Major royal jelly protein 3 modulates immune responses in vitro and in vivo

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Abstract

We have recently shown that royal jelly has potent antiallergic properties in a mouse model of immediate hypersensitivity. However, it is still unclear which components of royal jelly exhibit antiallergic activity. In this study, we have screened for antiallergic factors in royal jelly based on inhibition of IL-4 production by anti-CD3 stimulated spleen cells derived from OVA/alum-immunized mice. Using a series of column chromatographies, we purified a 70 kDa glycoprotein, major royal jelly protein 3 (MRJP3), that suppresses IL-4 production. In in vitro experiments, MRJP3 suppressed the production of not only IL-4 but also that of IL-2 and IFN-γ by T cells concomitant with inhibition of proliferation. The MRJP3-mediated suppression of IL-4 production was also evident when lymph node cells from OVA/alum-immunized mice were stimulated with OVA plus antigen presenting cells. We next examined the purified suppressive factor on OVA/alum-induced allergic responses in mice. Interestingly, in spite of the antigenicity of MRJP3 itself as an extraneous foreign protein, intraperitoneal administration of MRJP3 inhibited serum anti-OVA IgE and IgG1 levels in immunized mice. In addition, heat-treated soluble MRJP3 treatment reduced its antigenicity while maintaining its inhibitory effects on antibody responses to OVA. These results indicate that MRJP3 can exhibit potent immunoregulatory effects in vitro and in vivo. Furthermore, considering the intriguing immunomodulatory effects of MRJP3, it may be of clinical significance to design MRJP3-derived antiallergic peptides by identifying the associated polypeptide regions.

Keywords: IL-4; IgE; Royal jelly protein; Antiallergy; Antigenicity

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Introduction

Royal jelly (RJ) is secreted from the cephalic glands of worker honeybees (Apis mellifera L), and directs the development of honeybee larvae into queen bees (Brouwers et al., 1987). RJ is composed of proteins (12–15%), sugars (10–16%), lipids (3–6%), vitamins, and free amino acids, and has been used for medical and nutritional purposes in folk medicine (Howe et al., 1985). Recently, five types of major royal jelly proteins (MRJPs; MRJP1-5) have been characterized by cDNA cloning and sequencing (Albert et al., 1999; Klaudiny et al., 1994; Schmitzova et al., 1998). It was found that MRJPs belong to one protein family (60–70% amino acid sequence homology between the proteins) and account for over 80% of the proteins present in RJ.

The biological functions of some components of RJ have been described. The antibacterial activities of the 5.5 kDa protein royalisin, and trans-10-hydroxy-Δ2-decenoic acid found in RJ have been demonstrated against various Gram-positive and Gram-negative bacteria, respectively (Fujiwara et al., 1990; Genc and Aslan, 1999). These two antibacterial components are thought to contribute to host defenses in honeybees. One of the MRJPs, MRJP1, which exhibits a molecular mass of 57 kDa as a monomer or 350 kDa as a hexamer after gel-filtration chromatography, promoted the viability and proliferation of primary cultured rat hepatocytes with ED50 of 100 μg/ml (Kamakura et al., 2001; Kimura et al., 1995). In addition, peptides derived from MRJP1 as a result of gastrointestinal enzyme hydrolysis, possessed potent angiotensin I-converting enzyme inhibitory activity in the spontaneously hypertensive rat (Matui et al., 2002). Furthermore, others and ourselves have demonstrated that a soluble fraction of RJ shows antiallergic activities, including reduced antigen-specific IgE levels in the sera of allergic mice, although the substance(s) in RJ showing this activity remains unknown (Kataoka et al., 2001; Oka et al., 2001).

Th2 cells are immunologically the dominant cell type associated with several notable allergic reactions (Romagnani, 2000). Th2 cells secret interleukin-4 (IL-4), causing IgE class switching in B cells (Barner et al., 1998; Fallon et al., 2002; Swain et al., 1990). It has been reported that OVA/alum-sensitized allergic mice harbor a systemic Th2 response characterized by elevated production of Th2 cytokines and antigen-specific IgE synthesis (Brewer et al., 1996, 1999). We have examined here which component in RJ has potent antiallergic activity, and have identified MRJP3 as a factor that inhibits T cell-derived cytokine production in vitro. Furthermore, we have found that MRJP3 shows novel immunomodulatory effects in vivo.

Material and Methods

Mice and ovalbumin (OVA)/alum sensitization

Female BALB/c mice, age 6 to 8 wk, were purchased from Japan Charles River (Kanagawa, Japan). These mice received an intraperitoneal injection of 2 μg of OVA (Sigma, St. Louis, MO) with 3 mg of alum (Pierce, Rockford, IL) in phosphate buffered saline (PBS), three times at 7-day intervals.

Purification of CD4+ T cells

Purified CD4+ T cells were isolated from spleen cell preparations derived from OVA/alum-immunized mice as described previously (Okamoto et al., 2001). The purity of the fractionated CD4+ T cells was
confirmed to be >93% by flow cytometric analysis on an EPICS XL SYSTEM II (Coulter Electronics, Hialeah, FL) using rat anti-mouse CD4 mAb and FITC-conjugated F(ab')2 fragments of goat anti-rat IgG (Cedarlane, Ontario, Canada).

