Royal Jelly Inhibits the Production of Proinflammatory Cytokines by Activated Macrophages

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In this study, we have examined the anti-inflammatory actions of royal jelly (RJ) at a cytokine level. When supernatants of RJ suspensions were added to a culture of mouse peritoneal macrophages stimulated with lipopolysaccharide and IFN-γ, the production of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1, was efficiently inhibited in a dose-dependent manner without having cytotoxic effects on macrophages. This suggests that RJ contains factor(s) responsible for the suppression of proinflammatory cytokine secretion. We named the factor for honeybees RJ-derived anti-inflammatory factor (HBRJ-AIF), and further investigated the molecular aspects of it. Size fractionation study showed that HBRJ-AIF is composed of substances of low (<5 kDa) and high (>30 kDa) molecular weights, with the former being a major component. Chromatographic analysis showed that MRJP3 is one candidate for the HBRJ-AIF with high molecular weights. Thus, our results suggest that RJ has anti-inflammatory actions through inhibiting proinflammatory cytokine production by activated macrophages.

Key words: royal jelly (RJ); proinflammatory cytokine; macrophage; major royal jelly protein 3 (MRJP3)

Proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), and interleukin-6 (IL-6) play important roles in many physiological processes including the regulation of immune and inflammatory responses. For example, TNF-α is produced by activated macrophages in response to pathogenic and inflammatory cytokines and plays essential roles in host defense by limiting the spread of pathogenic organisms into the circulation.1,2 However, a variety of studies have shown that these proinflammatory cytokines contribute to the pathophysiology of a range of diseases, when they are expressed at sufficiently high concentrations. In rheumatoid arthritis (RA), it is now established that TNF-α, IL-1, and IL-6 are produced by the synovial membrane and are closely involved in the pathogenesis of the disease.3–6) Of these proinflammatory cytokines, it is suggested that TNF-α plays a key role in the manifestation of clinical symptoms of RA, since TNF-α is at the apex of a proinflammatory cytokine cascade and treatment of RA patients with anti-TNF-α antibodies resulted in the dramatic reduction in the disease activity.7,8) Thus, cytokines may be good therapeutic targets in inflammatory/autoimmune diseases.

Royal jelly (RJ) that is produced by the hypopharinceal and mandibular glands of worker honeybees is well known to be a necessary food for the growth of the queen honeybee. Since it contains a variety of free amino acids, sugars, minerals, and vitamins, RJ has been consumed as a dietary supplement. In addition, a number of biological and immuno-regulatory actions attributed to RJ have been reported. These include vasodilative and hypotensive activities,9) induction of decrease in serum cholesterol levels,10) antitumor activities,11) and protective activity against hemopoietic dysfunction in X-irradiated mice.12) We have shown that RJ has anti-allergic activities through inhibiting IgE production using immediate hypersensitivity mouse models.13) Recently, we have identified a 70-kDa glycoprotein, major royal jelly protein 3 (MRJP3), as a molecule that is responsible for inhibiting IgE and IgG1 responses in vivo.14) Furthermore, we have shown that RJ increases collagen production by normal hamster fibroblasts in the presence of ascorbic acid 2-O-α-glucoside.15)

To our knowledge, only one paper regarding anti-inflammatory actions of RJ has appeared in the literature to date. Fujii et al. reported that RJ inhibits capillary permeability in the acute phase and reduces granulation tissue formation in the chronic phase of inflammation observed in streptozotocin-diabetic rats, although the
mechanism of the anti-inflammatory actions of RJ remains to be identified. In this study, to substantiate the anti-inflammatory actions of RJ at a cytokine level, we have examined whether RJ inhibits the production of proinflammatory cytokines by activated macrophages.

Materials and Methods

Mice. BALB/c female mice, 8–12 wk of age, were purchased from Charles River Japan (Kanagawa, Japan). All animal experiments described in this article were conducted according to the guidelines by an Animal Experiments Review Board at our Institute.

Reagents. RJ that had been collected from Anhui in China was used throughout the experiments. We also tested RJ from Sao Paulo in Brazil in a separate experiment and obtained a similar result as we obtained using RJ from Anhui. RJ was suspended in sterile phosphate-buffered saline (PBS) at concentration of 50 mg/ml. The supernatant of the RJ suspension was passed through a 0.22 μm filter, and then divided into portions. The portions of the RJ supernatant were stored at −80°C until usage. The endotoxin content of RJ was <50 pg/mg as measured by the Limulus amebocyte lysate assay (Seikagaku Kogyo, Tokyo, Japan). Hereafter, the concentrations of the RJ supernatants refer to the concentrations of the RJ suspended in PBS.

