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A ROYAL JELLY AS A NEW POTENTIAL IMMUNOMODULATOR IN RATS AND MICE

LIDIJA ŠVER,[1] NADA ORŠOLIĆ,[2] ZORAN TADIĆ,[2] BELA NJARI,[3] IVICA VALPOTIĆ[1]* and IVAN BAŠIĆ[2]

[1]Department of Biology and [3]Department of Hygiene and Technology of Animal Foodstuffs, Veterinary Faculty, University of Zagreb, P.O. Box 190, 41000 Zagreb, Croatia and [2]Department of Animal Physiology, Faculty of Science, University of Zagreb, P.O. Box 933, 41000 Zagreb, Croatia

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Abstract—In order to study a possible immunomodulatory effect of the royal jelly (RJ) secreted by mandibular and hypopharingeal glands of the worker honeybee (*Apis mellifera* Linné.) we have used a well established rodent model. The CBA mice were given s.c. 0.1 ml of RJ, 7 days before, or immediately after, the immunization with sheep red blood cells (SRBC). The Y59 rats received i.m. 0.4 ml or i.v. 0.025 ml of RJ once or twice at 7 day intervals. Serum levels of total proteins and immunoglobulins in the rats that received RJ once or twice within a 2-week-period were significantly lower ($P \leqslant 0.05$) as compared with the nontreated animals. In mice which were immunized with 4×10^8 of SRBC 7 days after the application of RJ the number of plaque forming splenocytes was significantly higher ($P \leqslant 0.05$) than that in the controls. Both the weight of inguinal lymph node and the number of peripheral blood lymphocytes were increased ($P \leqslant 0.05$) in RJ-treated mice 3 or 5 days after the immunization, respectively. Neutrophils were decreased ($P \leqslant 0.05$) in the mice that were killed 5 or 10 days after the RJ treatment. Overall these results indicate that RJ exhibited immunomodulatory properties by stimulating antibody production and immunocompetent cell proliferation in mice or depressing humoral immune functions in rats. Both phenomena, though species-related in this model, could probably be reversed by changing the dose or the route of RJ application.

Key words: Immunomodulation, royal jelly, rat, mouse.

Résumé—Pour pouvoir étudier les possibles effets immunomodulateurs de la gelée royale (GR) secretée par les glandes sous-pharingées des abeilles (*Apis mellifera* Linné.), nous avons utilisé un modèle de rongeur bien établi. Des souris CBA ont reçu 0.1 ml s.c. de GR, immédiatement ou 7 jours aprés l'immunisation avec des globules rouges du sang ovin (GRSO). Des rats Y59 ont reçu 0.4 ml i.m. ou 0.0025 ml i.m. de GR, une ou deux fois à intervalle de 7 jours. Le niveau des protéines totales et des immunoglobulines dans le serum des rats qui ont reçu les GR, une ou deux fois pendant une période de 2 semaines a subi, en comparaison avec les animaux qui n'ont rien reçu, une diminution significative ($P \leqslant 0.05$) chez les souris immunisées avec 4×10^8 deGRSO, le nombre des splénocytes formant plaque d'hémolyse était significativement plus élevé ($P \leqslant 0.05$) par rapport aux témoins. Le poids des noeuds lymphatiques inguinaux et le nombre des lymphocytes sanguins périphériques a augmenté ($P \leqslant 0.05$) chez les souris traitées avec la CR, et sacrifiées 3 ou 5 jours après l'immunisation. Chez les souris sacrifiées 5 ou 10 jours après le traitement avec les GR, la quantité des neutrophiles a diminué ($P \leqslant 0.05$). En somme, les résultats indiquent que la GR, par sa stimulation de la production d'anticorps et la prolifération des cellules immunocompétentes chez les souris ou par la dépression des fonctions immunes humorales chez les rats, manifeste des qualités immunomodulatoires. Les deux phénomènes, quoique dans ce modèle liés à l'espèce, peuvent probablement être renversés par le changement de la dose et/ou de la voie d'application.

Mots-clefs: Immunomodulation, gelée royale, rat, souris.

*Author for correspondence.

