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Anti-allergic potential of oligomannose-coated liposome-entrapped Cry j 1 as immunotherapy for Japanese cedar pollinosis in mice

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ABSTRACT

Administration of oligomannose-coated liposomes (OMLs) in mice can induce Th1 immune responses against antigens entrapped in the OMLs. In the present study, we investigated the anti-allergic effect of treatment with oligomannose-coated liposomes (OMLs) with entrapped Cry j 1, a major allergen of Japanese cedar pollen (Cry j 1/OMLs), in Balb/c mice sensitized with Cry j 1. Pretreatment of unsensitized mice with Cry j 1/OMLs repressed the elevation of total and allergen-specific IgE levels in sera elicited in response to subsequent sensitization with Cry j 1. Cry j 1-specific IgG1 in sera also decreased in Cry j 1/OML-treated mice, while the levels of Cry j 1-specific IgG2a in these mice significantly increased after sensitization with Cry j 1. In addition, Cry j 1/OML-treated mice showed high IFN- γ production from spleen cells and low IL-4/IFN- γ and IL-5/IFN- γ ratios in response to *in vitro* stimulation with Cry j 1. These results indicate that allergen-specific Th1 immune responses predominate over Th2 responses and control IgE elevation in Cry j 1/OML-treated mice. The anti-allergic effect of Cry j 1/OML determined by suppression of serum IgE levels was also observed in Cry j 1-prensensitized mice after challenge with antigen. Thus, Cry j 1/OMLs may be useful for immunotherapeutic control of allergic reactions to Japanese cedar pollinosis.

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1. Introduction

Japanese cedar (*Cryptomeria japonica*; Cj) pollinosis is a common and serious type I allergic disease in Japan that is prevalent in 10 to 20% of the population and poses a significant social problem [1]. Cj pollinosis in humans is characterized by conjunctivitis and rhinitis during the spring cedar pollen season [2]. Specific immunotherapy has been applied for desensitization to type I allergic diseases, using stepwise subcutaneous administration of an escalating dose of the specific allergen to reach a state of immunological tolerance [3,4]. However, repeated administration of the allergen over a long period is inconvenient and high doses of allergen may cause side effects, including anaphylactic reactions [5]. Therefore, a new immunotherapeutic approach is required to treat and prevent Cj pollinosis.

Type I allergic diseases including Cj pollinosis are characterized by elevated immunoglobulin E (IgE) levels and mast cell degranulation that causes release of histamine and other chemical mediators of allergy [6]. The allergic reaction depends on allergen-specific T helper 2 (Th2) cells, which produce IL-4 and IL-5 and promote IgE synthesis [7]. IFN- γ produced by Th1 cells inhibits the development and activation of Th2 cells

and prevents IgE production [8,9]. Since patients with Cj pollinosis have high levels of allergen-specific IgE and T cell reactivity to Cry j 1 and/or Cry j 2 [5,10], which have been identified as major allergens in Cj pollen [11], induction of Th1 cells specific for Cry j 1 and/or Cry j 2 in Cj pollen may be a promising approach to interference with Cj pollinosis.

We have recently demonstrated that liposomes coated with the neoglycolipid constructed from mannitolose and dipalmitoylphosphatidylethanolamine (Man3-DPPE) (oligomannose-coated liposomes, OMLs) can induce a strong Th1 immune response against encapsulated antigens in mice, with significant IFN- γ production and suppressed IL-4 production following specific uptake of OMLs by antigen-presenting cells (APCs) such as macrophages [12,13]. In response to specific uptake of OMLs, APCs accumulate in the nearby lymphoid tissues [12,14] and undergo maturation with enhanced expression of MHC class II and co-stimulatory molecules (CD80 and CD86), suggesting that the OMLs have adjuvant activity [15,16]. Administration of OMLs also protects against subsequent protozoan infections and tumor inoculation when specific antigens are entrapped within the OMLs [13,14,17]. These findings led us to hypothesize that administration of OMLs with entrapped allergens may control allergic reactions in diseases such as Cj pollinosis by induction of allergen-specific Th1 immune responses.

To examine the anti-allergic effect of OMLs as adjuvant therapy for Cj pollinosis, we evaluated the efficacy of immunization with OMLs with entrapped Cry j (Cry j 1/OMLs) in BALB/c mice. Our results show that

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administration of Cry j 1/OMLs can control allergic immune responses in Cj pollinosis based on suppression of elevation of serum IgE levels.