Lipopolysaccharide (LPS) (E. coli 055:B5) was obtained from Difco Laboratories (Detroit, MI). Murine recombinant interferon-γ (IFN-γ) was prepared and purified in our laboratories. Cytokine (TNF-α and IL-6) standards for ELISA were obtained from BD PharMingen: for TNF-α capture and biotinylated detection were purchased from BD PharMingen (San Diego, CA). IL-1β was purchased from Cosmo Bio (Tokyo, Japan) and used as a positive control in an IL-1 bioassay. The following mAb pairs for ELISA capture and biotinylated detection were obtained from BD PharMingen: for TNF-α, G281-2626 and MP6-XT3; for IL-6, MP5-20F3 and MP5-32C11.

Cell cultures and stimulation. Murine peritoneal macrophages were elicited by intraperitoneal injection of 2 ml of 3% Brewer’s thioglycollate medium (Nissui Pharmaceutical, Tokyo, Japan) into the cavity of BALB/c mice. Peritoneal exudate cells were collected by lavage 3 to 4 days after injection. The cells were washed twice and plated onto 10-mm diameter plastic dishes (Nippon Becton Dickinson, Tokyo, Japan) at a density of 1 × 10^7 cells/dish in 10 ml of RPMI1640 medium (Nissui Pharmaceutical) containing 10% (v/v) FBS (Life Technologies, Grand Island, NY). After 2 h of incubation at 37°C in a humidified atmosphere of 5% CO₂ and 95% air, nonadherent cells were removed by rinsing. Then RPMI1640 medium containing 10% FBS was added to the adherent cells, and cells were recovered with a cell scraper (Nippon Becton Dickinson) and used as macrophages. Murine macrophage-like cell line, RAW264.7, was maintained in RPMI1640 medium containing 10% FBS.

For proinflammatory cytokine production, peritoneal macrophages or RAW264.7 cells were seeded at 5 × 10^4 cells per well in flat-bottom 96-well microtiter plates and were then stimulated with LPS (1 μg/ml) and IFN-γ (10 IU/ml) in the presence or absence of various concentrations of RJ for 48 h. After the incubation period, the culture supernatants were removed for the measurement of cytokines. Levels of TNF-α and IL-6 in the culture supernatants were measured by ELISA. The lower limits of detection were 50 pg/ml and 25 pg/ml for TNF-α and IL-6, respectively. Levels of IL-1 were measured by a bioassay using D10.G4.1 cells as described previously. Briefly, D10.G4.1 cells were seeded at 4 × 10^4 cells per well in flat-bottomed 96-well microtiter plates and were then cultured with test samples in the presence of Con A (1.25 μg/ml) for 72 h. After the incubation period, the proliferative response of D10.G4.1 cells was assessed as described below.

For measurements of cell proliferation, 20 μl of alamarBlue dye (Trek Diagnostic Systems, OH), a redox indicator, was added to each well of the microplates for the last 2 to 3 h of the incubation period. The alamarBlue assay has been shown to be used as an alternative to [3H]thymidine incorporation assay. Fluorescence intensity (FI) was measured at an excitation wavelength of 544 nm and an emission wavelength of 590 nm.

Size fractionation of RJ and purification of MRJP3. Five grams wet weight of fresh RJ were suspended in 100 ml of 200 mM Tris-HCl buffer, pH 8.0. 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, Tokyo, Japan) equilibrated with 200 mM Tris-HCl buffer, pH 8.0. Elution was done with a linear gradient of 0 to 0.4 M NaCl in 1,125 ml. Protein concentration was measured by the Bradford assay (Bio-Rad, Richmond, CA), using human serum albumin as a standard.

Fractionation of the RJ supernatant in terms of molecular weights was done by dialysis against PBS solution overnight at 4°C or by centrifugation (2,000 × g) using ultrafiltration membranes, an Ultrafree centrifugal filter device, with molecular weight cut-off of 30 kDa and 5 kDa (Millipore, Bedford, MA). To obtain a fraction containing substances with molecular masses higher than 30 kDa, the RJ supernatants concentrated with the 30 kDa cut-off membrane were reconstituted with PBS and were then re-spun. This process was repeated twice. After the centrifugation, the volume of the filtrate and concentrated solution was adjusted to be equal to the volume of the original RJ supernatant by adding PBS.
MRJP3 was purified from the RJ supernatant by chromatography on a DEAE-5PW column, a Resource Q column, a Heparin-5PW column, and a Superdex 200 gel-filtration column as described previously. The purity of MRJP3 was >97% as measured by SDS-PAGE under reducing conditions followed by estimation on an Image Master scanner (Amersham Biosciences, Piscataway, NJ).

