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Effect of Humic Acid on Humoral Immune Response and Phagocytosis

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Abstract: Humates are found wherever matter is being decomposed, or has been transposed as in the case of sediments. As these substances have different therapeutics properties, the possible effects of humic acid (HA) from humates, on the anti body response of *Brucella melitensis* (Rev-1) vaccine and phagocytosis in rat were studied. In this study 0.25, 0.50 and 1.0 % humic acid per kilo gram of feed were evaluated. Thirty six rats were divided into six equal groups. Group 1 (Control) injected with PBS, group 2 with Rev-1 alone, group 3 with 0.5% HA alone, group 4 with Rev-1 and 0.25% HA, group 5 with Rev-1 and 0.5% HA and group 6 with Rev-1 and 1% HA. The antibody titer and phagocytosis were measured by tube agglutination (TA) test, Rose Bengal (RB) test and phagocytosis with yeast respectively. Instead of serological tests, both tests were negative in groups 1 and 3. When the mean values of antibody titers of TA in the groups were compared, significant differences ($P < 0.05$ or 0.01) were found between each of 2 and 4, 5, 6 and also between groups 4 and 5. When the mean percentage of phagocytosis were compared between groups, significant differences ($P < 0.01$) were found between group 1 and 3, 4, 5, 6 and between group 4 and 3, 5, 6. In conclusion, present results suggest that all doses of HA had a significant stimulating effect on the antibody response of *Br. Melitensis* vaccine and phagocytic activity and that 0.5% was the most effective dose.

Key words: Humic acid · Antibody response · Rev-1 vaccine · Phagocytosis

INTRODUCTION

The widespread occurrence of dysfunctions of the immune system requires new approaches. Immunomodulation through natural or synthetic substances may be considered an alternative tool for the prevention and cure of infections [1-3] and of neoplastic diseases [4, 5]. Humic substances are widely spread in nature. They occur mainly in heavily degraded peat but also in all natural environments in which organic materials and microorganisms are, or have been present [6, 7]. Humic acids from peat contain a high amount of oxygenated groups. Humic acids extracted from leonardite and lignite showed a lower content of sugar-like components and polyethers. On the other hand, the aromatic structures are ubiquitous in all samples. Special physicochemical property of humic substances is responsible for some therapeutic reactions occurring in tissues [8]. The therapeutic properties of humates have been described as antibacterial, antitoxic, antiulcerogenic, antiarthritic, antiallergic, immunomodulatory and anti-inflammatory [9-11]. Joone *et al.* [12] have shown that bituminous-coalderived humate increases the proliferative response of PHA-stimulated MNL as well as monocyte-depleted human lymphocytes. This response was even more striking in the case of lymphocytes from HIV-infected patients and was therefore not limited to the *in vitro* setting. Similar effects were observed *ex vivo* following administration of 4 g humate per day to HIV positive individuals for 2 weeks. This increase can be attributed to increased production of IL-2, as well as expression of the IL-2 receptor (CD25) on lymphocytes. Humate therefore seems to enhance the activity of TH1 cells (IL-2-producing cells) whilst decreasing, at the same time, IL-10, a TH2 associated cytokine. Obminska-Domoradzka *et al.* [13, 14] found that the administration of Tolpa Peat Preparation TPP, a mixture rich in humic substances, to immunologically mature mice causes functional stimulation of the lymphatic system cells. Daily administration of 1 mg/kg TPP for 12 weeks enhanced the response to sheep erythrocytes significantly. Jankowski *et al.* [15] performed a randomized, double blind study to assess the therapeutic efficacy of TPP in 39 patients with recurrent respiratory tract infections. The phagocytic activity of granulocytes was significantly stimulated after six months of treatment. Riede *et al.* [16]

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tested three different low-molecular humic substances (two naturally occurring humates and one synthetically prepared humate) on neutrophil function. All of these substences were capable of stimulating certain functions of human neutrophils, such as the respiratory reaction with hydrogen peroxide as main product. It was suggested that the humic substances acted as signals to change dormant human neutrophils into activated cells.