2. Materials and methods

2.1. Animals

Female BALB/c mice of 5 to 6 weeks of age were purchased from Clea Japan (Tokyo, Japan). Until use in experiments at 6 to 7 weeks of age, the mice were housed under specific-pathogen-free conditions in the animal facility of the National Research Center for Protozoan Diseases at Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Japan. All mice were treated under the guiding principles for the care and use of research animals established by Obihiro University of Agriculture and Veterinary Medicine and by Tokai University.

2.2. Preparation of liposomes with entrapped allergen

Purified Cry j 1, a major allergen of Cj pollen, was purchased from Hayashibara Biochemical Laboratories Inc. (Okayama, Japan) and used without further purification. Dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylethanolamine (DPPE), and cholesterol were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Mannotriose (Man3) with the structure $\text{Man}\alpha 1\text{-6}(\text{Man}\alpha 1\text{-3})\text{Man}$ was obtained from Funakoshi. Neoglycolipid (Man3-DPPE) was prepared by conjugation of Man3 with DPPE [18]. Purification of the neoglycolipid from the crude lipid sample was performed by HPLC on a silica column (Wakosil 5SIL-120, 0.75×30 cm, Wako Pure Chemical, Tokyo, Japan). The purity and structure of the neoglycolipid were confirmed by HPLC and MALDI-TOF mass spectrometry (Autoflex, Bruker Daltonics, Bremen, Germany).

Liposomes with entrapped Cry j 1 were prepared as described previously with some modifications [12,13]. Briefly, a chloroform-methanol (2:1, v/v) solution containing 1.5 μmol of DPPC, 1.5 μmol of cholesterol, and 0.15 μmol of Man3-DPPE was added to a flask and evaporated to prepare a lipid film containing neoglycolipid. PBS (180 μl) containing Cry j 1 (900 $\mu\text{g}/\text{ml}$) was added to the dried lipid film and multilamellar vesicles were prepared by intense vortex dispersion. The vesicles were extruded five times through a polycarbonate membrane with 1- μm pores (Nucleopore, Pleasanton, CA, USA). Liposomes with entrapped Cry j 1 (Cry j 1/OMLs) were separated from free Cry j 1 by three rounds of washing in PBS with centrifugation at 4 °C (13,700 $\times g$, 30 min). The amount of entrapped antigen was measured using a modified Lowry protein assay reagent (Pierce, Rockford, IL, USA) in the presence of 4% sodium dodecyl sulfate (SDS), using bovine serum albumin as the standard.

2.3. Immunization and sensitization

Female BALB/c mice ($n=4$ to 5 per group) were intradermally immunized twice with 1 μg of Cry j 1 entrapped in OMLs (Cry j 1/OMLs), 1 μg of Cry j 1 adsorbed onto 0.4 mg of aluminum hydroxide (Pierce) (Cry j 1/alum), OMLs without entrapped Cry j 1 (empty OMLs), or PBS alone (control) at 1-week intervals (days 0 and 7). Two weeks after the last immunization, the mice were sensitized twice with intraperitoneal administration of 10 μg of Cry j 1 adsorbed onto 4 mg of alum at 1-week intervals (days 21 and 28). Sera were collected via the tail veins every week for quantification of total IgE and Cry j 1-specific IgE and IgG. Two weeks after the second sensitization (day 42), the mice were sacrificed for harvesting of spleen cells.

Alternatively, the mice were first sensitized with intraperitoneal administration of 10 μg of Cry j 1 adsorbed onto 4 mg of alum (day 0). The sensitized mice were then intradermally administrated with Cry j 1/OMLs or PBS at 1-week intervals for 2 weeks (days 7 and 14). Two weeks after the last immunization, the mice were subcutaneously challenged twice with 1 μg of Cry j 1 adsorbed onto alum at 1-week intervals (days

28 and 35). One week after the challenge (day 42), the mice were sacrificed for harvesting of spleen cells.

