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Anti-Allergic Properties of Humic Acids Isolated from Pine-Sphagnum-Cotton Sedge Peat

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The course administration of humic acids isolated by the sodium pyrophosphate method from pine-sphagnum-cotton sedge peat reduced the general anaphylaxis reaction in mice and guinea pigs immunized with ovalbumin and decreased serum content of IgG1 and IgE in mice. The serum from mice treated with humic acids and sensitized with ovalbumin did not increase the rate of degranulation of mast cells isolated from intact Wistar rats in the presence of ovalbumin in comparison with the serum of control animals.

Key Words: *humic acids; anaphylaxis; mast cell degranulation; ovalbumin*

The prevalence of allergic diseases in the world has acquired the scale of an epidemic and has become a serious medical and social problem. Allergic reactions are formed as a result of activation of mast cells that upon contact with allergen-specific IgE release such mediators as histamine, serotonin, and leukotrienes. The drugs used to relieve allergic reactions can be divided into two groups: H1-antihistamines that block histamine receptors and mast cell membrane stabilizers that prevent calcium entry into mast cells and inhibit their calcium-dependent degranulation. Pharmacological agents that would suppress the development of allergic reactions at the level of early mechanisms have not yet been created.

In recent years, growing interest was attracted to in the study of natural compounds such as peat and sapropel widely distributed throughout the earth's surface. The humic substances contained in peat and sapropel and mainly presented by humic and fulvic acids (80-90%) often become the objects of research in experimental medicine.

Antioxidant, hepatoprotective, adaptogenic, wound healing, and antibacterial activities of humic substances have been demonstrated [1]; they stimulate proliferation of activated lymphocytes, but at the same time suppress the production of TNFα, IL-1β, IL-6, and IL-10, as well as the classical and alternative pathways of complement activation [11].

Humates reduce paw edema in rats caused by carrageenan, allogeneic graft-versus-host reaction [10], hypersensitivity associated with cutaneous application of dinitrofluorobenzene [12], the level of C-reactive protein in osteoarthritis [8], the number of eosinophils in nasal smears of patients with allergic rhinitis, as well as skin tests for allergens [6]. At the same time, the toxic effects of humic substances in experimental animals after their peroral administration or skin application were not revealed [12].

Humic acids (HA) of the peat are nitrogen-containing high-molecular-weight compounds with a cyclic structure. They are a dark-colored mixture of organic, high-molecular-weight, aromatic, methoxy-containing hydroxy- and oxocarboxylic acids that have a common structure and some differences determined by their origin [2].

We have previously demonstrated that peat HA stimulated the production of nitric oxide by antigen-presenting cells that did not depend on endotoxin admixture, inhibited arginase activity, and increased secretion of some proinflammatory cytokines (IL-1β, IL-12, TNFα, and IFNγ) [4,5]. Since these substances

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shift the balance of cytokines towards the Th1-dependent immune response, we assume that HA sample under study can reduce the production of anti-inflammatory cytokines and, as a consequence, suppress Th2-dependent reactions associated with IgE production and histamine release.

Here we studied the effect of course administration of HA on the general anaphylaxis reaction, production of IgG1 and IgE, and the reaction of mast cell degranulation in animals sensitized with ovalbumin.

MATERIALS AND METHODS

The HA sample was obtained as follows. Peat samples obtained from the genetic center of the Vasyuganskoye peat deposit in the Bakcharsky district of the Tomsk region were air-dried at room temperature, ground in a rotary knife mill, sifted through a sieve with a hole diameter of 3 mm, and treated with 0.1 M sodium pyrophosphate (1:50-1:100 w/w). After constant stirring for 8 h in a R-100 reactor at 25-27°C, HA (liquid phase) was separated from the solid peat residues by vacuum filtration (suction filter), the extract was brought to pH 1-2 with hydrochloric acid, HA were precipitated by centrifugation, the precipitate was washed with water to pH 7.0 and dried at room temperature.

According to the botanical and gravimetric analysis, as well as by general technical characteristics, the selected sample was a pine-sphagnum-cotton sedge peat, the degree of decomposition of plant residues was 30-35%, the content of mineral impurities (total ash) did not exceed 7.2%, the content of bioactive HA was 13.2 wt%.