INTRODUCTION

The royal jelly (RJ), a principal food of the queen honey bee, is produced by the hypopharingeal and mandibular glands of the worker honey bees (*Apis mellifera* Linné.). The physical and chemical properties of the RJ and its constituents, such as free amino acids, proteins, sugars, fatty acids (mainly 10-hydroxy-2-decenoic acid; 10-HDA), minerals (mainly iron and calcium) and vitamins (thiamin, niacin, riboflavin) have been described in detail elsewhere [1–5]. However, there are only a few studies dealing with the influence of RJ on body and organ weights, and blood counts in experimental animals [6, 7]. In mice, a large dose of the RJ (1 ml/animal) caused a high rate of lethality, while smaller doses (0.1 ml or 0.01 ml) may be stressful but not lethal, and could cause the adrenal enlargement, gastrointestinal ulcers, and hypertrophy of lymphatic tissues [7]. Despite these investigations, which did not completely establish a biologically desirable (protective but not toxic) activity for RJ, it is certain that the immunopharmacological [8], or therapeutic [9] and *in vivo* antitumor effects [10] can be ascribed to 10-HDA or to sole RJ, respectively.

The *in vitro* antitumor activity of the RJ has been attributed to 10-HDA [11]. Due to organic acids (mainly 10-HDA), acidity and proteins (mainly royalisin), the RJ exhibited bacteriostatic [12–14] and antimicrobial activities [15, 16]. In addition, the RJ enhanced the cell-mediated immune response in the irradiated mice [9], and the synthetic 10-HDA stimulated phagocytosis by mononuclear and phagocytic cells in the immunosuppressed mice [17]. Since later reports have highlighted the potential of RJ (and 10-HDA) for the immunorestoration of the cellular immune functions in the immunocompromised mice, it is likely to assume that it would also be capable of enhancing immuneresponsiveness in normal animals.

In the present study, we extend these observations and test the putative immunogenicity of RJ (at least documented for the induced immunological abnormalities in the murine model) in an attempt to stimulate both humoral and cellular immune responses in mice and rats.

MATERIALS AND METHODS

Animals

Twenty-four 5-month-old Y59 rats, weighing 280–350 g, were used in Experiment 1, and thirty-five 4-month-old CBA mice, weighing 20–25 g, were used in Experiments 2 and 3 (see *Experimental design*).

Royal Jelly

Samples of RJ were collected, during the period from June to July, when the larvae of queen honey bees were 3 days old, and were kept frozen at $-10°C$ until used.

Experimental design

Experiment 1. 0.4 ml or 0.025 ml of RJ were given to Y59 rats i.m. or i.v., respectively, once (at Day 0) or twice (at Day 0 and Day 7 or at Day 0 and Day 12). Animals were anesthetized with ether and killed at Day 14 or Day 16 after the first injection.

Experiment 2. 0.1 ml of the RJ was injected s.c. to CBA mice 7 days before or immediately after immunization with 4×10^8 sheep red blood cells (SRBC) in 0.5 ml of

Hank's balanced salt solution (HBSS). Animals were euthanized four days after immunization with SRBC. Immediately following this, spleen was removed and placed into HBSS. The single cell suspension of splenocytes was prepared by gently teasing the tissue into small fragments and passing the cells through a wire mesh. The viability of splenocytes was determined by the Trypan blue exclusion test.

Experiment 3. 0.1 ml of RJ was injected s.c. to CBA mice. Animals were killed at Day 3, Day 5, or Day 10 after application.

Serum protein determination

Blood samples of Y59 rats were taken as soon after euthanasia as feasible. Total serum proteins were determined by the biuret method [18] using a spectrophotometer (Shimadzu, UV-160) at a wavelength of 546 nm. Estimates of total serum immunoglobulins (Igs) were performed by semimicroelectrophoresis on gelatinized cellulose acetate strips (Cellogel™, "Chemetron", Milano, Italy) as detailed earlier [19–21].