2.4. Determination of antibodies in sera

Total IgE levels in mouse sera were measured by a sandwich enzyme-linked immunosorbent assay (ELISA). Flat-bottom 96-well plates were precoated with 100 μl of affinity purified goat anti-mouse IgE (Bethyl Laboratories, Montgomery, TX, USA) (10 $\mu\text{g}/\text{ml}$) overnight at 4 °C. The plates were washed three times with PBS containing 0.05% Tween 20 (PBS-T) and then blocked with PBS containing 1% bovine serum albumin (BSA-PBS) for 1 h at room temperature. After washing with PBS-T, 100 μl of serum samples diluted 1:100 with BSA-PBS were added to duplicate wells and incubated at 37 °C for 1 h. After washing with PBS-T, the plates were incubated at 37 °C for 1 h with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgE (Bethyl Laboratories) adjusted to 0.2 $\mu\text{g}/\text{ml}$ with BSA-PBS. The plates were washed extensively with PBS-T, and then 100 μl of TMB (3,3',5,5'-tetramethylbenzidine) substrate buffer was added to each well. After incubation at room temperature for 30 min, 1 M phosphoric acid was added to each well in 100- μl aliquots to stop the reaction and the absorbance at 450 nm was measured using a plate reader. Total IgE levels were calculated on a standard curve generated for mouse reference serum (Bethyl Laboratories).

For the antigen-specific antibody assay, flat-bottom 96-well plates were precoated with 50 μl of Cry j 1 (2.5 $\mu\text{g}/\text{ml}$) overnight at 4 °C. The plates were blocked with BSA-PBS and 2-fold serial dilutions of serum samples were dispensed in the wells. After incubation for 1 h, the plates were washed with PBS-T and the levels of IgG1 or IgG2a in the samples were determined using rabbit anti-mouse IgG1 or rabbit anti-mouse IgG2a antibody (Zymed Laboratories, San Francisco, CA) followed by HRP-conjugated goat anti-rabbit antibody (MBL, Nagoya, Japan). HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-mouse IgE were used for detection of antigen-specific total IgG and IgE, respectively. After washing with PBS-T, the plates were incubated with o-phenylenediamine dihydrochloride (Sigma-Aldrich). The reactions were stopped with 1 M HCl and absorbance at 490 nm was measured.

2.5. In vitro stimulation of spleen cells

A single-cell suspension was prepared from the spleen of each mouse and then hemolyzed in a lysing buffer (0.75% NH_4Cl and 0.017 M Tris-HCl, pH 7.4). After washing with RPMI 1640 medium (Sigma), the cells were plated in 96-well microplates at 2×10^6 cells/200 μl /well in RPMI 1640 supplemented with 10% fetal bovine serum and penicillin/streptomycin. The cells were cultured with or without 50 $\mu\text{g}/\text{ml}$ of Cry j 1. After incubation for 72 h at 37 °C, the supernatants were collected and assayed for cytokine concentrations.

2.6. Cytokine analyses

Th1 and Th2 cytokines including interleukin (IL)-4, IL-5, and interferon gamma ($\text{IFN-}\gamma$) in the culture supernatants were simultaneously quantified using mice Th1/Th2 cytokine and inflammatory cytometric bead array (CBA) kits (BD Biosciences Pharmingen, San Diego, CA, USA). Premixed microbeads were plated in MultiScreen 96-well filtration plates (Millipore Corporation, Cork, BV, Ireland). Supernatant was diluted 10 times with Assay Diluent (BD Biosciences) and added to each well in 50- μl aliquots, and then the plates were incubated in the dark for 1 h at room temperature. After addition of 50 μl of phycoerythrin-conjugated antibodies against the cytokines, the plates were incubated in the dark for 1 h at room temperature and then washed by centrifugation at $240 \times g$ for 1 min. The resulting pellets were resuspended in 150 μl of washing buffer (BD Biosciences). A FACSCalibur flow cytometer (BD Biosciences) was calibrated with setup beads and 3000 events were acquired for each sample. The

concentrations of cytokines were determined from their fluorescent intensities using the standard reference curves in the CBA software (BD Biosciences).

2.7. Statistical analysis

Data are expressed as the mean $\pm$ standard error (SE). Two-tailed non-parametric multiple comparisons with a Bonferroni correction following a Kruskal–Wallis test were used for multiple comparisons between the control group and other groups. A Welch *t* test was used for direct comparison between two groups. A value of $P < 0.05$ was considered to indicate a significant difference.

