10, whose amounts can be controlled by the chitin treatment, remain to be elucidated.

Chitin, a polymer of N-acetyl-D-glucosamine, is an essential component of fungal cell walls and has been the target of antifungal drugs (nikkomycin Z, polyoxin D (29, 30)). Chitin is similar in structure to cellulose and has no mammalian counterpart. Following cellulose, chitin is the second most abundant polysaccharide found in nature. It comprises the horny substance in the exoskeleton of crabs, shrimp, and insects as well as the cell walls of fungi. Chitosan (deacetylated chitin) is nontoxic, nonallergenic, biodegradable, and biocompatible, and has been used in the development of a number of prostheses (artificial skin, contact lens, surgical sutures) that are widely used in medical and veterinary practice (31, 32). When chitin particles prepared as described above, essentially 100% N-acetyl-D-glucosamine, are administered to mice, they bind to the Mφ plasma membrane mannose receptors (33). These receptors serve to mediate the interiorization of the chitin particles that, eventually, are degraded by lysozyme and N-acetyl-β-D-glucosaminidase that are present in the Mφ of humans and experimental animals (34, 35). Therefore, chitin preparations of nonmicrobial origin represent very attractive and highly effective products for the down-regulation of allergic inflammation. The oral administration for therapeutic/prophylactic purposes should most likely be the route of choice for children who suffer from allergic diseases.

In allergic animal models, the administration of IFN-γ or IL-12 inhibits Th2-driven inflammation (6–8). Exogenous IL-12 induces IFN-γ production, which is the most effective cytokine to down-regulate Th2 responses (7). The mechanisms for IFN-γ production are comprehensive. Recently, an additional IFN-γ-inducing factor (IL-18) was found to contribute to IFN-γ production (36). We have confirmed that chitin induces Mφ to produce IL-12, IL-18, and TNF-α, all of which act synergistically to induce IFN-γ production by NK cells (9). Interestingly, chitin-stimulated Mφ also produce IL-10, which up-regulates NK cell IFN-γ production (9). It is, therefore, reasonable to speculate that amounts of exogenous IL-12 far greater than physiological ranges are required to induce effective levels of IFN-γ production. Excessive doses of IL-12 would be associated with the toxic side effects (37–39).

Recently, it has been reported that treatment with live or HK bacteria, including M. bovis BCG, Mycobacterium vaccae, and Listeria monocytogenes, results in down-regulation of Th2 responses in various allergic mouse models (40–42). These bacterial suspensions are known to induce Th1 immune responses. Several bacterial components that induce Th1 cytokines have also been identified. Endotoxin, superantigen exotoxins, and mycobacterial DNA with CpG motifs are able to induce IL-12/TNF-α/IL-18 by Mφ (43–45), suggesting usefulness of these components for the down-regulation of allergic inflammation (17, 46, 47). However, the toxic side effects of these components are of great concern (48). In the present studies, mice given chitin orally showed no visible adverse effects. Interestingly, their body weight tended to increase slightly compared with mice receiving saline (Table III).

The activity of chitin to induce Th1 cytokine production in vitro is found to be relatively stable under acidic conditions, although deacetylation can occur and deacetylated chitin (chitosan) does not induce Th1 cytokines (10). We found that the activity is unchanged after treatment with 0.1 M hydrochloric acid at 37°C for 4 h (data not shown). In addition, it is established that 1–10 μm poly(β-L-lactic-coglycolic acid) particles and polystyrene particles administered directly into the intestines following a laparotomy are readily internalized by Peyer’s patch M cells (49, 50). One would expect, therefore, that a part of the chitin particles given orally are taken up by M cells and translocated to the pocket region containing Mφ and other leukocytes. During chitin mobilization in intraepithelial lymphoid tissues, some cells would produce Th1 cytokines. However, exact mechanisms of mucosal immunity in the small intestines stimulated by chitin remain to be elucidated.

Nonpathogenic and pathogenic facultative intracellular bacteria (for example, L. monocytogenes and M. tuberculosis) invade and survive in Mφ, and only highly activated Mφ can kill these bacteria. Since lifestyles in industrialized countries tend to create new environments that eliminate/reduce bacteria (51), Mφ activation levels that are promoted by such environments are substantially diminished. Studies using germfree experimental animals appear to support this hypothesis (52). Our study suggests that oral administration of chitin may be a substitute for such bacterial exposures.

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