Enumeration of plaque forming cells (PFC)

The local hemolytic assay for the assessment of antibody-producing cells among murine splenocytes was essentially performed as described by Jerne and Nordin [22]. The suspension of splenocytes (0.025 ml) was mixed with 0.5 ml of agar and 0.5 ml of SRBC, and placed onto agar on a glass slide. After incubation for 1 h at 37°C the complement was added, and slides were additionally incubated for 2 h at 37°C. Then the complement

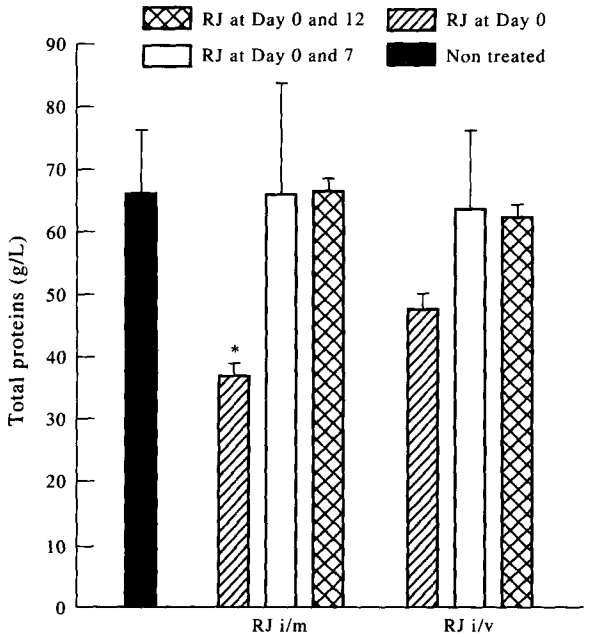

Fig. 1. Mean (±SD) levels of total serum proteins in Y59 rats injected with either 0.4 ml (i.m.) or 0.025 ml (i.v.) of the RJ once (at Day 0) or twice (at Day 0 and Day 7 or at Day 0 and Day 12). Groups comprised 3 rats each. Animals were killed at Day 14 or Day 16 after the first injection. *Significantly ($P \leq 0.05$) different from the nontreated controls.

was removed by a vacuum pump and the PFC were counted on at least three slides per group under the light microscope.

Weighing of lymphatic tissues

Immediately following the euthanasia of CBA mice, the spleen and single right superficial inguinal lymph node were removed, and weighed using a torsion balance.

Blood parameters

Blood samples were taken from CBA mice immediately after the euthanasia into heparinized tubes. Differential blood cell counts and the enumeration of leukocytes (lymphocytes, monocytes, and granulocytes) were performed by the standard method.

Statistics

The significance of differences between means of the groups were tested by the paired Student's t test using a statistical package, the Statgraph (Statgraphs Inc., Version 4.2). Results were considered statistically significant if $P \leqslant 0.05$.

RESULTS AND DISCUSSION

The concentration of total proteins in serum of rats that received the RJ i.m. once (at Day 0) was significantly lower ($P \leqslant 0.05$) as compared to that in nontreated animals

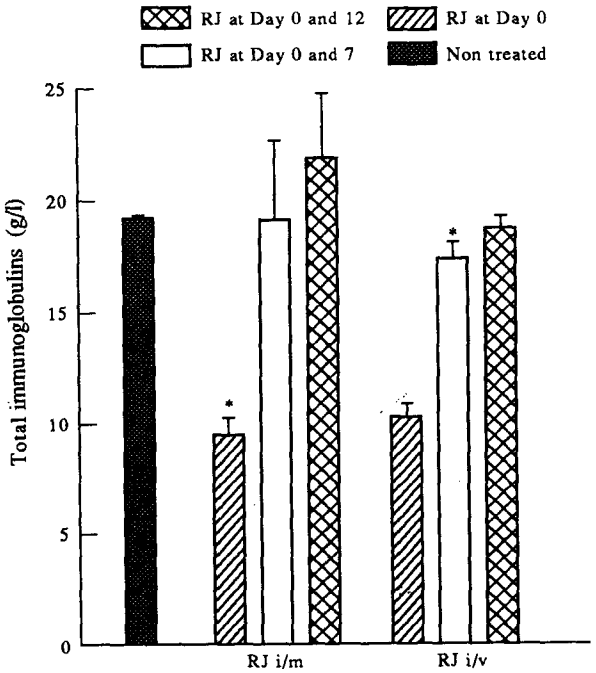

Fig. 2. Mean (±SD) concentrations of total serum immunoglobulins in Y59 rats injected with either 0.4 ml (i.m.) or 0.025 ml (i.v.) of the RJ once (at Day 0) or twice (at Day 0 and Day 7 or at Day 0 and Day 12). Groups comprised 3 rats each. Animals were killed at Day 14 or Day 16 after the first injection. *Significantly ($P \leqslant 0.05$) different from the nontreated controls.