3. Results

3.1. Pretreatment with Cry j 1/OMLs represses total IgE in sera after sensitization with Cry j 1

To evaluate the anti-allergic potential of administration of Cry j 1/OMLs, mice were immunized intradermally with 1 μ g of Cry j 1 entrapped in OMLs (Cry j 1/OML), 1 μ g of Cry j 1 adsorbed on alum (Cry j 1/alum), OMLs without entrapped allergen (empty OML), or PBS at 1-week intervals for 3 weeks and then sensitized intraperitoneally with 10 μ g of Cry j 1 adsorbed on alum at 1-week intervals for 2 weeks. As an unprimed control, mice were treated intradermally with PBS three times and then inoculated intraperitoneally with PBS twice. Sera were collected weekly from each mouse and total IgE in each sample was determined. As shown as Fig. 1A, there was a marked increase in total IgE in sera at 1 week after the second Cry j 1 sensitization in PBS-treated mice ($4,580 \pm 400$ μ g/ml). The total IgE in sera also increased in Cry j 1/alum-treated mice after Cry j 1 sensitization. In contrast, total IgE in sera of Cry j 1/OML-treated mice did not change after sensitization and remained lower than that in PBS-treated mice until day 42 (1900 ± 410 μ g/ml).

Comparison of total IgE levels at 2 weeks after the last sensitization (day 42) among the groups of mice (Fig. 1B) indicated that total IgE in sera of Cry j 1/OML-treated mice was significantly lower than that in sera from PBS- and Cry j 1/alum-treated mice. Furthermore, there was no significant difference in total IgE levels between Cry j 1/OML-treated mice and control unprimed mice. Mice that received empty OMLs had much higher total IgE than Cry j 1/OML-treated mice after Cry j 1 sensitization (4220 ± 320 μ g/ml) and the levels were similar to those of PBS- and Cry j 1/alum-treated mice ($P > 0.3$ and 0.1 , respectively). These data show that immunization with Cry j 1/OMLs repressed IgE elevation in sera elicited by subsequent Cry j 1-sensitization, whereas empty OMLs did not exert this effect.

3.2. Increased Cry j 1-specific IgG2a in sera of mice treated with Cry j 1/OMLs

The titers of Cry j 1-specific IgE in sera on day 42 in Cry j 1/OML-treated mice were lower than those for Cry j 1/alum- and PBS-treated mice after sensitization with Cry j 1 (Fig. 2). The serum levels of Cry j 1-specific IgG1 and IgG2a were measured to evaluate the *in vivo* immune response after sensitization. Serum Cry j 1-specific IgG also decreased significantly in Cry j 1/OML-treated mice. The titers of Cry j 1-specific IgG1 in Cry j 1/OML-treated mice were about 10- to 40-fold lower and those for Cry j 1-specific IgG2a were about 40-fold higher compared to the respective titers in PBS- and Cry j 1/alum-treated mice (Fig. 2).

3.3. Effect of administration of Cry j 1/OMLs on cytokine production

Cytokine production from spleen cells obtained at 2 weeks after the second sensitization with Cry j 1/alum (day 42) was measured to examine induction of Th1 and Th2 cells. Spleen cells from Cry j 1/OML-treated mice showed significantly higher production of IFN- γ (2123 ± 279 pg/ml) compared to those from unprimed mice in response to *in*