Statistical analysis. Results were analyzed by the Students unpaired t test. P-values less than 0.05 were considered statistically significant.

Results

RJ inhibits the production of proinflammatory cytokines by LPS/IFN-γ-stimulated macrophages

To investigate the anti-inflammatory effects of RJ, RJ was suspended in PBS at various concentrations and supernatants of the RJ suspensions were added to the culture of mouse peritoneal macrophages in the presence of 1 µg/ml of LPS and 10 IU/ml of IFN-γ. As shown in Fig. 1, peritoneal macrophages secreted TNF-α, IL-6, and IL-1 into the culture fluid in response to LPS and IFN-γ. Interestingly, the production of these proinflammatory cytokines was dose-dependently inhibited by the addition of the RJ supernatants to the culture (Fig. 1). Of the three proinflammatory cytokines, TNF-α production was most efficiently inhibited by the RJ supernatants. At 5 mg/ml of the RJ supernatant, TNF-α was almost completely inhibited (Fig. 1A). The inhibition of cytokine production was not due to a cytotoxic effect on macrophages, since the growth of macrophages was not inhibited, but rather augmented by the addition of the RJ supernatants (Fig. 1D).

We next examined whether RJ could inhibit the production of proinflammatory cytokines by a murine macrophage cell line, RAW264.7. As shown in Fig. 2A and C, the RJ supernatants inhibited the production of both TNF-α and IL-1 by LPS/IFN-γ-stimulated RAW264.7 cells in a dose-dependent manner. At 5 mg/ml of the RJ supernatant, the production of both TNF-α and IL-1 was inhibited by 70% of PBS control. However, IL-6 production by LPS/IFN-γ-stimulated RAW264.7 cells was not inhibited, but rather augmented by the addition of the RJ supernatants to the culture (Fig. 2B). At 2.5 mg/ml of the RJ supernatant, IL-6 production was augmented to 1.7-fold of PBS control. This was not due to the promotion of cell growth, since the RJ supernatants had little or no effects on the growth of RAW264.7 cells at concentrations less than 2.5 mg/ml (Fig. 2D). We then examined whether the augmentation of IL-6 production by the RJ supernatants could be observed when RAW264.7 cells were stimulated with LPS alone. As shown in Fig. 3A, TNF-α production by LPS-stimulated RAW264.7 cells was efficiently inhibited regardless of the presence of IFN-γ, although the levels of TNF-α produced by LPS/IFN-γ-stimulated RAW264.7 cells were significantly higher than those produced by RAW264.7 cells stimulated with LPS alone (p < 0.05). Interestingly, in contrast to the stimulation with both LPS and IFN-γ, IL-6 production by LPS-stimulated RAW264.7 cells was inhibited by the RJ supernatants in a dose-dependent manner, although the reason is not clear (Fig. 3B).

Thus, these results indicate that RJ inhibits the production of proinflammatory cytokines by activated macrophages. These results further suggest that RJ contains factor(s) responsible for the suppression of proinflammatory cytokine secretion. Since this is the first report to show the presence of anti-inflammatory factor(s) in RJ, we named the factor for honeybees RJ-derived anti-inflammatory factor (HBRJ-AIF).

HBRJ-AIF is composed of substances of both low and high molecular weights

To characterize molecular aspects of HBRJ-AIF, the
RJ supernatant was dialyzed against PBS or fractionated by centrifugation using ultrafiltration membranes with molecular cut-off 30 kDa and 5 kDa. As shown in Fig. 4A-C, the inhibitory activities for both TNF-α/C11 and IL-6 production were largely abrogated by the dialysis. These results suggest that most of HBRJ-AIF activities are attributable to substance(s) with low molecular mass. Presence of HBRJ-AIF with low molecular masses was also confirmed by the fractionation of the RJ supernatant using ultrafiltration membranes. Most of HBRJ-AIF activities remained in the filtrate solution of 5 kDa cut-off membrane (Fig. 4A-C). However, a significant inhibition of both TNF-α and IL-6 production was still observed with two-fold dilutions of the dialyzed RJ supernatant compared with the control PBS culture (p < 0.01), suggesting that HBRJ-AIF with high molecular masses exists in the RJ supernatant. As shown in Fig. 4A-C, the cytokine inhibitory activities in the filtrate solution of the 30 kDa cut-off membrane were comparable with those observed in the filtrate solution of the 5 kDa cut-off membrane. These results suggest that substances with molecular mass range of from 5 to
30 kDa are not responsible for the inhibition of the cytokine production. To examine whether a fraction containing substances with molecular masses higher than 30 kDa have the cytokine inhibitory activities, the RJ supernatants were concentrated by ultrafiltration using the 30 kDa cut-off membrane. To remove thoroughly substances with molecular masses lower than the molecular cut-off, the concentrated RJ supernatants were reconstituted with PBS and were then re-spun, and this process was repeated again. As shown in Fig. 4D, the fraction containing substances with molecular masses higher than 30 kDa significantly inhibited TNF-α production by LPS/IFN-γ-stimulated peritoneal macrophages. Taken together, these findings suggest that HBRJ-AIF is composed of substances of both low (≤5 kDa) and high (>30 kDa) molecular masses, with the former being a major component.