Cytokine production in vitro

Spleens were removed from the OVA/alum-immunized mice. Single-cell suspensions were prepared in RPMI 1640 medium containing 10% FCS and 5 × 10\(^{-5}\) M 2-ME. The spleen cells (5 × 10\(^5\) cells/well) or purified CD4\(^+\) T cells (2 × 10\(^5\) cells/well) were incubated for 40 h at 37 °C with RJ-derived samples or control PBS on culture plates pre-coated with anti-CD3 mAb (5 μg/ml)(Cedarlane Laboratories Limited, Ontario, Canada). The supernatants were harvested for the determination of the cytokine levels using ELISA. Proliferation of the spleen cells and T cells was also measured after 40 h of culture as described below. In some experiments, lymph node cells (5 × 10\(^5\) cells/well) from OVA/alum-immunized mice were stimulated for 72 h with OVA (200 μg/ml) plus 50 μg/ml of mitomycin C (Wako Pure Chemical Industries, Osaka, Japan)-treated spleen cells (6 × 10\(^6\) cells/well), from normal BALB/c mice.

Neutralization of MRJP3 activity in vitro

A monoclonal Ab (mAb), which reacts specifically with MRJP3, was produced in our laboratories from a hybridoma cell line generated from splenocytes of a BALB/c mouse hyperimmunized with purified MRJP3. Cell culture supernatants from hybridoma colonies were screened by ELISA against MRJP3, and hybridoma cells from positive wells were subjected to cloning twice. Anti-MRJP3 mAbs (#7–9D, IgG1) were purified according to standard methods (Harlow and Lane, 1998).

MRJP3 (125 μg/ml) was pre-incubated with anti-MRJP3 mAb or isotype-matched control Ab (150 μg/ml, anti-human IFN-α mAb, which was prepared in our laboratories) for 1 h at 4 °C. Spleen cells (5 × 10\(^5\) cells/well) from OVA/alum-immunized mice were stimulated with immobilized anti-CD3 mAb (5 μg/ml) in the presence of the mixtures of MRJP3 and anti-MRJP3 mAb, or MRJP3 and control mAb for 40 h at 37 °C. Culture supernatants were collected for IL-4 ELISA.

Cell proliferation and viability

Cell proliferation was measured using alamarBlue™ dye (Trek Diagnostic Systems, OH), a redox indicator, according to the manufacturer’s instructions. Fluorescence Intensity (FI) was measured at an excitation wavelength of 544 nm and an emission wavelength of 590 nm. Trypan blue exclusion was used to determine the viabilities of the cultured cells by counting the number of dead (stained) and live cells.

Purification of natural MRJP3

Five grams wet weight of fresh RJ were suspended in 100 ml of 200 mM Tris–HCl buffer, pH 8.0 (buffer A), and dialyzed against 1-liter of buffer A at 4 °C. The supernatant of the RJ suspension was collected by centrifugation at 10,000 × g for 15 min at 4 °C, and was passed through a 0.22 μm filter unit. The RJ supernatant was loaded on a DEAE-5PW column (54.4 ml gel) (Tosoh Co., Tokyo,
Japan) equilibrated with buffer A. The IL-4 production suppressive activity was eluted with a linear gradient of 0 to 0.3 M NaCl in 1088 ml. The pooled active fractions were diluted two-fold with buffer A and applied to a Resource Q column (6 ml gel) (Amersham Bioscience, Piscataway, NJ) equilibrated with buffer A. The elution was carried out with a linear gradient from 0 to 0.5 M NaCl in 120 ml. The eluted active fractions were pooled and loaded on to a Heparin-5PW column (3.3 ml gel) (Tosoh Co.) equilibrated with buffer A. The active fractions were eluted with a linear gradient of 0 to 1 M NaCl in 49.5 ml. The pooled active fractions were then concentrated to a volume of 2 ml (about 10-fold) by Ultrafree 5 (Millipore, Bedford, MA). The sample obtained was applied onto a Superdex 200 gel-filtration column (16 × 60 cm) (Amersham Bioscience) equilibrated with 1.5 times concentrated PBS buffer. Column calibration was performed using thyroglobulin, ferritin, aldolase, albumin, and ovalubumin. After a series of column chromatographies, the purity of MRJP3 fractionated at a molecular mass of 70 kDa was >97% as determined by SDS-PAGE under reducing conditions followed by estimation on an Image Master scanner (Amersham Biosciences). Protein concentration was determined by the Bradford assay (Bio-Rad, Richmond, CA), using human serum albumin as a standard. The level of endotoxin in the purified MRJP3 was always less than 2 pg/µg protein, as determined by the Limulus Amebocyte assay (Seikagakukogyo, Tokyo, Japan). N-terminal amino acid sequence analysis was performed using an Applied Biosystems model 477A protein sequencer after SDS-PAGE, and electrotransfer of the proteins to Immobilon-P polyvinylidene difluoride membrane (Millipore).