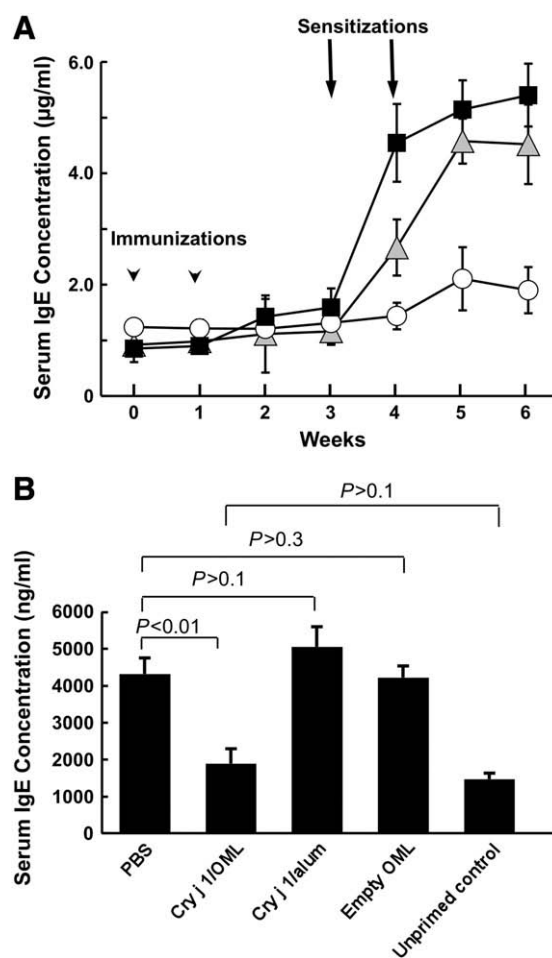


Fig. 1. Effect of administration of Cry j 1/OMLs on serum IgE levels in BALB/c mice. BALB/c mice ($n = 5$ per group) were immunized with Cry j 1/OMLs, Cry j 1/alum, empty OMLs, or PBS, and then sensitized with 10 μ g of Cry j 1 adsorbed on alum. For the unprimed control, mice were treated with PBS and then inoculated with PBS. A. Time course of total IgE concentration in sera. Triangles, squares, and circles indicate the total IgE concentration in sera from PBS-, Cry j 1/alum-, and Cry j 1/OML-treated mice, respectively. The time points of immunization and sensitization are indicated by arrowheads and arrows, respectively. Each value is the mean $\pm$ SE. B. Total IgE levels in sera at 2 weeks after the second sensitization. Each value is the mean $\pm$ SE.

vitro stimulation with Cry j 1 (Fig. 3). In contrast, IFN- γ secretion from spleen cells of PBS- and Cry j 1/alum-treated mice was lower than that for Cry j 1/OML-treated mice. IL-4 production did not differ significantly among spleen cells from mice treated with PBS, Cry j 1/OMLs and Cry j 1/alum, and from unprimed mice. In contrast, secretion of IL-5 from spleen cells of Cry j 1/OML-treated mice was slightly lower than those from spleen cells from PBS- and Cry j 1/alum-treated mice. The production of the immunosuppressive cytokine, IL-10, from spleen cells of Cry j 1/OML-treated mice was slightly higher than those from spleen cells from PBS- and Cry j 1/alum-treated mice (Fig. 3). The IL4/IFN- γ , IL-5/IFN- γ , and IL-10/IFN- γ ratios were then calculated to evaluate the Th1/Th2 balance (Fig. 4). Compared to mice treated with PBS, these ratios were significantly lower in Cry j 1/OML-treated mice ($P < 0.05$) and much higher in Cry j 1/alum-treated mice.

3.4. Suppression of total IgE elevation elicited in response to challenge with allergen in Cry j 1-presensitized mice by treatment with Cry j 1/OMLs

To examine the anti-allergic effect of OMLs in allergen-sensitized mice, Balb/c mice were first sensitized intraperitoneally with 10 μ g of Cry j 1 adsorbed on alum. The presensitized mice were immunized with Cry j 1/OML or PBS, and then challenged subcutaneously with 1 μ g of Cry j 1 adsorbed on alum. As shown in Fig. 5A, the levels of total

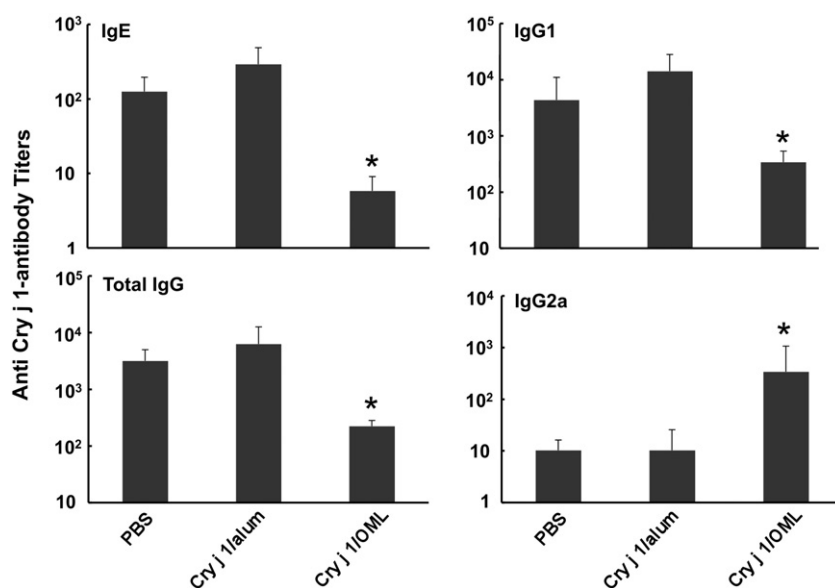


Fig. 2. Effects of administration of Cry j 1/OMLs on Cry j 1-specific IgE, IgG, IgG1, and IgG2a antibodies in mice sera. The titers given are half-maximal values of 2-fold serial dilutions performed in duplicate for serum samples for individual mice ($n=5$). Each value is the mean $\pm$ SE. * $P<0.05$ vs. PBS-treated mice.

