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Suppressive Effect of Honey on Antigen/Mitogen Stimulated Murine T Cell Proliferation

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Abstract

It has been reported that the intraperitoneal administration of honey suppressed the induction of antigen-specific humoral antibody response in mice as determined by passive cutaneous anaphylaxis and Ouchterlony double immunodiffusion tests. Hence, we studied the effect of honey on induction of antigen-specific IgG antibody response and found suppression in mice as determined by ELISA and also T cell proliferation *in vitro*. T cell proliferation induced by antigens such as ovalbumin, diphtheria toxoid and tetanus toxoid, as well as the mitogens concanavalin A and phytohemagglutinin, was significantly suppressed by various doses of natural and commercial honeys, as evaluated by ^{3}H-thymidine incorporation. Furthermore, the suppressive effect of honey was also confirmed on antigen- or mitogen-induced T cell proliferative responses using splenic T cells from different haplotypes of mice. The results obtained in this work confirm the antiproliferative activity of honey.

Keywords: Honey, immunosuppression, mitogens, antigens, T cell proliferation.

Introduction

Immunomodulatory activity of compounds has been evaluated by examining their effects on immune response *in vivo* and T cell proliferation *in vitro*. There is a large body of literature pertaining to the influence of natural or synthetic pharmaceutical compounds on *in vitro* T cell proliferative responses. For example, steroids such as fluorohydrocortisone and methylprednisolone, suppress phytohemagglutinin (PHA) stimulated T cell blastogenesis (Langhoff et al., 1985). The immunosuppressive, long chain amphiphilic molecules were found to be antiproliferative (Coy et al., 1990). Cannabinoids and cocaine have been found to suppress mitogen-induced T cell proliferation both in human and murine systems (Luo et al., 1992). Also, it was found that interferon-beta produced by human placental trophoblast cells show an antiproliferative effect against mitogen stimulated and resting lymphocytes (Zdravkovic et al., 1994). It was reported that an extract of the fern *Polypodium leucotomos* can inhibit human peripheral blood mononuclear cell proliferation (Rayward et al., 1997). Microcolin A, a lipopeptide extracted from the marine blue green alga, *Lyngbya majuscula*, was a potent immunosuppressive and antiproliferative agent (Zhang et al., 1997). Several immunosuppressive compounds exhibit specific down-regulatory mechanisms affecting either T cells or macrophages. For example, agents like cyclosporin A, FK506, rapamycin (Wu et al., 1991; Sigal & Dumont, 1992), dexamethasone (Baus et al., 1996), hydrocortisone (Robertson et al., 1981), and d-penicillamine (Lipsky & Ziff, 1980) suppress T cell activation directly, while others, like 15-deoxyspergualin (Hoeger et al., 1994) and antimalarials (Lipsky, 1988) interfer with the antigen processing by macrophages. Further, *Withania somnifera* root extract was found to suppress T cell proliferation in mice by affecting antigen processing and presentation (Srinivasulu, 1999).

Earlier, we have reported that intraperitoneal administration of honey in low doses suppresses the induction of ovalbumin-specific murine humoral antibody responses, as determined by passive cutaneous anaphylaxis (PCA) and Ouchterlony double immunodiffusion (Duddukuri et al., 1997). The substance also suppresses ovalbumin-specific IgG subclasses and other classes of antibody response as evaluated by ELISA (Duddukuri et al., 2001). Furthermore, it was

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reported that oral intake of honey could cause allergic reactions in human beings due to the presence of pollen and insect allergens (Florido-Lopez et al., 1995). However, the prevalence of allergic symptoms is rare in pollen sensitive patients who are orally challenged with honey compared to placebo group (Kiistala et al., 1995). In this study, to understand and further substantiate these immunological parameters and to examine the role of honey on the immune system, *in vitro* experiments were conducted for studying the effects of honey on T cell proliferative responsiveness against different antigens or mitogens.

Materials and methods

Mice

Eight-week-old female BALB/c mice, (H-2^d), C57BL/6 (H-2^b) and CBA/J (H-2^k), were obtained from the animal facility at the National Institute of Immunology, New Delhi, India.