SDS-PAGE

The protein composition of various fractions was estimated by SDS-PAGE (4–20% or 10% gels) (Daiichi pure chemicals, Tokyo, Japan) according to the method of Laemmli (Laemmli, 1970). Protein bands were detected after staining with Coomassie Brilliant Blue R-250. The percentage purity of MRJP3 protein was estimated by densitometric scanning.

MRJP3 administration

MRJP3 (0.5, 5, 50 µg/mouse, n = 5) was administered intraperitoneally two days before the first and again six hours before each OVA/alum-immunization. In some cases, MRJP3 heated for 20 min at 100 °C or digested with pronase was administered according to the same protocol. Control group mice were injected with PBS. The supernatant of a whole RJ suspension (corresponding to 50 µg of raw RJ) was administered as a positive control. Mouse sera and spleens were collected 7 days after the last OVA/alum immunization.

Measurement of serum Igs and cytokines

Serum OVA-specific IgG1 and IgE levels were determined by ELISA. For this purpose, 96-well plates were coated with OVA (5 µg/well) for 2 h at 37 °C. The standards for the OVA-specific IgG1 and IgE ELISA were prepared from the sera of mice immunized with OVA that had been tentatively quantified for IgG1 (128,000 U/ml) and IgE (640 U/ml), respectively. Serum IgG1 specific to natural and heat-treated MRJP3 was also determined by ELISA. Microtiter wells were coated with natural or heat-treated MRJP3 for 2 h at 37 °C. Biotinylated anti-mouse IgG1 and biotinylated anti-mouse IgE Abs were
purchased from BD PharMingen (San Diego, CA). In some experiments, mouse IgG1 (S1-68.1, BD PharMingen) and mouse IgE (IgE-3, BD PharMingen) were used as standards.

Cytokine standards and mAbs used for the cytokine (IL-2, IL-4, and IFN-\(\gamma\)) ELISAs were obtained from BD PharMingen. The mouse IFN-\(\gamma\) standard and rabbit anti-mouse IFN-\(\gamma\) pAb were prepared at our laboratories (Okamoto et al., 1999). The lower limits of detection, as determined using standard titration curves, were as follows: for IL-2, 25 pg/ml; IL-4, 12.5 pg/ml; IFN-\(\gamma\), 0.2 IU/ml.

**Enzymatic digestion**

Purified MRJP3 (1 mg/ml) was incubated with pronase (50 \(\mu\)g/ml) at 37 °C for 4 h, and then the mixture was heated for 5 min at 100 °C to inactivate the enzyme. The extent of digestion was determined by SDS-PAGE. N-deglycosylation of MRJP3 (20 \(\mu\)g) was performed by boiling in the presence of 0.4% SDS and 5 mM DTT, and then the denatured MRJP3 was incubated with 0.6 units of peptide N-glycosidase F (Boehringer Mannheim GmbH, Germany) in 10 mM sodium phosphate buffer (pH 6.0) containing 1% Nonidet P-40 for 18 h at 30 °C. To confirm the removal of the N-linked oligosaccharide chain, the reaction samples were subjected to SDS-PAGE under reducing conditions. To determine the internal peptide sequences of MRJP3, peptides obtained by trypsin digestion were separated by HPLC on a reverse-phase C18 column (Vydac, Hesperia, CA) at a flow rate of 0.8 ml/min with a linear gradient of 0 to 100% CH\(_3\)CN containing 0.1% trifluoroacetate. The isolated peptide fragments were then subjected to protein sequence analysis.

**Results**

**Purification of IL-4 production-suppressive factor from RJ**

As demonstrated in our previous and present studies, the PBS-soluble fractions of RJ suspensions show antiallergic activity, such as a down-regulation of anti-OVA IgE levels in OVA/alum-immunized mice (Kataoka et al., 2001). We therefore attempted to isolate the responsible antiallergic substance(s) from the supernatants of RJ suspensions. It has been shown that the cytokine IL-4 is required for the proliferation and differentiation of Th2 cells, and for the production of IgE antibodies, both of which events are associated with a number of allergic responses (Romagnani, 2000; Fallon et al., 2002; Swain et al., 1990; Barner et al., 1998). Spleen cells from OVA/alum-immunized BALB/c mice, but not those from normal mice, produced detectable levels of IL-4 in response to stimulation with anti-CD3 mAb (data not shown). Thus, we have screened for the antiallergic activity present in RJ suspensions by searching for an activity that suppresses IL-4 production by anti-CD3-stimulated spleen cells obtained from OVA/alum-immunized mice.