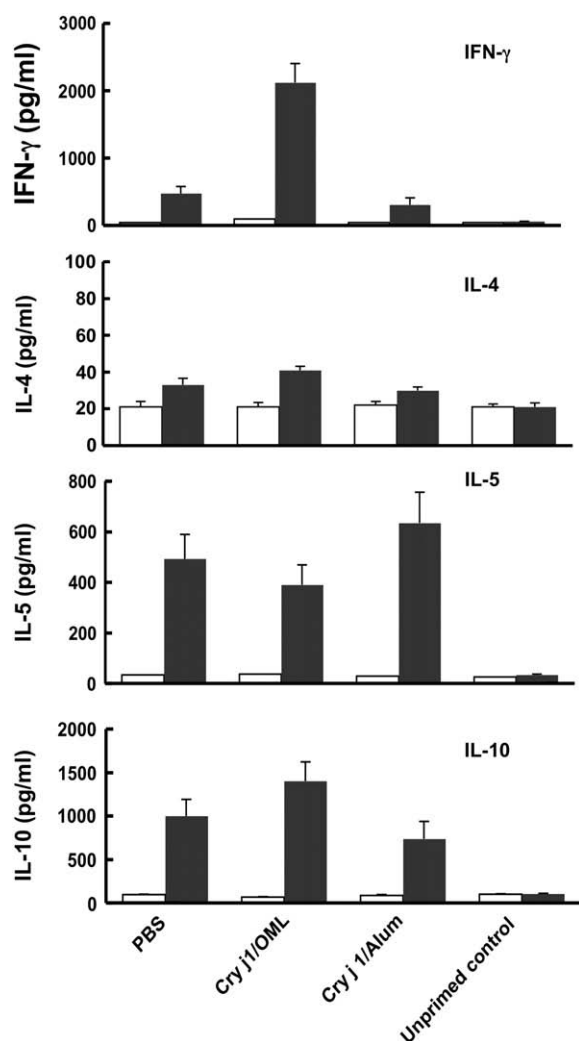


Fig. 3. Effect of Cry j 1/OMLs on production of cytokines from spleen cells in response to *in vitro* stimulation. Mice were sacrificed at 2 weeks after the second sensitization (day 42) and the spleen cells were cultured with (gray bars) or without (white bars) Cry j 1 for 72 h. Culture supernatants were assayed for cytokine production by CBA kits using a flow cytometry. Each value represents the mean $\pm$ SE.

IgE increased significantly in sera of PBS-received mice in response to the subcutaneous challenge with Cry j 1. In contrast, there was only slight elevation of serum IgE levels in Cry j 1/OML-treated mice after the challenge. The IL-5/IFN- γ ratio was lower in Cry j 1/OML-treated mice compared to PBS-treated mice (Fig. 5B), based on measurement of cytokine production from spleen cells at one week after the last challenge with Cry j 1/alum (day 42).

4. Discussion

We have shown previously that administration of a protozoan-specific antigen entrapped in OMLs induces Th1 immune responses

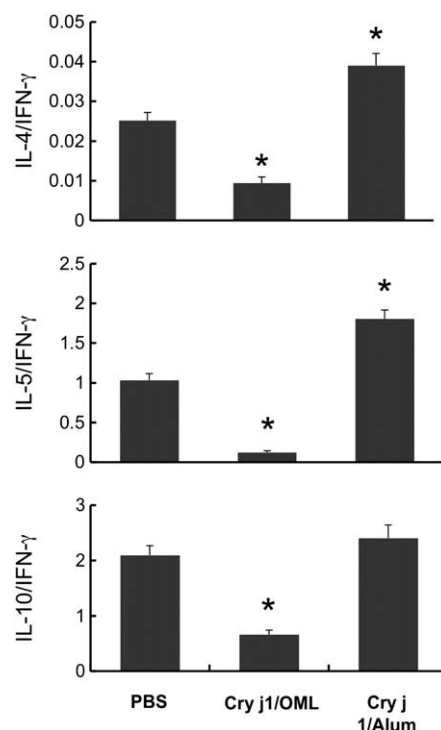


Fig. 4. Effect of administration of Cry j 1/OMLs on the IL-4/IFN- γ , IL-5/IFN- γ , and IL-10/IFN- γ ratios for cytokines produced from spleen cells in response to *in vitro* stimulation with Cry j 1. Each value is the mean $\pm$ SE. * $P<0.05$ vs. PBS-treated mice.