Reagents and media

Ovalbumin (OVA), diphtheria toxoid (DT), tetanus toxoid (TT) (as antigens), concanavalin A (Con A), phytohemagglutinin (PHA) (as mitogens), RPMI-1640 medium and fetal calf serum (FCS) were obtained from Sigma Chemical Co., USA. RPMI 1640 medium is supplemented with 2 mM glutamine, gentamycin (40 µg/ml), streptomycin (50 µg/ml), and 10% FCS.

Honey sources

Andhra Pradesh Girijan Cooperative Corporation's rockbee honey, Apiary and Dabur honeys are the commercial sources, while rockbee (*Apis dorsata*) and thodithi (*A. mellifera*) are natural sources which were obtained from Nallamalai forests, Andhra Pradesh, India. Honey is appropriately diluted in RPMI medium and then filter-sterilized using 0.22 µm Nunc filters prior to testing immunological activity on T cell proliferation.

Determination of IgG antibody response by ELISA

Total IgG levels in antisera from both control and test groups of mice were assayed by ELISA. Briefly, the wells of the 96-well microtiter plate were coated with 100 µl of OVA (100 ng/well) in carbonate buffer (pH 9.6) and incubated overnight at 4 °C. After incubation, the wells were washed three-times with PBS containing 0.05% Tween 20 (PBS-Tween). The nonspecific binding sites in the microtiter wells were blocked by incubating with 300 µl of 3% skim milk powder in PBS (Anikspray, Hindustan Lever Limited, India) for 10–12 h at room temperature. After washing the plate, the wells were further incubated with 100 µl of diluted sera in PBS (1 : 400) in triplicate for 1 h at 37 °C. The unbound serum constituents were washed off and the levels of IgG were measured by incubating with 100 µl of horseradish peroxidase conjugated goat antimouse IgG (Sigma Chemical Co., USA) at a dilution of 1 : 1000 for 1 h at 37 °C. Finally, the unbound conjugate was washed with PBS-Tween and 100 µl of freshly prepared substrate solution (10 ml of 1.5 M citrate phosphate buffer, pH 5.0, containing 4.0 mg of orthophenylene diamine and 10 µl H_2O_2) was added. The reaction was stopped after 5 min by adding 50 µl of 8 N H_2SO_4. The color developed was read at 490 nm using an automatic microplate reader (BioRad, model 550). The data expressed were the mean optical density (OD) of the triplicates.

Immunization protocol

For testing the effect of honey on antigen-induced T cell proliferation, mice were subcutaneously injected with OVA, DT or TT in presence of Freund's complete adjuvant (FCA) on day 0, into the hind footpad, and a booster dose was given on day 8 with Freund's incomplete adjuvant (IFA). On day 12, mice were sacrificed and spleens were removed. Spleens obtained from the normal (unimmunized) mice were used as controls.

T cell proliferation assay

Spleens were aseptically removed either from the normal or antigen primed mice, ruptured in a Petri dish in RPMI medium to obtain single cell suspension, and centrifuged at 1000 rpm for 10 min. The pellet was resuspended in medium and washed twice with media. Red blood cells were removed by incubating with 1 ml of 0.9% NH_4Cl for 1 min, at 37 °C. The cells were centrifuged and washed three-times with RPMI to remove NH_4Cl traces. B cells were removed by the panning technique, coating the Petri dish with goat anti-mouse immunoglobulins. The unbound cells (Ig-negative cells) were carefully resuspended in RPMI with 10% FCS. The viability of cells was checked with Trypan blue exclusion.

Cultures were carried out in sterile microtiter plates (Nunc, USA) with 96 flat bottom wells. All the reagents used were filter sterilized using 0.22 µm Nunc millipore filters prior to adding into the wells. For each individual assay, the cultures were done in triplicate, with each well containing 2×10^5 cells in 250 µl RPMI medium. Cells were stimulated with antigens/mitogens in the absence or presence of different doses of honey, i.e., 0.01, 0.1, 1.0, and 2.5 µl. Cultures were incubated for 72 h at 37 °C in a humidified 5% CO_2 modular chamber. The last 18 h, the cultures were incubated with tritiated thymidine (0.5 µCi/well). After the incubation period, cells were harvested on glass fiber filters using a Nunc cell harvester, and thymidine incorporation was determined with a liquid scintillation counter (Wallac 1410, Pharmacia). Data expressed in counts per minute (cpm) are the means of triplicates of control or test cultures.