The molecular weight distribution of humic acids was assessed by size exclusion HPLC using a Dionex Ultimate 3000 chromatographic system (Thermo Fisher Scientific). The number-average molecular weight (Mn) of the obtained HA was 6110.2 Da, the weight-average molecular weight (Mw) was 22,783.9 Da, the peak molecular weight (Mp) was 11,798.9 Da, and the polydispersity (p) was 3.7.

The elemental composition and atomic ratios of elements (C, H, N, and O) in the humic acid molecule were determined by the combustion method on a EuroEA 300 elemental analyzer (EuroVector), the oxygen content was determined by the difference. The atomic fractions of elements were as follows: carbon (C) 38.57±0.48%$_{atom}$, hydrogen (H) 42.46±0.44%$_{atom}$, nitrogen (N) 1.40±0.02%$_{atom}$, oxygen (O) 17.57±0.22%$_{atom}$. The atomic ratio H/C of 1.10 indicated that the molecule of the studied HA has an aromatic hydrocarbon backbone with aliphatic chains of up to 10 carbon atoms, the atomic ratio O/C of 0.46 attested to high content of oxygen-containing functional groups, the atomic ratio of C/N was equal to 27.55 and was the mean value of nitrogen content in the HA molecule.

The experiments were carried out on outbred CD1 mice, outbred Wistar rats at the age of 8-10 weeks and guinea pigs at the age of 6 weeks, obtained from the Department of Experimental Biological Models of the E. D. Goldberg Research Institute of Pharmacology and Regenerative Medicine. The experiments were approved by the Committee for the Humane Treatment of Animals of the E. D. Goldberg Research Institute of Pharmacology and Regenerative Medicine (protocol No. 98122015). All procedures were carried out in accordance with GOST 33215-2014 (Rules for the Equipment of the Premises and Organization of Procedures for Working with Laboratory Animals).

For modeling Th2-dependent immune response, the mice were injected twice with an interval of 3 weeks under the skin of the thigh with 100 μg ovalbumin (OVA) and 5 mg aluminum hydroxide (both from Sigma) in 0.1 ml normal saline as a solvent. HA was injected intraperitoneally in a dose of 1 mg/kg body weight in 0.1 ml once a day over 5 days before each OVA injection and over 5 days after the second immunization (a total of 15 injections). Control mice were injected with 0.1 ml saline. In 7 days after the last immunization, the mice were intravenously challenged with OVA (10 μg in 0.1 ml saline) for induction of general anaphylaxis reaction, or the blood was taken from the heart of animals anesthetized with carbon dioxide, and the serum was prepared by centrifugation and the concentration of immunoglobulins or indirect mast cell degranulation were measured (IMCD).

The severity of anaphylactic shock was assessed by animal mortality (%) within 2-4 h.

The content of IgG1 and IgE in the blood serum of mice was measured the ELISA using appropriate test systems (Invitrogen).

The indirect degranulation response was evaluated in mast cells isolated from the peritoneal cavity of intact Wistar rats. A suspension of mast cells, mouse blood serum, and OVA in a concentration that does not cause spontaneous degranulation were applied in equal proportions to degreased and stained with neutral red glasses, covered with glass, and incubated for 15 min at 37°C. The number of cells with different degrees of degranulation was counted (per 100 cells) under a light microscope. The degranulation index (DI) was determined by the formula [3]:

$$(1a+2b+3c+4d)/100,$$

where a, b, c, d are the numbers of cells with mild, moderate, sharp and complete degranulation, respectively.

For modeling anaphylactic reaction in guinea pigs, the animals were orally immunizing with 1%

OVA solution in normal saline in a dose of 0.5 ml per 250 g body weight over 3 days. Starting from the first day of immunization, guinea pigs of the experimental group were intraperitoneally injected with HA in a dose of 1 g/kg body weight in saline once a day over 7 days; controls received an equivalent volume of saline. In 14 days after the first immunization, guinea pigs were injected intravenously with OVA solution in saline in a dose of 1 mg per 300 g body weight. The Weigle anaphylactic index (AI) was calculated by the formula [3]:

$$[(n\times4)+(n_1\times3)+(n_2\times2)+(n_3\times1)+(n_4\times0)/n+n_1+n_2+n_3+n_4],$$

where n is the number of guinea pigs that have died and n_1, n_2, n_3, and n_4 are the number of animals with severe, moderate, and mild shock, and without shock, respectively.