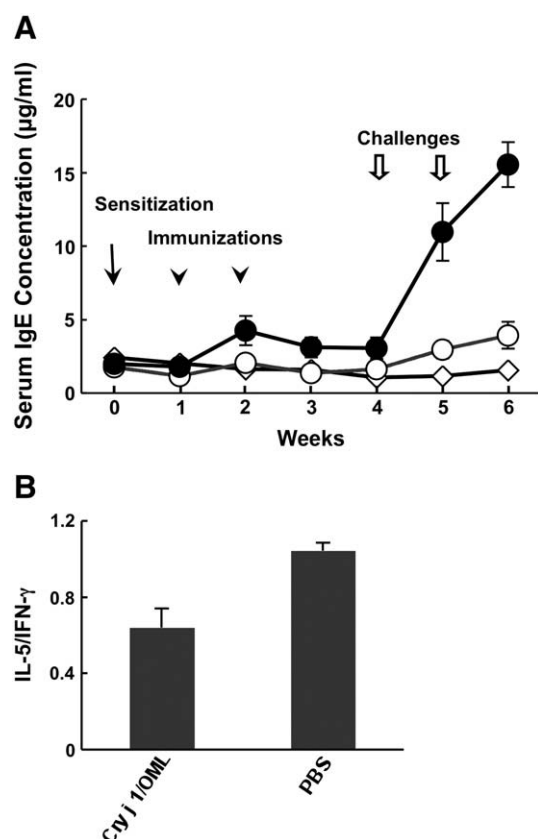


Fig. 5. Effect of administration of Cry j 1/OMLs on serum IgE levels in BALB/c mice presensitized with Cry j 1. BALB/c mice were sensitized with intraperitoneal administration of 10 µg of Cry j 1 adsorbed on alum. The presensitized mice were then immunized with Cry j 1/OMLs or PBS. Two weeks after the last immunization, the mice were challenged twice with 1 µg of Cry j 1 adsorbed on alum. Closed and open circles and diamonds indicate the total IgE concentration in sera from PBS- and Cry j 1/OML-treated mice and unprimed control mice, respectively. A. Concentration of total IgE in sera. The time points of sensitization, immunization, and challenge are indicated by an arrow, arrowheads, and open arrows, respectively. Each value is the mean $\pm$ SE. B. The IL5/IFN- γ ratio for cytokines produced from spleen cells in response to *in vitro* stimulation with Cry j 1. Spleen cells were prepared from each mouse 1 week after the last challenge with Cry j 1, and were cultured with or without Cry j 1 for 72 h.

specific for the entrapped antigen and protects against subsequent infections with protozoans such as *Leishmania major* and *Neospora caninum* [13,17]. The aim of the present study was to examine whether administration of an OML-entrapped allergen can control an allergic reaction such as that in Cj pollinosis in BALB/c mice, since the induction of allergen-specific Th1 cells is a promising approach for interfering with an allergic reaction. We found that pretreatment of unsensitized mice with OMLs containing 1 µg of Cry j 1 (Cry j 1/OMLs) repressed the elevation of total and allergen-specific IgE elicited by subsequent intraperitoneal sensitization with 10 µg of Cry j 1 adsorbed on alum (Fig. 1A). Treatment of Cry j 1-presensitized mice with Cry j 1/OMLs also suppressed the elevation of IgE levels elicited in response to challenge with the allergens (Fig. 5A). Since type I allergic diseases including Cj pollinosis are characterized by elevated IgE levels [6], OML-entrapped allergens may be useful for control of these diseases.