First, fresh RJ was suspended in Tris–HCl buffer and supernatants of the suspension were collected. The supernatants were loaded onto a DEAE-5PW ion-exchange column against a NaCl gradient (Fig. 1A). The major IL-4 production-suppressive activity eluted at about 0.1 M NaCl as a single peak. The active fractions were pooled and were loaded onto a Resource Q ion-exchange column to concentrate the activity (Fig. 1B). A strong IL-4 production-suppressive activity eluted at 0.1 M NaCl as in DEAE-5PW chromatography. The elution pattern of the suppressive activity coincided with a wavelength reading at both 214 and 280 nm, suggesting that the desired factors showing IL-4 production-suppressive activity
Fig. 1. Purification of MRJP3 as a IL-4 production-suppressive factor from RJ suspension. (A) DEAE-5PW HPLC of the supernatant obtained from a suspension of RJ corresponding to 5 g of the raw material. The activity was eluted with a linear gradient of NaCl from 0 to 0.3 M. IL-4 production by spleen cells stimulated with anti-CD3 mAb is expressed relative to that of cells stimulated with control PBS buffer. (B) Resource Q HPLC of the pooled active fractions from DEAE-5PW. (C) Heparin-5PW HPLC of the pooled active fractions from Resource Q. The activity was eluted with a linear gradient of NaCl from 0 to 0.5 M. (D) Superdex 200 gel-filtration chromatography of the pooled active fractions from Heparin-5PW. The column was eluted at a flow rate of 0.9 ml/min with 1.5 times concentration of PBS buffer. Control levels (100%) of IL-4 production in A, B, and C were 720 pg/ml, 693 pg/ml, and 762 pg/ml, respectively.
was protein in nature. Next, the active fractions were loaded onto a Heparin-5PW column (Fig. 1C). The suppressive activity eluted at 0.2 M (minor fraction) and 0.4 M (major fraction) NaCl. Because the specific activity of the major active fractions was four times higher than that of minor active fractions (data not shown), only the major active fractions were collected. The minor active fractions were found to be derived from a 55 kDa protein (>95% purity) that has an identical N-terminal amino acid sequence with that of major royal jelly protein 3 (MRJP3) described below. The major active fractions isolated by Heparin-5PW column chromatography were then concentrated and loaded onto a Superdex 200 gel-filtration column (Fig. 1D). Small amounts of protein aggregates were observed in the void fraction. The aggregate was found to be derived from the 70 kDa MRJP3 described below as determined by N-terminal amino acid analysis. The activity eluted with the pooled major 70 kDa fraction and with the pooled negligible 210 kDa minor fraction as calculated from the elution profile of protein standards (Fig. 1D).

Reducing SDS-PAGE analysis of both active fractions (70 and 210 kDa) obtained after Superdex 200 column chromatography indicated a 70 kDa single band (>97% purity). The 70 kDa fractions showed the presence not only of the 70 kDa band, but also a certain amount of two approximately 210 kDa bands under non-reducing conditions of SDS-PAGE (Fig. 2A). Regarding SDS-PAGE analysis of the 210 kDa fraction, both the 70 and 210 kDa bands were also observed under non-reducing conditions (data not shown). The N-terminal amino acid sequences of the 70 and 210 kDa proteins were homologous and determined to be AAVNHQRKSA that perfectly matched that of MRJP3 (positions 1–10) (Schmitzova et al., 1998; Albert et al., 1999). Thus, we considered that the 210 kDa protein consists of a disulfide-bonded or nondisulfide-bonded trimer of the 70 kDa MRJP3 molecule. In addition to the molecular mass and N-terminal amino acid sequence, the amino acid

Fig. 2. SDS-PAGE analysis of purified MRJP3 glycoprotein. (A) Reducing and non-reducing SDS-PAGE (4–20% gels) analysis of purified MRJP3. Lanes 1 and 2 were analyzed under reducing and non-reducing conditions, respectively. (B) Purified natural MRJP3 was incubated with or without peptide N-glycosidase F for 18 h at 30 °C. The deglycosylated proteins were separated by SDS-PAGE in 4–20% gradient gels under reducing conditions and stained with Coomassie Brilliant Blue. Lanes 3 and 4 are untreated MRJP3 and peptide N-glycosidase F-treated MRJP3, respectively.
sequences of two internal peptides obtained by trypsin digestion of the 70 kDa molecule were analyzed and found to be LTVGESFT (positions 223–231) and YHNQNA\(\text{G}\)NQN (positions 402–411, within the repetitive regions of a QNXX motif) of the MRJP3 protein (Schmitzova et al., 1998; Albert et al., 1999). These results further support the above conclusion that the 70 kDa protein is MRJP3. Although both the 70 and 210 kDa fractions showed IL-4 production-suppressive activity, the specific activity of the 210 kDa minor fraction was comparable to or slightly higher than that of the 70 kDa fraction (data not shown). Since it was considered difficult to obtain only the 70 kDa protein because of its tendency to form the 210 kDa trimer, we used the major active fractions with a molecular mass of 70 kDa as purified MRJP3 in the following experiments. Finally, after the series of column chromatographies described earlier, 30 mg of purified MRJP3 was obtained after starting from 1057 mg of PBS-soluble proteins (corresponding to 25 g of raw RJ), as summarized in Table 1.

