Immuno- and Phagocytosis-Modulating Properties of Low Doses of B_1-Aflatoxin and Benzene

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Benzene or benzene-dissolved B_1-aflatoxin in low doses promotes an increase of the Thy-1^+ cell count in the bone marrow of mice and an enhancement of the thymus-dependent immune response. *In vitro* aflatoxin and benzene are unable to induce the expression of Thy-1-antigen in bone marrow T-precursors.

Key Words: *B_1-aflatoxin; benzene; immune response; phagocytosis*

It is known that low concentrations of the aromatic compounds B_1-aflatoxin and benzene are widespread in the environment and are potentially hazardous for man and animals [4,6]. Therefore, it seems to be important to study the effect of low doses of these substances on the indexes of specific and nonspecific defense. The importance of the problem is also underscored by the similarity of the mechanisms underlying the damage caused by benzene and radiation [4].

The aim of the present study was to investigate the effect of low doses of B_1-aflatoxin and benzene on the immune response and phagocytosis in mice.

MATERIALS AND METHODS

In vivo experiments were carried out on 248 male CBA mice weighing 14-16 g. Benzene (Reanal, Hungary) and B_1-aflatoxin (manufactured at the Institute of Nutrition, Russian Academy of Medical Sciences, Moscow), dissolved in benzene (10.5 μg/ml), were emulsified in physiological saline in dilutions 10^{-4}, 10^{-8}, and 10^{-16} and administered per os via a probe over 10 days. Control animals received physiological saline following the same scheme. The mice were then divided into 4 groups. The 1st group was intravenously immunized with sheep erythrocytes (SE) (2×10^6 cells) or with Vi-antigen (0.001 μg/mouse), and on day 4 post-immunization the number of IgM-antibody-producing cells (APC) was determined in the spleen of each mouse.

For determination of APC to Vi-antigen, SE were loaded with this antigen. The final concentration of antigen was 20 μg/ml. The unbound Vi-antigen was washed out of erythrocytes with physiological saline at least 8 times. The number of APC was counted per 10^6 karyocytes of the spleen.

The phagocytic activity of peritoneal neutrophils was measured in nonimmunized mice of the 2nd group [2]. The activity of neutrophils was also assessed *in vitro*. Exudates for *in vivo* and *in vitro* assessment of phagocytosis were obtained 2.5 h after a 10% sterile peptone solution was injected intraperitoneally. The final concentration of cells (neutrophils comprising 98% of them) in the exudates was 12.5×10^6/ml. A one-day *St. aureus* cul-

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TABLE 1. Effect of B_1–Aflatoxin and Benzene on Immune Response to Sheep Erythrocytes in Mice ($M \pm m$)

Preparation	Number of IgM-APC per 10^6 karyocytes in spleen after administration of preparations in dilutions		
	10^{-4}	10^{-8}	10^{-16}
B_1–aflatoxin in benzene	28.4±4.6**	34.2±7.8**	37.6±3.3*
Benzene	18.5±7.0	43.2±8.9*	26.6±4.8**
Physiological saline (control)	15.0±1.0	15.0±1.0	15.0±1.0

Note. Here and in Table 2: one and two asterisks show reliable differences (for $p<0.01$ and $p<0.05$, respectively) from the control.

ture with a final concentration of 250×10^6/ml served as the test microbe. The phagocytic (granulopectic) index - the percentage of neutrophils involved in phagocytosis - and the phagocyte count - the mean number of microbial cells per leukocyte - were determined. A minimum of 900 to 1000 neutrophils were counted.

In nonimmunized animals of the 3rd group the function of macrophages [3,7,9] was assessed as the rate of dye elimination from the blood flow. The dye was injected in the caudal vein as a mixture of India ink suspension (Pelikan, Germany) and 3% gelatin (2:3) in a dose of 0.1 ml/mouse. Blood samples were taken from the retroorbital sinus after 10 sec, 1 and 2 min, and then at 5-min intervals until the dye disappeared from the blood flow. The presence of ink in the samples was detected at 670 nm using calibration curves. The clearance rate constant (K) and the clearance index (K_1) were determined: $K = log_2 / T_{1/2}$, where $T_{1/2}$ is the half-time of dye disappearance [3]; and $K_1 = (logC_1 - logC_2) / T$ [9], where C_1 and C_2, respectively, are the maximum and the minimum concentrations of the dye (in μg) in the sample, and T is the time of dye elimination (min).