Cry j 1-specific IgG1 was significantly reduced but Cry j 1-specific IgG2a increased in sera of Cry j 1/OML-immunized mice. Since production of IgG1 and IgG2a in mice is mediated by Th2 and Th1 immune responses, respectively [9,19], our results indicate that a Cry j 1-specific Th1 response was induced over a Th2 response in Cry j 1/OML-treated mice, even after Cry j 1-sensitization. This is supported by the cytokine production from spleen cells of Cry j 1-sensitized mice in response to *in vitro* stimulation with Cry j 1. Spleenocytes from Cry j 1/OML-treated mice secreted much more IFN- γ than those from

control mice in response to *in vitro* stimulation with the allergen, indicating that allergen-specific Th1 cells were induced in the treated mice. The IL4/IFN- γ and IL-5/IFN- γ ratios for cytokines secreted from spleen cells, which reflect the Th1/Th2 balance [20,21], were also lower in the Cry j 1/OML-treated mice than in PBS- and Cry j 1/alum-treated mice. Since the level of IgE is positively regulated by Th2 cytokines [19], repression of serum IgE elevation in Cry j 1/OML-treated mice may be due to Th1-skewing of the immune response.

It has been shown that allergen-specific IL-10 producing cells are present at a higher frequency in nonallergic individuals compared to allergic subjects [22]. The principle function of IL-10 is to limit the magnitude of immune responses. In addition, IL-10 promotes the differentiation of regulatory T cells (Treg) [23], which play a key role in preventing immune responses to harmless aeroantigens [24]. Treg produce IL-10 and suppress production of IgE and Th2 cells [25]. Since splenocytes from Cry j 1/OML-treated mice produced much more IL-10 than those from control mice in response to *in vitro* stimulation with Cry j 1, antigen-specific Treg may be more effectively induced in Cry j 1/OML-treated mice. Since immune responses in healthy and allergic individuals are a fine balance between allergen-specific Treg and Th2 [23,25], the induction of Treg may also contribute to the anti-allergic effect of the OMLs. Direct evidence for induction of allergen-specific Treg by Cry j 1/OMLs is required for further understanding of the anti-allergic potential of OMLs.

Chemically modified or mutant recombinant allergens and synthetic peptides of T-cell epitopes have been evaluated as immunotherapeutic agents for allergic diseases [20,26,27], because these derivatives may be safer than the native allergens. Repeated subcutaneous administration of Cry-consensus peptide inhibited the production of anti-Cry j 1 IgE and significantly enhanced anti-Cry j 1 IgG2a [20,21]. However, a higher dose (1 mg/mouse) of the peptide was required to suppress IgE levels and to induce a Th1 immune response. Our study demonstrated that administration of Cry j 1 at only 1 µg/mouse could control IgE elevation in sera and induce Th1 immune responses when the allergen was entrapped in OMLs. In contrast, the same dose of Cry j 1 accelerated elevation of IgE in sera and Th2-skewing of the immune response when the allergen was adsorbed onto alum. We have previously examined the required amount of antigens in OMLs for induction of cellular immune responses, and shown that the immune response increased in a dose-dependent manner and that administration of 1 µg of antigen/OMLs per mouse is sufficient to induce a maximal cellular immune response [12]. Therefore, administration of a much lower allergen dose in OMLs may cause IgE elevation, although experiments on the dose dependency were not carried out in this study. OMLs consist of innocuous materials that are distributed ubiquitously in vertebrates and cause no damage to the skin at the injection site [28]. Therefore, OMLs have advantages for immunotherapy as a safe vaccine vehicle and can induce Th1 immune responses specific for an entrapped allergen, even at a low allergen dose.

In conclusion, our results show that immunization with OMLs with entrapped Cry j 1 inhibits the IgE elevation elicited by Cry j 1 administration in both unsensitized mice and Cry j 1-presensitized mice. This inhibitory effect might occur through a shift from a Th2 immune response to an allergen-specific Th1 immune response. Therefore, OML-encased allergens may serve as immunotherapeutic agents to control type I allergic diseases, including Cj pollinosis. Further evaluation of the physiological and therapeutic potential of OMLs requires histological and clinical studies in an *in vivo* disease model in animals. Clarification of whether a long-term memory response can be induced by Cry j 1/OMLs is also required to use OMLs as immunotherapeutic agents, although we showed that the anti-allergic effect of Cry j 1/OMLs persists for at least 6 weeks after the first immunization. Cj pollinosis is also a serious concern in dogs [29] and treatment with an OML-encased allergen may also be useful for control of allergies to Cj pollen in veterinary medicine, as well as in human disease.

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