In the present study, the effect of humic acid on humoral immune response induced by *Brucella melitensis* (Rev-1) vaccine and phagocytic activity of mononuclear cells in rat were investigated.

MATERIALS AND METHODS

Animal Experiments: For this experiment, 36 male Wistar albino rats with 8 weeks old and similar live weight were used. They kept under quarantine for 1 week and the rats, which had no loose weight, were included in this study.

Diet and Management: Rats were fed *ad libitum* consumption with the conventional pellet diet. During the experiment temperature and humidity were maintained at 20-24 °C and 58%, respectively. All rats were exposed to 12:12 light: Dark cycle. Cages were cleaned twice a week and fresh water was always available via glass bottles with rubber nipples [17].

Humic Acid Administration: In this study Farmagulator® Dry (Farmavet Turkey) was used as source of Humic acid (HA). HA was administered orally during 12 weeks. Experimental groups were include 3 different doses of HA, 0.25, 0.50 and 1.0 % per kg of feed.

Vaccination: In this study, *Brucella melitensis* (Rev-1) vaccine (Razi vaccine manufacture institute, Iran) was prepared according to the manufacturer recommendations and rats were injected subcutaneously at the 10^{th} week of study.

Study Design: Six experimental groups were designed and in each group, there were 6 rats. Group 1 (normal control) rats were injected with 1 ml phosphate buffer saline (PBS) subcutaneously. Group 2 (vaccine control) rats were injected with 0.1 ml Rev-1 (Razi vaccine manufacture institute, Iran) with 0.9 ml PBS. Group 3 (HA control) rats were injected with 1 ml PBS + 0.5% HA per kg feed. Group 4 rats were injected with 0.1 ml Rev-1 with 0.9 ml PBS + 0.25% HA per kg feed. Group 5 rats were injected with 0.1 ml Rev-1 with 0.9 ml PBS + 0.5% HA per kg feed. Group 6 rats were injected with 0.1 ml Rev-1 with 0.9 ml PBS + 1% HA per kg feed.

Sample Collection: At the end of the week 12, blood samples took from heart under anesthesia. Blood samples were collected into with and with out heparin vacutainers. Serum of heparin free samples obtained following centrifugation at 3000 g for 15 min at 20 °C and stored at-20°C until analysis be done for Wright agglutination method [18-20].

Serological Tests: The RB plate agglutination test was performed by placing one drop of serum on a plate and then adding one drop of RB reagent (Razi Vaccine Manufacture, Iran). After mixing, the appearance of agglutination indicated a positive reaction. The Tube Agglutination (TA) test was done according to the procedure of Alton *et al.* [18]. A 12 tube serial dilution of serum was prepared using 10% phenolized sodium chloride solution. A standard *Br. melitensis* antigen was used [18].

PBMC Isolation: PBMCs were isolated using standard Ficoll-Paque gradient centrifugation according to the instructions of the manufacturer Pharmacia, Freiburg, Germany. Briefly, 4 ml of Ficoll-Paque gradient was pipetted into two 15-ml centrifuge tubes. The heparinized blood was diluted 1:1 in PBS and carefully layered over the Ficoll-Paque gradient. The tubes were centrifuged for 30 min at 1500 g. The cell interface layer was harvested carefully and the cells were washed twice in PBS and resuspended in RPMI 1640 supplemented with penicillin (50 U/ml)-streptomycin (50 g/ml) [21].

Yeast Particles: Frozen stored bakers' yeast (Saccharomyces cerevisiae) was autoclaved in PBS at 120 °C for 15 min. after autoclaving, the yeast particles were washed in PBS and stored at 4°C. On the day of experiment the yeast particles were washed in Hanks balanced salt solution (HBSS) and counted using a hemacytometer, mixes with RPMI 1640 containing 10% rat fresh serum and incubated 30 min in 37 °C. Yeast particles opsonized with rat fresh serum components. The yeast particles in RPMI 1640 centrifuged 10 min in 1500 g. Then the sediments were washed twice in HBSS. Sediments were solved in serum free RPMI 1640 and diluted to 10^7/ml [21, 22].