In the lymphoid organs of nonimmunized animals of the 4th group the Thy-1^+ cell count was determined. The number of Thy-1^+ cells was counted in the complement-dependent cytotoxic test [1] with the aid of antiserum against the cerebral cortex tissue of CBA mice. The antiserum was absorbed by a homogenate of mouse liver, by murine erythrocytes, and by SE [1] and diluted 1:50. In the presence of fresh guinea pig complement (1:3) the antibrain serum in this concentration caused death of 88.0±1.3% of thymocytes and did not interact with the bone marrow cells of CBA mice. Not less than 200 cells, the viability of which was assessed with 0.2% aqueous solution of Trypan Blue (Sigma), were counted in each sample. The experiment was repeated at least 2-3 times.

The *in vitro* expression of Thy-1 antigen, induced by B_1-aflatoxin or benzene (in dilutions 10^{-8} and 10^{-16}), was also measured in T precursors with the aid of antibrain serum (1:50) in the complement-dependent cytotoxic test after a 30-min exposure of the preparations to bone marrow cells at 37°C.

RESULTS

As is shown in Table 1, benzene and B_1-aflatoxin dissolved in benzene in the studied doses diversely affected the thymus-dependent immune response to SE. Benzene in a 10^{-4} dilution (44 μg/mouse) reduced the Thy-1^+ splenocyte count 3.8-fold without altering the immune response (Table 2). Benzene in a 10^{-8} dilution (4.4×10^{-3} mg/mouse) raised the number of APC 2.9-fold, irrespective of a 2.6-fold reduction of the number of Thy-1^+ cells in the spleen, while in a dilution of 10^{-16}, when normalization of the Thy-1^+ splenocyte count was already observed, it enhanced the immune response just 1.8-fold. The benzene solution of B_1-aflatoxin, the concentration of which (for dilutions of 10^{-4}, 10^{-8}, and 10^{-16} it was 0.5×10^{-3}, 0.5×10^{-7}, and 0.5×10^{-15} μg/mouse, respectively) was 88,000 times lower than the concentration of the solvent, in all the above-mentioned doses enhanced the immune response without altering the Thy-1^+ splenocyte count (Tables 1 and 2).

In the case of B_1-aflatoxin or benzene in a dilution of 10^{-8} the immune response to thymus-independent Vi-antigen was unchanged: the number of APC was 8.0±0.3 and 9.4±0.5, respectively, vs. 9.1±0.7 in the control (15 mice being examined in each group).

Studies of the *in vivo* effect of B_1-aflatoxin and benzene on the function of macrophages demonstrated that the activity of macrophages did not alter: in the case of B_1-aflatoxin or benzene in dilutions of 10^{-4}, 10^{-8}, and 10^{-16} the clearance rate constant for India ink did not depend on the concentration of the preparations and varied from 0.011±0.0013 to 0.012±0.0007 and from 0.014± ±0.0015 to 0.016±0.003, respectively, vs. 0.011± ±0.0017 in the control; the clearance index was also unchanged and varied from 0.014±0.002 to

TABLE 2. Effect of B_1–Aflatoxin and Benzene on Thy–1^+ Cell Count in the Bone Marrow, Spleen, and Thymus ($M \pm m$)

Preparation	Thy-1^+ cell count in lymphoid organ (cytotoxicity index of antibrain serum, %) after administration of preparations in dilutions								
	10^{-4}			10^{-8}			10^{-16}		
	BM	S	T	BM	S	T	BM	S	T
B_1–aflatoxin in benzene	15.6±2.6*	20.0±2.8	71.9±3.2	8.8±2.0*	23.7±3.0	88.3±2.3	8.3±1.9*	24.7±3.0	84.8±2.5
Benzene	14.9±2.5*	5.2±1.5*	83.8±2.6	25.6±3.0*	8.9±2.0*	84.6±2.5	17.9±2.7*	19.3±2.8	77.1±3.0
Physiological saline (control)	0	20.0±2.8	84.7±2.5	1.5±0.8	22.9±3.0	85.5±2.4	1.3±0.8	21.5±2.9	80.1±2.8

Note. BM: bone marrow; S: spleen; T: thymus. The cells of bone marrow and spleen were treated with 0.65% NH_4Cl for removal of erythrocytes. The viability of the bone marrow and spleen cells in Hanks' solution in the presence of complement (1:3) was 85–90% and the viability of thymocytes 90–95%.

0.016±0.002 and from 0.017±0.003 to 0.018± ±0.003, respectively, vs. 0.017±0.0019 in the control (8 to 9 mice being examined in each group).

After the animals were given the preparations in dilutions of 10^{-4}, 10^{-8}, and 10^{-16}, the number of Thy-1^+ cells in the bone marrow increased from 0-1.5% in the control to 8.3-25.6%. The effect of benzene was more pronounced (Table 2).