The data were processed statistically using Statistica 13.3 software (StatSoft, Inc.); the Student's *t* test was applied. Normality of distribution was verified using the Shapiro—Wilk test. For each sample, the arithmetic mean (*X*) and error of the mean (*m*) were calculated. The data for anaphylactic shock are presented as relative coefficients. The differences were significant at $p<0.05$.

RESULTS

The dynamic balance of Th1 and Th2 is an integral part of the regulation of the immune response. Th1 cytokines (IFNγ, IL-2, and IL-12) tend to induce proinflammatory reactions responsible for the destruction of intracellular parasites and the maintenance of autoimmune reactions. Th2-type cytokines (IL-4, IL-5, and IL-13) are associated with IgE production and eosinophilic response in atopy, and IL-10 has a significant anti-inflammatory response.

Many researchers consider allergy as a shift of the immune response towards the Th2 and are exploring the ways to redirect the allergic Th2 response towards the increase in Th1 in order to reduce the manifestation of atopy, for example, by using high doses of the allergen [7] or mycobacterial vaccines [9].

As the studied HA sample has the ability to stimulate the Th1-dependent response and the production of proinflammatory cytokines [4,5], it was interesting to study its anti-allergic properties.

Course administration of the studied HA sample reduced the severity of the of general anaphylaxis reaction. In mice treated with HA, mortality decreased by 50% and characteristic signs of the reaction (scratching, convulsions, lateral posture) were less pronounced (Table 1). In guinea pigs receiving course treatment with HA, the Weigle index of systemic anaphylaxis reaction was 0.9 (1 animal developed moderate shock, 7 developed mild shock, and 2 had no anaphylactic reaction). In the control group, all animals showed signs of anaphylactic shock and the Weigle index was 2.4 (severe and moderate shock was observed in 4 and 6 guinea pigs, respectively) (Table 1). Thus, course administration of HA from pine-sphagnum-cotton sedge peat led to inhibition of the intensity of the systemic anaphylactic reaction in mice and guinea pigs.

TABLE 1. Effect of Course Administration of HA from Pine-Sphagnum-Cotton Sedge Peat on Mortality of Outbred CD1 Mice during Anaphylactic Shock and Intensity of Anaphylactic Shock in Guinea Pigs

Parameter	Control (saline)	Experimental group (HA sample)
Number of animals	10	10
Mortality, % (mice)	80	40
Weigle anaphylactic index (guinea pigs)	2.4	0.9

TABLE 2. Influence of Course Administration of HA from Pine-Sphagnum-Cotton Sedge Peat on Serum Content of IgG1 and IgE in Outbred CD1 Mice Immunized with OVA (*X*±*m*; *n*=5)

Concentration	Control (saline)	Experimental group (HA sample)
IgG1, μg/ml	2004.61±98.92	924.88±86.97*
IgE, ng/ml	13.37±0.24	10.48±0.44*

TABLE 3. Effect of Course Administration of HA from Pine-Sphagnum-Cotton Sedge Peat on Antigen-Depended Degranulation Index of Mast Cells (*X*±*m*; *n*=5)

Group	HA in the presence of blood serum	
	+saline	+OVA
Control (saline)	0.040±0.007	0.094±0.009*
Experimental (HA)	0.110±0.009	0.148±0.021

Note. *$p<0.05$.

In addition, course administration of the studied sample of HA reduced the serum content of IgE (by 1.3 times) and IgG (by 2.1 times) in mice immunized with OVA (Table 2).

Incubation of mast cells with the serum from mice sensitized with OVA and receiving a course of the studied HA in the presence of OVA did not significantly change the index of degranulation (Table 3). The blood serum of mice treated with saline in the presence of antigen caused a 2-fold increase of the degranulation index.

Thus, it has been experimentally established that HA of pine-sphagnum-cotton sedge peat from the Vas-

yuganskoye deposit of the Bakcharsky district of the Tomsk region have antiallergic effect due to suppression of IgE and IgG1 synthesis and stabilization of mast cell membranes, which leads to inhibition of the pathological reactions associated with the production and release of allergy mediators, and thus can expand the arsenal of low-toxic herbal anti-allergic agents.

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