Judging from the putative amino acid sequence of MRJP3, it has been suggested that MRJP3 may contain one N-glycosylation site in its structure (Klaudiny et al., 1994; Schmitzova et al., 1998). As expected, purified MRJP3 treated with peptide N-glycosidase F migrated as a 66-kDa band on SDS-PAGE under reducing conditions, indicating that MRJP3 is a glycoprotein with a molecular mass of 70 kDa (Fig. 2B).

**Biological activities of MRJP3 in vitro**

Purified MRJP3 suppressed IL-4 production by anti-CD3 mAb-stimulated spleen cells in a dose-dependent manner with ED\(_{50}\) of 50–100 \(\mu\)g/ml (Fig. 3A). The MRJP3-mediated inhibitory activity was almost completely lost after treatment with pronase (Fig. 3B). The activity was, however, unchanged by boiling for 5 min. Boiling for over 20 min was required for complete inactivation, but even after boiling, MRJP3 remained soluble, suggesting that MRJP3 is a heat-stable protein compared with other common proteins (data not shown). In addition, the MRJP3-mediated inhibitory activity on IL-4 production was completely neutralized by addition of anti-MRJP3 mAb (#7–9D) (Fig. 3C). Suppression of proliferative responses by MRJP3 was also abrogated by the addition of mAb #7–9D to the cultures (data not shown). These results together with the findings that the mAb #7–9D specifically recognizes the 70 kDa MRJP3 band on Western blotting analysis (data not shown).

<table>
<thead>
<tr>
<th>Purification steps</th>
<th>Volume, ml</th>
<th>Total protein, mg</th>
<th>MRJP3 monomer content(^a), %</th>
<th>Total MRJP3 monomer, mg</th>
<th>Yield, %</th>
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<tr>
<td>RJ suspension sup</td>
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<td>1057</td>
<td>6.2</td>
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<td>100</td>
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<tr>
<td>DEAE-5PW pooled fraction</td>
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<td>39.9</td>
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<td>42.2</td>
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<td>52</td>
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<tr>
<td>Heparin-5PW pooled fraction</td>
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<td>38</td>
<td>67.5</td>
<td>26</td>
<td>39</td>
</tr>
<tr>
<td>Superdex 200 pooled fraction</td>
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<td>30</td>
<td>&gt;80.0(^b)</td>
<td>24</td>
<td>36</td>
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</tbody>
</table>

\(^a\) MRJP3 monomer content was estimated by densitometer scanner after SDS-PAGE under non-reducing conditions.

\(^b\) Purity of MRJP3 was >97% under reducing SDS-PAGE conditions.
shown) confirm that MRJP3 is the molecule responsible for the IL-4 production-suppressive activity present in RJ.

We next investigated the effects of MRJP3 on in vitro cytokine production by lymph node cells from OVA/alum-immunized mice upon stimulation with OVA plus antigen presenting cells (APCs). As a result, MRJP3 significantly reduced IL-4 production under these physiologically-relevant stimulatory conditions, but the level of inhibition at 200 μg/ml of MRJP3 was about 30%, which is lower than that observed with anti-CD3 stimulation (about 80%) (Fig. 3A and D). This result suggests that MRJP3 suppresses IL-4 production regardless of the T-cell stimulus. Furthermore, this finding suggests that different routes of T cell stimulation may affect the degree of inhibitory activity by MRJP3.
MRJP3 acts directly on T cells and suppresses the production of several cytokines

We next examined the effects of MRJP3 on the production of other cytokines besides IL-4, and investigated what cells are targeted by MRJP3 in vitro. For this purpose, CD4⁺ T cells were purified from spleen cells of OVA/alum-immunized mice and were stimulated with immobilized anti-CD3 mAb in the presence or absence of MRJP3. As shown in Fig. 4A, MRJP3 down-regulated IL-2, IL-4, and IFN-γ production to a similar extent, in association with the suppression of CD4⁺ T cell proliferative responses. As shown in Fig. 4B, the down-regulation of T cell proliferation induced by MRJP3 at the doses used did not seem to be due to direct cytotoxicity on T cells because the cell viability was unaffected by the presence of MRJP3. Thus, the MRJP3-mediated suppressive effect on T cell proliferation was likely to reflect decreased levels of the growth factors, IL-2 and IL-4, required for T cell proliferation. These results indicate that MRJP3 directly acts on T cells without being cytotoxic.

![Fig. 4. Cytokine production suppressive activity of purified MRJP3 without any cytotoxicity.](image)

(A) Purified CD4⁺ T cells (2 × 10⁵ cell/well) from OVA/alum-immunized mice were stimulated with plate-bound anti-CD3 mAb (5 μg/ml) in the presence of MRJP3. After 40 h culture, IL-2, IL-4, and IFN-γ in the supernatants were measured by ELISA. (B) At the time that the culture supernatants were collected, proliferative responses were measured using alamarBlue™ dye and cell viability was determined by trypan blue exclusion. Results are expressed as mean ± SD of triplicate cultures. Data are representative of two separate experiments. *, Significantly different, p < 0.05 compared with control PBS (Student’s t test).