Results and discussion

As shown in Figure 1, the OVA-specific primary, secondary and tertiary IgG antibody responses were significantly suppressed by intraperitoneal administration of honey as determined by ELISA, and is in support with our earlier observation of suppressing the IgE antibody response as evaluated by PCA. Even small aliquots, viz., 0.01, 0.1, 1.0 and 2.5 µl of honey, have been found to suppress *in vitro* T cell proliferative responses to different antigens, namely, OVA, DT or TT (Fig. 2). This indicates that the suppression of T cell proliferation was induced by honey but not due to its cytotoxicity, which was also demonstrated by the trypan blue exclusion test. Furthermore, honey has shown consistency in suppressing T cell proliferation in a dose-dependent manner, and a 1.0 µl dose was found as optimum and used for further assays. Honey is also found to suppress T cell proliferative responses induced by two doses of the above antigens (data not shown). Moreover, in addition to antigen-induced T cell proliferation, mitogen [Con A (5.0 µg) and PHA (1.0 µg)] induced T cell proliferative responses were also found to be significantly suppressed with rockbee honey (Fig. 3), implying the possibility of affecting the T cells. Similarly, T cell proliferative responses induced by antigens were found to be suppressed with different sources of natural and commercial honeys (data not shown). Furthermore, the suppressive effect of honey was found on T cell proliferation using the splenic T cells from different haplotypes of mice, namely, H-2^d, H-2^b and H-2^k, inferring a suppressive effect across genetic variation (Fig. 4). All these results clearly indicate the consistency of the antiproliferative effect of honey.

The suppressive nature of honey may be comparable with that of gold compounds that can inhibit antigen and mitogen-induced human lymphocyte proliferation (Lipsky & Ziff, 1977), and also 1,25α-dihydroxyvitamin D_3 (calcitriol), a biologically active metabolite of vitamin D, that inhibits both lectin- and antigen-driven human T cell proliferation (Rigby et al., 1985). It is reported that immunosuppressive agents like cyclosoporin A, rapamycin and FK506 inhibit both antigen- as well as mitogen-induced T cell proliferative responsiveness (Wu et al., 1991; Sigal & Dumont, 1992), whereas 15-deoxyspergualin and *Withania somnifera* root extract specifically suppress the macrophage function, but not the mitogen-induced T cell proliferative response (Hoeger et al., 1994; Srinivasulu, 1999). Hence, as honey tested at different doses inhibited T cell proliferation against antigens as well as mitogens (Figs. 2, 3), it can, therefore, be presumed that honey may be suppressing the immune response by acting on T cells.

In contrast to our findings of suppressing the antibody response as well as T cell proliferation by honey, it has been reported elsewhere that the oral intake of honey could cause allergic reactions in human beings. This may be due to the digested fragments of allergens causing allergic reactions, while putative immunosuppressant may become inactivated during the process of digestion, or it may be insufficient to induce significant suppressive activity. However, the rare