In vitro treatment of the bone marrow cells with B_1-aflatoxin or benzene in concentrations not producing a direct cytotoxic effect (10^{-8} and 10^{-16}) did not induce the expression of Thy-1 antigen in T precursors: the cytotoxicity index varied within the control values: 0-1.5±0.8%. Introduction of the test preparation to mice did not affect the T-cell count in the thymus (Table 2).

Benzene (10^{-8}) did not alter the phagocytic activity of neutrophils *in vitro* or *in vivo*: the phagocytic index was 26.0±2.0 and 23.1±2.1%, respectively, vs. 23.2±2.2 and 23.8±2.2% in the control. On the other hand, B_1-aflatoxin in the same dilution (10^{-8}) enhanced the phagocytic activity of neutrophils: the *in vivo* and *in vitro* phagocytic index was, respectively, 49.1±3.8 and 38.3±2.0% vs. 23.2±2.2 and 23.8±2.2 in the control ($p<0.01$). In the *in vivo* and *in vitro* experiments the phagocyte count did not change under the influence of the preparations and varied from 1.8±0.07 to 2.0±0.08 (versus 1.8±0.08-1.9±0.07 in the control). In each group the phagocytic activity of neutrophils was assessed using cells derived from 5-7 mice. The experiment was repeated not less than 2 times.

Our findings demonstrate that the per os administration of low doses of B_1-aflatoxin or benzene over a short (10 days) time results in a pronounced immunostimulation.

Meanwhile, it is worthy of note that the immunostimulation induced by benzene (but not by B_1-aflatoxin) is accompanied by a decrease in the number of Thy-1^+ splenocytes, and that not a reduction, but, on the contrary, an enhancement of the immune response goes along with the drop of the Thy-1^+ cell count. This phenomenon is typical not only of T-cell but also of macrophage deficiency, since a decrease in the number of macrophages is also attended by stimulation of the thymus-dependent immune response [8]. Thus, the quantitative deficiency of the immunocompetent cells does not directly correlate with their function.

Stimulation of a thymus-dependent, but not of a thymus-independent immune response is indicative of the involvement of the T rather than the B system in the process. The involvement of the T system is realized nonspecifically, probably, by corticosteroid-induced migration of T cells to the bone marrow [10] with a subsequent accelerated differentiation of the brainstem hemopoietic cells under their influence [5], by activation of T- and B-cell cooperation, and by an enhancement of the immune response. This postulate is corroborated by the appearance of Thy-1^+ cells in the bone marrow after mice were fed B_1-aflatoxin or benzene but not after the bone marrow cells were directly treated *in vitro* with the preparations.

Along with the above, the immunostimulating effect of benzene seems likely to be realized by suppression of T suppressors (which are highly sensitive to benzene [4]), followed by activation of T helpers.

The *in vivo* and *in vitro* enhancement of the phagocytic activity of neutrophils in the case of B_1-aflatoxin, but not in the case of benzene, attests to the nonuniformity of the population of neutrophils and is evidently due to the presence of B_1-aflatoxin-receptors on the membranes of some neutrophils.

B_1-aflatoxin and benzene were tested for short-term administration. In order to better approximate the real ecological situation, it is necessary that the

time of the organism's exposure to the preparations be prolonged.

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ONCOLOGY

Increased Resistance to P3-X63-Ag8.653-MOPC Plasmacytoma after Exposure to Repeated Stress

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The antitumor effect of multiply repeated stress is demonstrated. The maximal inhibitory effect on tumor growth is achieved by a moderate physical load (swimming), the efficacy of immobilization stress is somewhat lower, while intensive physical load is considerably less effective.

Key Words: *chronic stress; tumor growth*

Reports that exposure to stress before transplantation or induction of a tumor may have an antitumor effect are of great interest [1-5,7]. The inhibition of tumor growth has been achieved by electrical shock [3], cold stress [4], handling [7], and periodic separation of newborn mice from the mother [5]. The effect has been demonstrated on various models of experimental tumor process: MSV virus inoculation [3], transplantation of Gross virus-induced lymphoma [4], Walker 256 carcinoma [7], and Ehrlich ascitic carcinoma [5]. However, there are also some reports that stress stimulates [8,9] or has no effect [6] on tumor growth.

Bearing in mind that the present data on this topic are contradictory and limited, in the present study we attempted to develop an experimental model for studying the effect of different kinds of stress in the pretransplantation period on antitumor resistance.

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MATERIALS AND METHODS

The experiments were carried out on 164 female BALB/c mice aged 6 to 8 months. The P3-X63-

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