Fig. 5. Effects of MRJP3 administration on allergic reactions in OVA/alum-immunized mice. BALB/c mice received an intraperitoneal injection of OVA with alum three times at 7-day intervals. MRJP3 (0.5, 5, 50 μg/mouse, n = 5) was administered intraperitoneally two days before and six hours before each OVA/alum-sensitization, as described in Materials and Methods. Control group mice were injected with PBS. As a control for antiallergic effects, the supernatant of an RJ suspension (corresponding to 50 μg of raw RJ) was injected. Seven days after the final OVA/alum-sensitization, serum was collected. Serum anti-OVA IgE (A), anti-OVA IgG1 (B), and anti-MRJP3 IgG1 (C) levels were determined by ELISA. 5 μg/body (n = 5) of natural MRJP3 (no treatment), heat-treated soluble MRJP3 (heat), or pronase-treated MRJP3 (pronase) were administered according to the same protocol described above (D). In the inactivated-pronase administered group (pronase control group), there were no significant differences when compared with the PBS control group (data not shown). Serum anti-OVA IgE (D, top), anti-MRJP3 IgG1 (D, middle) and total IgG1 (D, bottom) levels were determined by ELISA. Each point in the graphs represents an individual mouse. The horizontal bar denotes the average within each group. NT, not tested. *, Significantly different, p < 0.05 compared with the control group treated with PBS (Mann-Whitney U test).
The effect of MRJP3 administration on IgE and IgG1 production in a mouse model of allergy

The effect of MRJP3 on IgE and IgG1 production in vivo was examined. For this purpose, BALB/c mice were immunized three times (weekly) with OVA in alum adjuvant (OVA/alum). These mice are well known to exhibit up-regulation of Th2 cytokines, and serum anti-OVA IgG1 and IgE levels. Three doses of MRJP3 (0.5, 5, 50 μg/mouse) were injected six times intraperitoneally on day 2 before and on the day of each immunization with OVA/alum. All mice in each group showed neither body weight loss
nor were there any deaths throughout the experiment (data not shown). In agreement with the previous report, supernatants of RJ suspension significantly reduced serum OVA-specific anti-IgE and IgG1 levels (Fig. 5A and B). MRJP3 (5 and 50 \( \mu \)g/mouse) significantly suppressed serum OVA-specific anti-IgE levels dose-dependently (Fig. 5A). MRJP3 administration also clearly reduced anti-OVA IgG1 levels in the serum (Fig. 5B) \((p < 0.05)\). These results suggest that MRJP3 administration suppresses antibody responses against OVA antigen in vivo.

Because natural MRJP3 produced by the honey bee is a protein foreign to mice, it can easily be speculated that intraperitoneal injection of MRJP3 into mice would cause antibody production against MRJP3 itself. As expected, the administration of MRJP3 induced high amounts of anti-MRJP3 IgG1 Ab in a dose-dependent manner (Fig. 5C). It is of note that MRJP3 reduced antibody production (IgE and IgG1) against another allergen, OVA, which was injected with alum adjuvant, regardless of the antibody production against MRJP3 itself. In other words, MRJP3 administration exhibited both an antiallergic action against OVA and antigenicity in OVA/alum-immunized mice.

If the peptide regions on MRJP3 responsible for its antiallergic action and those responsible for its antigenicity are different, a partial conformational change in MRJP3 caused by heating may modify its antigenicity and/or its antiallergic activities (Davis and Williams, 1998; Baur et al., 1996). To investigate this possibility, we administered heat-treated MRJP3 (5 \( \mu \)g/mouse) intraperitoneally into OVA/alum-immunized mice. MRJP3 treated for 20 min at 100 °C did not form any insoluble aggregate. In addition, compared with natural MRJP3, heat-treated MRJP3 had a 5 to 6-fold reduced OD as determined by direct ELISA using anti-natural MRJP3 IgGs, probably because of the partial conformational change in heat-treated MRJP3 (data not shown). Surprisingly, heat-treated MRJP3 administration significantly suppressed anti-OVA IgE levels in immunized mice comparable with the activity observed in the untreated MRJP3 group. In contrast, the administration of the heat-treated MRJP3 reduced to one-fifth the serum titer of anti-MRJP3 IgG1 compared with that in the untreated MRJP3 group (Fig. 5D top and middle). In addition, total IgG1 levels were also markedly down-regulated in the heat-treated MRJP3 group to levels comparable to those in the PBS control group (Fig. 5D bottom). By comparison with the pronase-treated MRJP3 group, we confirmed that antiallergic activities were restricted to undigested MRJP3 proteins (natural and heat-treated MRJP3), but not to the pronase-digested MRJP3 (Fig. 5D). These findings suggest that the peptide regions responsible for the antiallergic action on natural MRJP3 may be different from those associated with antigenicity. Thus, identifying the regions showing antiallergic activities present in MRJP3 polypeptides may lead to designing a clinically useful peptide that exhibits antiallergic activity with considerably reduced antigenicity.