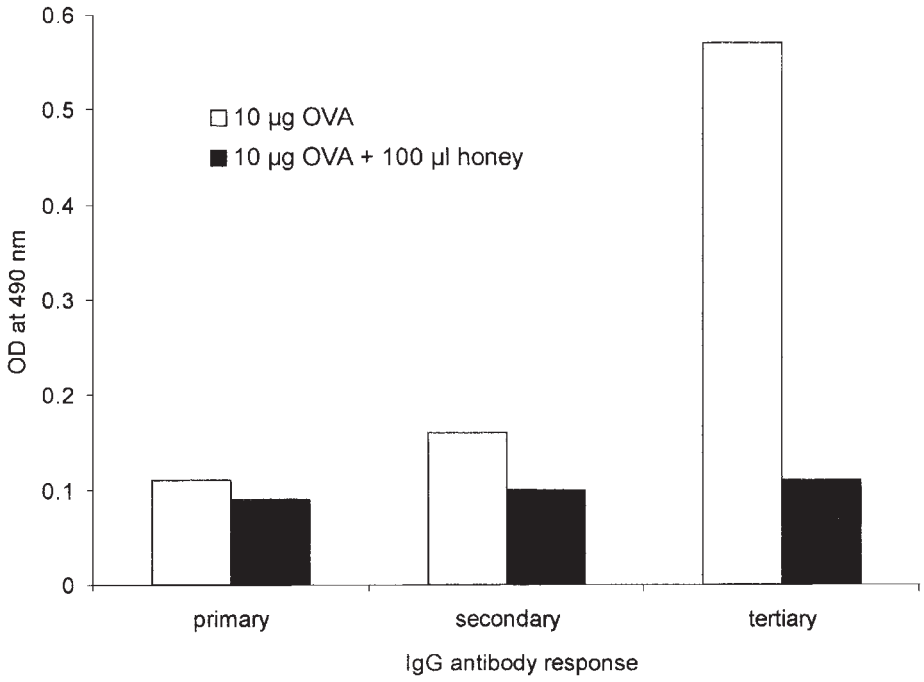

Figure 1. Down-regulatory effect of honey on antigen-specific total IgG antibody response. BALB/c mice were immunized with 10 µg OVA or OVA plus 100 µl honey on day 0. Booster doses were given on days 21 and 35. The mice were bled on days 14, 28 and 42. Sera were separated and measured the total IgG antibody responses by ELISA.

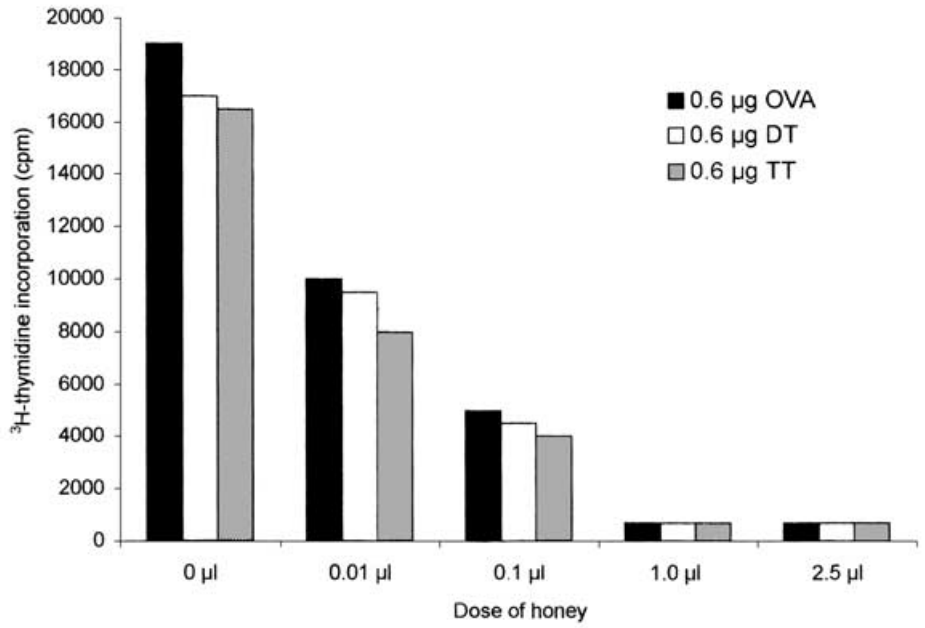

Figure 2. Suppressive effect of honey on antigen induced murine T cell proliferation. BALB/c mice were immunized with 1 µg OVA, 3 µg DT or TT in CFA on day 0. Booster doses were given on day 8 in IFA. The mice were sacrificed on day 12 for their spleens. T cell proliferation assays were conducted with splenic T cells stimulated *in vitro* with 0.6 µg each of OVA/DT/TT in absence or presence of different doses of honey.