**MRJP3 inhibits the production of TNF-α by LPS/IFN-γ-stimulated macrophages**

To identify the HBRJ-AIF with high molecular mass, the RJ supernatant dialyzed against 200 mM Tris-HCl buffer was put on a DEAE-5PW column. Fractions corresponding to each protein peak were pooled as shown in Fig. 5A, and the pooled fractions were added to the culture of peritoneal macrophages at a protein concentration of 250 μg/ml in the presence of LPS and IFN-γ. As shown in Fig. 5B, pooled fraction 3 significantly inhibited TNF-α production by LPS/IFN-γ-stimulated peritoneal macrophages. Regarding IL-6 production, no significant inhibition was observed at any pooled fractions (Fig. 5C). Since we had observed that MRJP3 is eluted at pooled fraction 3 (0.1 M NaCl) on the DEAE-5PW column,14 we examined whether purified MRJP3 could inhibit TNF-α production by LPS/IFN-γ-activated peritoneal macrophages. As shown in Fig. 6A and B, MRJP3 inhibited TNF-α production by LPS/IFN-γ-stimulated macrophages in a dose-dependent manner, while MRJP3 had no effect on IL-6 production. The growth of the macrophages was not affected by the addition of MRJP3 (Fig. 6C). These results suggest that MRJP3 is one candidate for HBRJ-AIF with high molecular masses.

**Discussion**

In this study, we have shown that RJ inhibits the production of proinflammatory cytokines, such as TNF-α, IL-6, and IL-1, by macrophages stimulated with LPS or with LPS plus IFN-γ. The inhibition does not seem to be caused by cytotoxic effects on macrophages, since the growth of peritoneal macrophages was not inhibited, but rather augmented by the addition of RJ.

It has been shown that TNF-α can bind to the glycans of uromodulin and its binding is inhibited by diacetylcystobiose and trimannose.19) Since RJ contains lots of mannose-rich sugar chains, we examined whether the RJ supernatants would affect the TNF-α ELISA system.

Our results showed that the RJ supernatants at concentrations from 0.156 mg to 12.5 mg/ml had no effect on the binding of TNF-α to both capture and biotinylated detection mAbs in the TNF-α ELISA system (data not shown).

IL-10 has been shown to have potent anti-inflammatory properties and to inhibit the synthesis of proinflammatory cytokines by human monocytes stimulated with LPS.20) However, it seems unlikely that RJ inhibits the production of proinflammatory cytokines via induction of IL-10 by the activated macrophages. Our preliminary study showed that RJ had no effect on IL-10 production by LPS/IFN-γ-stimulated peritoneal macrophages (data not shown). These results suggest
that RJ directly acts on the activated macrophages to inhibit proinflammatory cytokine production.

Of the three proinflammatory cytokines, TNF-α was inhibited most efficiently by the RJ supernatant. Since TNF-α is capable of initiating a cascade of proinflammatory cytokines,7 this efficient inhibition of TNF-α production rather than IL-1 and IL-6 production in vitro may lead to effective anti-inflammatory actions of RJ in vivo. In fact, a dramatic reduction in disease activity was observed in RA and Crohn’s disease after treatment of patients with neutralizing anti-TNF-α antibody.8,21 Furthermore, it has been reported that clinical trials of RA patients with anti-TNF-α antibody resulted in a rapid decrease of the elevated serum IL-6 levels.22 However, the mechanism by which the RJ supernatants increased IL-6 production by LPS/IFN-γ-stimulated RAW264.7 cells in spite of efficient inhibition of TNF-α production remains unclear. Since IL-6 production by LPS-stimulated RAW264.7 cells was inhibited dose-dependently by the addition of RJ, co-stimulation of RAW264.7 cells with both LPS and IFN-γ in the presence of RJ might transmit unusual activation signals leading to an expression of IL-6 gene in this malignant cell line. Further study should test this possibility.