**Discussion**

MRJP3 cDNA has been cloned as a member of the major protein family in RJ (Klaudiny et al., 1994). The C-terminal region of the MRJP3 protein has extensive repetitive regions consisting of XQNXX pentapeptides, as we also confirmed by the internal amino acid sequence analysis (Albert et al., 1999; Schmitzova et al., 1998). However, the biological functions and potential applications of MRJP3 remain undefined. In this study, we have purified natural MRJP3 from RJ based on its activity inhibiting in vitro cytokine production. Furthermore, using an allergic mouse model, we have described antiallergic activities for MRJP3 in vivo.
MRJP3 markedly inhibited IL-2, IL-4, and IFN-γ production by anti-CD3 mAb-stimulated purified splenic T cells as well as whole spleen cells, while inhibitory effects of MRJP3 on cytokine production by lymph node cells in response to OVA plus APCs were also observed, albeit to a lesser extent. It has been reported that the TCR/CD3-mediated activation signal provided by anti-CD3 mAb may be qualitatively distinct from that provided by antigen plus APCs (Constant et al., 1995). In fact, APC could play additional roles as a source of cytokines and costimulatory signals (Lenschow et al., 1996; Ohshima and Delespesse, 1997). These results suggest that MRJP3 may inhibit stimulatory signals through the TCR/CD3 complex, but not through TCR/CD3-independent pathway. In this regard, the suppressive activity of MRJP3 resembles that of nonmitogenic CD3 antibody that shows protective effects in T cell-mediated disease, such as graft-versus-host disease and autoimmune disease by blocking stimulatory signals generated from the TCR/CD3 complex (Von Herrath et al., 2002).

It has recently been reported that certain high-mannose oligosaccharides can bind to IL-2 and subsequently inhibit IL-2 responses (Fukushima et al., 2001). MRJP3 also contains a N-linked oligosaccharide, as demonstrated in the present study. It is possible that the oligosaccharide on MRJP3 may be of the high-mannose type, which is often produced by insect cells. However, the mechanism underlying the inhibitory effects of MRJP3 on T cell responses seems to be different from those mediated by high-mannose type oligosaccharides because of the following two reasons. First, pronase digestion and heat denaturation of MRJP3 almost completely abrogated its activity in vitro. The oligosaccharide structure is usually stable despite these treatments. Second, the pattern of suppression of IL-4 production by T cells from OVA/alum-sensitized mice was not always correlated with that of IL-2, but rather correlated well with that of T cell proliferation (data not shown). In addition, it has not been demonstrated so far that the high-mannose oligosaccharide inhibits IL-4 production. These observations are consistent with the previous report that IL-2 is not required for IL-4 production by primed T cells from OVA/alum-immunized mice (Yang and HayGlass, 1993). Taking these results together, we consider that MRJP3 protein but not the oligosaccharide on MRJP3, contributes to the cytokine production-suppressive activity exhibited by MRJP3.

To examine whether MRJP3-mediated suppression of cytokine production is due to the consequence of reduced T cell proliferation, in our preliminary experiments, we have added exogenous IL-2 (250–1000 pg/ml, comparable amounts to those produced endogenously) into the spleen cell cultures in the presence or absence of MRJP3. As a result, exogenous IL-2 did not up-regulate the proliferative response regardless of the presence of MRJP3, although the exogenous IL-2 slightly increased the production of IL-4 (20% increase). The failure of exogenous IL-2 to stimulate the proliferative response suggests that T cells from OVA/alum-immunized mice may require little or no IL-2 for their proliferation when both IL-2 and IL-4 are present in the cultures. This also led us to speculate that endogenously produced IL-4 may be mainly used as a growth factor for those T cells. This speculation was supported by other studies and our observations that proliferative response of T cells from OVA/alum-immunized mice was closely correlated with the levels of IL-4 but not IL-2 in the cultures, as mentioned above (Yang and HayGlass, 1993). We were therefore interested to see if exogenous IL-4 overcomes the MRJP3-induced suppression of proliferative response and IL-2 production. As expected, addition of exogenous IL-4 (250–1000 pg/ml, comparable amounts to those produced endogenously) to the spleen cell cultures dose-dependently enhanced both proliferative responses and IL-2 and IFN-γ production in the absence of MRJP3, and fully restored the MRJP3-induced reduction in proliferation and cytokine production to the levels comparable to those observed in the absence of MRJP3. These results suggest that MRJP3 inhibits IL-4 production from T cells, resulting in the inhibition of the Th2 cell proliferation.
and subsequent suppression of several cytokine productions. Together, these findings support the view that MRJP3 exhibits antiallergic activities via inhibition of IL-4 production. Intrapertitoneal injection of MRJP3 into OVA/alum-immunized mice suppressed serum anti-OVA allergen specific IgE and IgG1 levels, and simultaneously up-regulated anti-MRJP3 IgG1 and total IgG1 levels (Fig. 5A–C). These results were surprising and interesting, because the antibody response against OVA was inhibited in spite of the fact that both OVA and MRJP3 are antigenic foreign proteins. Thus, these MRJP3-mediated bystander effects are different from a specific down-modulation of the immune response to an allergen, which is observed after administration of the allergen (allergen-specific immunotherapy), such as treatment with phospholipase A2 (the major allergen in bee venom) for bee venom (Ebner, 1999; Ferreira et al., 2002; Jutel et al., 1995). A similar bystander immunomodulation was observed by Von Garnier et al. (Von Garnier et al., 2002). They showed that the specific IgE response to OVA was down-regulated by intraperitoneal injection of phospholipase A2. Thus, the intensity of certain allergic responses might be interfered with bystander effects of coimmunization with an unrelated antigen. At present, although we cannot clarify the mechanism underlying these MRJP3-mediated novel biphasic immunomodulatory effects on antibody production (antigenic and antiallergic), the specific suppression of anti-OVA antibody production by MRJP3 in mice cannot be explained solely by a comprehensive inhibition of Th2 cytokine production by T cells as shown in vitro experiments. In fact, as well as IL-4, MRJP3 inhibited the production of Th1 cytokines by T cells in vitro. Furthermore, heat-treated MRJP3 lost its inhibitory activity on IL-4 production in vitro, while it still exhibited antiallergic activity in vivo. We may need to examine the effects of MRJP3-mediated antiallergic functions in mice immunized with alum plus allergens other than OVA. It is also necessary to investigate other immune responses modulated by MRJP3, such as antibody production by B cells.