Phagocytosis Assay: A sterile glass coverslip placed in each well of a multiwell plate.1 ml of PBMC suspension, at 10^5/ml, added to each well and Incubated at 37°C for 2 h. RPMI 1640 medium removed and PBMCs washed again with RPMI 1640 medium. 1 ml RPMI medium added to each well and incubated for 2 h at 37°C. 100 µl yeast

suspension added to per plate and plates Incubated for 1 h at 37°C in a 5% CO_2 humidified incubator. PBMCs washed twice gently with RPMI 1640 medium and 1 ml 1% w/v tannic acid solution were added. Then wash with RPMI 1640 medium. Coverslip covered with a drop of heat-inactivated FBS and dried in air. Cells were stained with May-Grünwald freshly diluted 1: 2 with buffer, for 5 min. then coverslips rinsed in buffer. PBMCs stained in Giemsa solution, freshly diluted with buffer, for 15 min and rinse in buffer. At last coverslips inverted on microscope slides and observed at 100 magnification (Nikon microscope, Japan). On each coverslip PBMCs were counted until at least 100 PBMCs were scored. At access phagocytosis, the number of ingested yeast particles per counted PBMCs was determined [21, 22].

Statistical Analysis: Results are expressed as mean ±/S.E.M. Multiple comparisons were performed by ANOVA and followed by the Tukey honestly significant difference (HSD) test. In all analyses, the level of significance was set to (P</0.05 or 0.01).

RESULTS

Serological Study: Table 1 shows the means and standard error means of antibody titers against *Br. melitensis* vaccine (Rev-1) alone (group 2), Rev-1 with 0.25% HA (group 4), Rev-1 with 0.5% HA (group 5) and Rev-1 with 1% HA (group 6) using TA and RB. None of the serological tests was positive in the first and third groups (normal control and HA control, respectively). However, the tests were positive in the rest of the groups (2, 4, 5 and 6). When the mean values of antibody titers of TA were compared, significant differences were found between each of 2 and 4 (P= 0.045), 2 and 5 (P < 0.01) and 2 and 6 (P < 0.01). A significant difference was also found when the mean antibody titer of group 4 was compared with group 5 (P= 0.016).

Phagocytosis Assay: Table 2 shows the percentages mean and standard error means of phagocytic activity of PBMCs. When the percentage mean of phagocytosis of experiment groups were compared, significant differences were found between group 3 and groups 1, 2, 4, 6 (P< 0.01). A significant difference was also found when the percentage mean of phagocytosis of group 4 was compared with groups 5 and 6 (P< 0.01). Comparison of percentage means of phagocytosis of groups 1 with 2 (P=0.993), also 3 with 5 (P= 0.993) and 6 (P=0.342) shows no significant difference (Table 2).

Table 1: Effect of Humic Acid on the antibody titer of *Brucella Melitensis* antigen (Rev-1) in rats. Three different doses (0.25%, 0.5%, 1%) of HA were used. The values are expressed as mean ± SEM

Groups	Factor	Rose Bengal	Wright
1	PBS	-	-
2	Rev-1	+	1:193 ± 13.3 [a]
3	HA 0.5%	-	-
4	HA 0.25% +Rev-1	+	1:186 ± 26.6 [b]
5	HA 0.5% +Rev-1	+	1:293 ± 26.6 [c]
6	HA 1% +Rev-1	+	1:266 ± 33.7 [bc]

Values with different superscript differ significantly (P<0.05)

Table 2: Effect of Humic Acid on the Phagocytosis in rats. Three different doses (0.25%, 0.5%, 1%) of HA were used. The values are expressed as mean ± SEM