(Fig. 1). The level of Igs in the RJ-treated rats was also decreased ($P \leqslant 0.05$) regardless of the route of application (either i.m. or i.v.) or the number of injections (doses) given within a 7-day-period (at Day 0 or at Days 0 and 7) (Fig. 2). There is evidence to suggest the implication of an immunoregulative role for fatty acids from the RJ for murine monocytes/macrophages [17]. It seems likely that 10-HDA (and probably other fatty acids) selectively bind to the cell membrane of B lymphocytes and, thus, could interfere with antibody secretion by these cells. Previous studies have shown that the malonic acid from the RJ acts as an inhibitor of the succinoxidase system in nearly all types of cells [11]. However, the mechanism by which the dicarboxylic acids inhibit antibody formation has not yet been elucidated.

In mice immunized with SRBC 7 days after s.c. application of the RJ the number of splenic PFC was significantly higher ($P \leqslant 0.05$) than that in the controls (Fig. 3). However, this was not true for the simultaneous application of both antigens, i.e. the RJ and SRBC.

An increase in weight of inguinal lymph node ($P \leqslant 0.05$) in the RJ-treated mice was recorded between Day 3 and Day 10 after treatment (Fig. 4). Consistent with this observation is an early report of Grad *et al.* [7] who also found heavier lymphatic tissues (thymus, spleen, lymph nodes) in mice treated with RJ. However, the application of the RJ did not affect the weight of spleen in our experiment, which is in contrast to those findings.

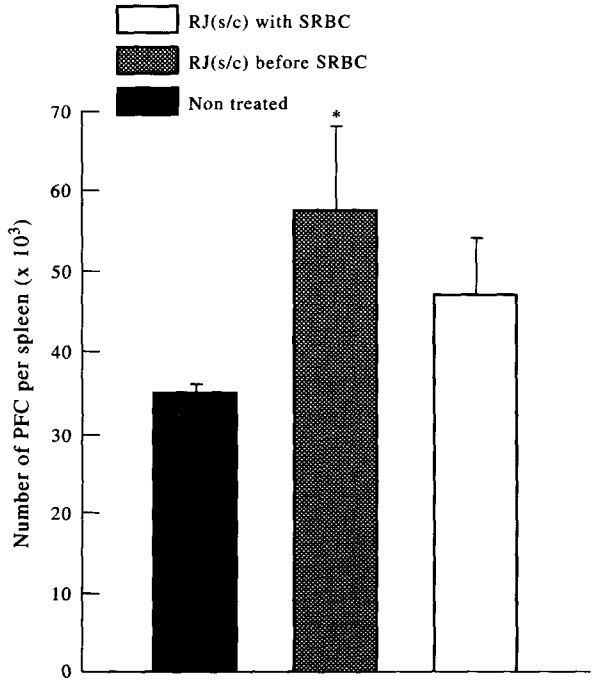

Fig. 3. Mean (±SD) numbers of splenic PFC in CBA mice s.c. injected with 0.1 ml of RJ 7 days before or immediately after immunization with 4×10^8 SRBC in 0.5 ml of HBSS. Groups comprised 5 mice each. Animals were killed 4 days after immunization with SRBC. *Significantly ($P \leqslant 0.05$) different from the nontreated controls.

It is possible that in mice, the RJ acts as an immunostimulator presumably due to its ability to alter the normal immune cell proliferation (locally in at least inguinal lymph nodes, i.e. near the *site* of application of the immunogen), thus allowing efficient mobilization of immune response (at least of humoral type, i.e. an increase in antibody-producing splenocytes following the immunization with RJ) to the xenoantigen, such as SRBC. The number of peripheral blood lymphocytes was also increased ($P \leqslant 0.05$) in the RJ-treated mice 5 days after immunization (Fig. 5). This finding could be related to an early study dealing with the RJ-induced restoration of cellular immunity in immunosuppressed mice [9]. However, there is also evidence for the facilitation of serum hemolysin formation in normal mice following 10-HDA-treatment [8], indicating that RJ (or one of its components) may have a damaging effect on erythrocytes. We observed a significantly decreased percentage of neutrophils ($P \leqslant 0.05$) in the RJ-treated mice at Days 5 and 10 post immunization (Fig. 5), which could be ascribed to the selective activation of leukocyte subsets by the RJ.