Our data showed that considerable differences exist in the effective doses of MRJP3 between in vitro and in vivo. A possible explanation for this result could be the difference in the Th2 differentiation states of the responding T cells. In in vitro cytokine production experiments, we have used splenocytes or lymph node cells isolated from mice that had been immunized with OVA/alum three times. Thus, it is considered that T cells from these mice have highly differentiated toward a Th2 phenotype (a high IL-4-producing phenotype). On the other hand, in in vivo experiments, because MRJP3 was administered into mice on 2 days before and on the day of each weekly immunization with OVA/alum, T cells in these mice would be exposed to MRJP3 before and during the initial phase of their differentiation to the Th2 phenotype. Furthermore, it is well known that IL-4 is responsible for the differentiation of Th2 cells and is produced in larger quantities by highly differentiated T cells toward a Th2 phenotype than by naïve or immature Th2 cells (Fallon et al., 2002; Swain et al., 1990). For these reasons, it is probable that even small amounts of MRJP3 would exhibit the inhibitory effect on IL-4 production by naïve or immature Th2 cells, resulting in a limited development into mature effector cells secreting high amounts of Th2 cytokines. This interpretation could explain our observation that MRJP3 was effective at a lower dose in vivo than in vitro, although evidence for such a mechanism is currently lacking.

Natural MRJP3 administration showed both antiallergic and antigenic actions in OVA/alum-immunized mice, while heat-treated MRJP3 exhibited only antiallergic actions. Heat-treated MRJP3 considerably reduced its antigenicity, as suggested by the decrease in total IgG1 levels almost equivalent to that of the PBS control group. These results might be explained by the possibility that the antigenic region in MRJP3 is entrapped in the interior of the molecule by heat treatment-induced conformational changes (Baur et al., 1996; Constant et al., 1995). Alternatively, conformational changes in MRJP3 induced by heating could cause the peptide region showing antigenicity to be more readily attacked by proteases in
vivo. These findings also imply that the regions exhibiting antiallergic activities exist on MRJP3 and are different from those associated with antigenicity. Thus, our next goal for safer potential immunotherapy using MRJP3 is to design a MRJP3-derived synthetic peptide that has potent anti-allergic activity without inducing antigenic responses. We might be able to achieve this by identification of the active site with antiallergic activity among the polypeptides derived from natural MRJP3 and by synthesis of the active site-related peptide.

We have screened for IL-4 production-suppressive factors present in RJ and have identified MRJP3 that shows immunosuppressive effects by inhibiting the production of several relevant cytokines in vitro, and also shows immunomodulatory effects in a mouse model of allergy. Therefore, at present we believe that MRJP3 plays a major role in the antiallergic activity of RJ. However, we cannot exclude the possibility that other anti-allergic factors still exist in RJ because administration of the supernatants obtained from 50 μg of RJ, which contains approximately 2 μg of MRJP3, reduced anti-allergic antibody responses equivalent to that obtained by 50 μg/mouse of MRJP3. The experiments are now underway to test this possibility.

Conclusion

In summary, we isolated a royal jelly protein with immunosuppressive activity and identified it to be a protein MRJP3. We showed that the administration of MRJP3 inhibits IgE and IgG1 responses to OVA in spite of the antigenicity of MRJP3. In addition, the finding that heat-treated MRJP3 injection was inhibitory but not antigenic suggests that a peptide which retains the biological activity without the antigenicity could be isolated. Therefore, the results presented in this study and further research on identifying the active sites could lead to new insights into immunoregulatory approaches such as allergen desensitization using MRJP3 protein or its derived peptide.

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