In this study, we have shown that HBRJ-AIF is composed of substances of low and high molecular masses. DEAE-5PW chromatography of proteins present in RJ suggested that a protein eluted at the position corresponding to MRJP3 is responsible for HBRJ-AIF with high molecular mass. Indeed, purified MRJP3 inhibited TNF-α production by LPS/IFN-γ-stimulated peritoneal macrophages in a dose-dependent manner. MRJP3 is a highly polymorphic protein and belongs to a large protein family designated the major royal jelly proteins (MRJPs) that accounts for 82–90% of total larval jelly proteins.23,24 In this family, five species of proteins (MRJP1-5) with molecular masses in the range of 49–87 kDa have been identified by cDNA cloning and sequencing.23 Physiological functions of MRJPs in honeybees remain unknown. Since MRJP2, MRJP3, and MRJP5 contain extensive repetitive regions consisting of high amounts of nitrogen-rich amino acids, it is presumed that they have a storage function of bio-logically accessible nitrogen that is required for the fast developing organism.24 Recently we have shown that MRJP3 inhibits the production of IL-2, IL-4 and IFN-γ by anti-CD3 mAb-stimulated T cells in vitro.14 Furthermore, MRJP3 inhibited IgE production in vivo when MRJP3 was administered intraperitoneally to OVA/Alum-immunized BALB/c mice.14 These results suggest that MRJP3 has immunosuppressive functions in a mammalian immune system.

We have previously shown that the MRJP3-mediated inhibitory action on IL-4 production by anti-CD3 mAb-stimulated spleen cells was neutralized by addition of anti-MRJP3 mAb (#7-9D). We then examined whether the mAb #7-9D could abrogate the MRJP3-mediated inhibition of TNF-α production by LPS/IFN-γ-stimulated peritoneal macrophages. Contrary to our expectation, the MRJP3-mediated inhibitory action on TNF-α production was not neutralized by the mAb #7-9D (data not shown). This raises the possibility that the epitope on the MRJP3 molecule that is recognized by the mAb #7-9D is not responsible for suppressing TNF-α production by macrophages, while the same epitope is essential for suppressing IL-4 production by T cells. However, further experiments are necessary to understand the molecular mechanisms of the MRJP3-mediated inhibitory action on cytokine production.

Furthermore, the results of dialysis experiments on the RJ supernatant suggested the presence of substance(s) of high molecular masses that inhibit IL-6 production. Protein(s) other than MRJP3 may be responsible for the inhibition of IL-6 production by activated macrophages. In addition, experiments to identify HBRJ-AIF with low molecular masses are now underway.

Fujii et al. reported that oral administration of RJ to streptozotocin-diabetic rats inhibits capillary permeability in the acute phase, reduces granulation tissue formation in the later phase of inflammation, and
shortens the healing period in desquamated skin lesions, although the mechanism of the anti-inflammatory actions of RJ remains to be determined.\textsuperscript{16} Since it has been shown that TNF-α and IL-6 play roles in the vascular permeability and granulation tissue formation during cutaneous wound healing, respectively,\textsuperscript{25,26} it seems likely that the anti-inflammatory actions of RJ observed in the streptozotocin-diabetic rats are attributable to down-regulation of these proinflammatory cytokines.

Very recently, we have shown that oral administration of RJ inhibits the development of atopic dermatitis-like skin lesions in NC/Nga mice induced by repeated application of picryl chloride.\textsuperscript{27} In this study, in addition to the significant decrease in the total skin severity scores, we could observe histologically the decrease in hypertrophy, hyperkeratosis, and infiltration of the epidermis and corium by inflammatory cells in the RJ-administered mice. These results suggest that RJ has anti-inflammatory actions \textit{in vivo} as observed with streptozotocin-induced diabetic rats.

In summary, we have shown here that RJ efficiently inhibits the production of proinflammatory cytokines including TNF-α, IL-6 and IL-1 by LPS/IFN-γ-activated macrophages. Our preliminary study showed that 2.5 mg/ml of the RJ supernatant suppressed the production of prostaglandin E\textsubscript{2}, another inflammatory mediator, by 63% compared with that produced by LPS/IFN-γ-stimulated control peritoneal macrophages (data not shown). RJ neither had cytotoxic effects on macrophages \textit{in vitro} as shown in this study, nor did it cause weight loss when administered orally to mice.\textsuperscript{13,27} Thus, our results suggest that RJ is an effective dietary supplement for the improvement of quality of life in the autoimmune diseases, such as RA and inflammatory bowel diseases.

References