In conclusion, data from the present study demonstrate, in a rodent model, the existence of consistent (and at least short-term) stimulation of nonspecific and specific (to SRBC) immune reactivity following RJ application. Further studies on the modulatory effect of RJ *in vitro* should add to our knowledge of the relevance of this potential immunomodulator in enhancement of different immune functions and disease resistance. Also, it would be appropriate to check for any altered effects of RJ on hematopoetic cells and hematopoesis in this model.

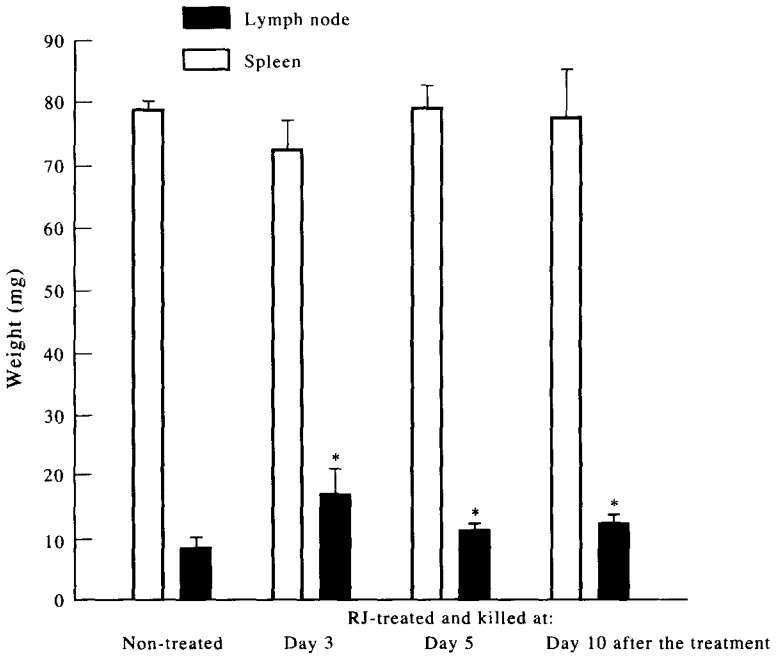

Fig. 4. Mean (±SD) weight of spleen and inguinal lymph node in CBA mice s.c. injected with 0.1 ml of RJ. Groups comprised 5 mice each. Animals were killed at Day 3, Day 5 or Day 10 after the application of RJ. *Significantly ($P \leqslant 0.05$) different from the nontreated controls.

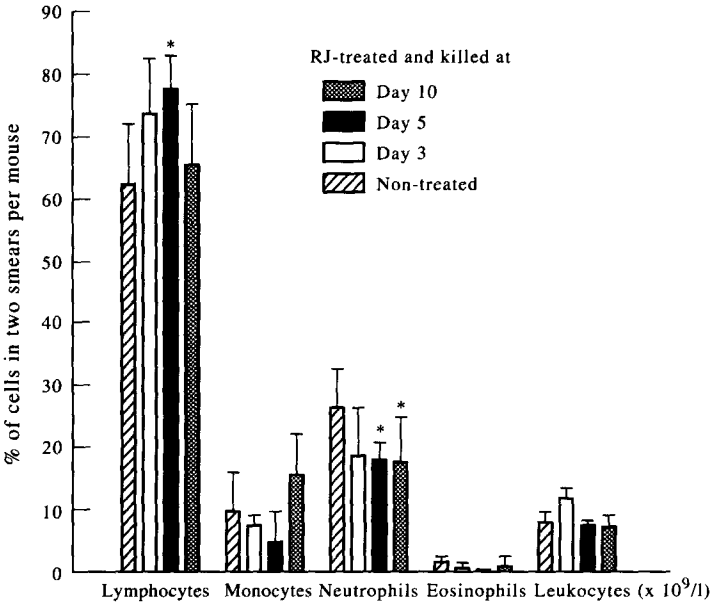

Fig. 5. Mean (±SD) values for percentages of lymphocytes, monocytes and granulocytes, and number of leukocytes in the periferal blood of CBA mice s.c. injected with 0.1 ml of the RJ. Groups comprised 5 mice each. Animals were killed at Day 3, Day 5 or Day 10 after the application of the RJ. *Significantly ($P \leqslant 0.05$) different from the nontreated controls.

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