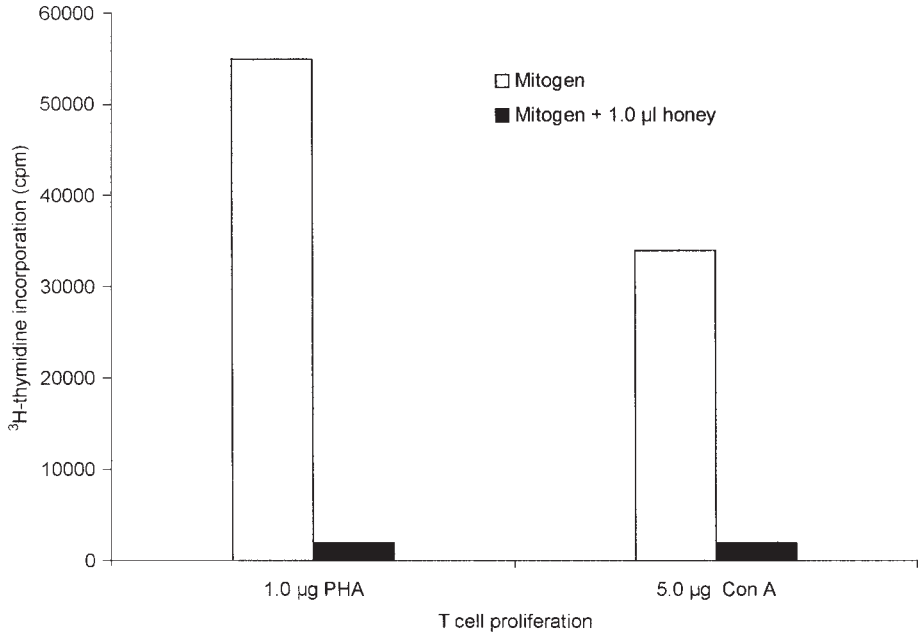

Figure 3. Down-regulatory effect of honey on mitogen-induced murine T cell proliferative response. T cell proliferation assays were conducted with splenic T cells obtained from naive BALB/c mice and stimulated *in vitro* with 1 µg PHA or 5 µg Con A in presence or absence of honey.

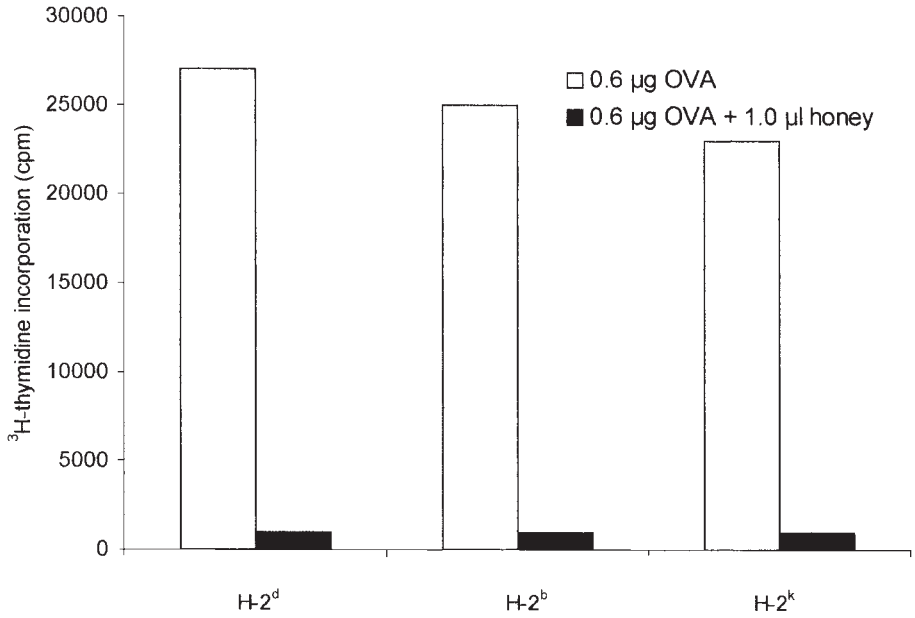

Figure 4. Inhibitory effect of honey on T cell proliferative responses of the indicated strains of mice. The same immunization protocol as that of Figure 2 was followed, T cells from different haplotypes of mice were stimulated *in vitro* with OVA in presence or absence of honey.

occurrence of allergic symptoms in pollen-sensitive subjects challenged with honey have also been reported, which may correlation with our results. This may possibly be explained by the immunosuppressive property of honey, which was further evidenced by the inhibitory activity of honey on murine T cell proliferation stimulated by antigens and mitogens.

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