Group	Factor	Phagocytosis
1	PBS	14.66 ± 1.08 [a]
2	Rev-1	15.16 ± 1.01 [a]
3	0.5% HA	49.33 ± 2.06 [c]
4	HA 0.25% +Rev-1	30.66± 1.14 [b]
5	HA 0.5% +Rev-1	50 ± 1.52 [c]
6	1% HA+Rev-1	51.33 ± 2.23 [c]

Values with different superscript differ significantly (P<0.01)

DISCUSSION

Humic Acid and its related compounds have been applied successfully for a variety of therapeutic effects [23]. In alternative medicine, it is a long time that HA was known to improve the function of the immune system [11]. 50 different compounds have been identified with in humic matters [24]. Most of these are carboxylic acids and ordinary physiological metabolites without evidence of any toxic compound in the product mixture [25]. The results presented in this study demonstrated that the HA dose dependently potentiated the antibody response against *Br. melitensis* (Rev-1) vaccine and the phagocytic activities of mononuclear cells of rats. The administration of HA gave a significantly higher titer when compared to the group 2 (Rev-1 alone), (P < 0.05) and an increased phagocytosis percentage of mononuclear cells in comparison with group 1 (normal control). Present study as well as pervious studies show that HA have a revival effect on immune responses. Dekker and Melden [26] had an extensive study on therapeutic effects of HA in various conditions. According to their study Oxihumate (99% purified HA) increased the proliferative response of phytohemagglutinin (PHA) stimulated lymphocytes in a dose-related manner. Also, oxihumate increased the expression of CD25 significantly on PHA-stimulated cells. Oxihumate caused a statistically significant increase in IL-2 and IL-4 production by stimulated lymphocytes. A slight but not significant increase in IL-6 production by

oxihumate was also observed. An increase in IL-2 is necessary for cell-mediated (T_H1-type) immunity, as well as an increase in IL-4 and IL-6, is necessary for humoral (T_H2-type) immunity, indicate that this compound can be an effective immunostimulant which may prove suitable for application by patients suffering from viral and bacterial infections. Oxihumate increased LTB4 production by PMA-stimulated human lymphocytes more than 300% compared to the untreated controls at all 3 concentrations tested. The role of leukotrienes in the immune response is less clear. Various researchers [26] have reported that LTB4 can act as a multifunctional regulator of cytokine production and that it is able to stimulate both T_H1 and T_H2 subpopulations. LTB4 not only plays an important role in the induction of the production of IL-2 but also enhances the activity of cytotoxic T-cells and this could possibly explain the increase in IL-2 production as well as the increase in cytotoxicity of oxihumate-treated lymphocyte cultures. Dekker and Melden [26] reported that chickens treated with 100 mg oxihumate/kg/day showed a two to four fold increase in titers against Newcastle disease. This result indicates the ability of this compound to stimulate the humoral immune response. This is the only available report about effect of HA on humoral immune response. Obminska-Domoradzka *et al.* [13, 14] found that the administration of Tolpa Peat, a mixture rich in humic substances, to immunologically mature mice causes functional stimulation of the lymphatic system cells. Daily administration of 1 mg/kg TPP for 12 weeks enhanced the response to sheep erythrocytes significantly. Humic acid also increase the number and functional activity of macrophages, neutrophils and killer T-cells, in laboratory experiments [16]. It was found that Tolpa Peat administered to healthy volunteers in doses of 100 to 300 mg/day for 14 days evoked the stimulation of the phagocytic and bactericidal activity of the granulocytes [27]. Van Rensburg *et al.* [25, 28] found that this product stimulates lymphocyte proliferation, important for cell mediated immunity necessary to cope with opportunistic infections, by increasing the production of the Interleukin-2 (IL). Jooné *et al.* [12] found that oxihumate increased the *in vitro* proliferative response of PHA-stimulated human lymphocytes, from a concentration of 20 μg oxihumate/ml and upwards.

In conclusion according to founding of present study, HA dose dependently can modulate the antibody response a phagocytic activities of mononuclear cells and 0.5%/kg feed was the most effective dose. However this warrants further evaluation of humic substances on other vaccines in different species and evaluation of molecular aspects of phagocytosis in presence of HA.

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