Effect of Pine Resin and Biopin Ointment on T and B Cell Immunity

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We studied the effects of pine resin and Biopin ointment used for the therapy of burns, wounds, purulent and inflammatory diseases on differentiation of T lymphocyte precursors, lymphocyte proliferation in the thymus and spleen, and production of tumor necrosis factor and interleukins 1, 2, and 6 by splenocytes and peripheral blood leukocytes. The wound-healing effect of these preparations is determined by their pronounced immunomodulatory properties.

Key Words: *pine resin; Biopin ointment; wound-healing effect; immunomodulation*

Extensive thermal and mechanical damages to the skin and subcutaneous tissues are accompanied by immunodeficiency, which depends on the severity of injuries and is associated with impaired cytokine synthesis and qualitative and quantitative changes in cell immunity [2,3]. Proliferation and maturation of immune cells are induced by mitotic factors. Various inflammatory mediators, including interleukins (IL-1, IL-2, and IL-6) and tumor necrosis factor (TNF) produced by macrophages, play an important role in these processes.

IL-1 is an inducible protein produced by monocytes and macrophages in response to tissue damages or microbial invasion. This cytokine is involved in protective acute-phase reactions [4]. IL-1 is normally absent in the circulation, but it can appear in the blood during severe infections. Plasma IL-1 concentration correlates with the rise in body temperature and contents of other cytokines. Maximum IL-1 concentrations were detected in patients with septic fever. IL-1 promotes migration of neutrophils into damaged regions, activates these cells, induces synthesis of IL-8 and other cytokines, and stimulates chemotaxis, degranulation, and production of reactive oxygen species. IL-1 stimulates proliferation of fibroblasts and production of prostaglandins, growth factors, and cytokines, including colony-stimulating factors, interleukins, and interferon.

In vivo systemic and *in vitro* cellular effects of TNF are similar to those of IL-1. IL-2 is produced by IL-1-sensitive T lymphocytes, potentiates their blast transformation in response to mitogens, and stimulates production of interferon responsible for immunoglobulin secretion. Receptors for IL-2 are present on membranes of various cells, including T helper cells.

IL-6 produced by T and B cells, activated Kupffer cells, macrophages, and enterocytes [5] is involved in the pathogenesis of immunodeficiency. Production of IL-6 is initiated by traumas or endotoxins released by gram-negative microorganisms, it peaks on days 3-4, and directly depends on the area of damages. IL-6 potentiates the effects of IL-1 and TNF, induces the release of acute-phase proteins, stimulates proliferation of B cells, affects the hypothalamus, and causes fever. Blood IL-6 concentration increases at the early stage after traumas in patients with high risk of sepsis and, therefore, serves as a prognostic factor [6].

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Here we studied the effects of pine resin (PR) and Biopin ointment (BO) used for the therapy of burns, wounds (phase I of wound process), purulent and inflammatory diseases of the skin and subcutaneous fat [1] on lymphocyte proliferation and cytokine production.

MATERIALS AND METHODS

In vivo effects of PR and BO were studied on 230 male CBA mice weighing 16-18 g and obtained from the Rappolovo nursery (Russian Academy of Sciences). Each group (n=46) included 5 animals. Thymogen and interleukin-1β (IL-1) were used as reference agents. Test preparations were dissolved in physiological saline and injected intraperitoneally in doses of 0.5, 5, 50, 500, and 5000 μg/kg. Control mice received an equivalent volume of physiological saline. The animals were decapitated 4 and 24 h postinjection. Lymphoid organs were dissected, bone marrow cells, thymocytes, and splenocytess were transferred into 96-well plates. The effects of preparations on differentiation of T cell precursors were studied on isolated bone marrow cells by expression of Thy-1 antigen using anti-Thy-1 serum (complement-dependent cytotoxic test). The effect of the test preparations on proliferation of lymphocytes in the thymus and spleen stimulated

TABLE 1. *In Vivo* Effects of Preparations on Proliferation of Lymphocytes in Mouse Thymus and Spleen (Incorporation of ^{3}H-Thymidine, Decays/Min, $M\pm m$, n=5)

Organ; preparation dose, μg/kg	PR	BO	Thymogen	IL-1
Thymus				
4 h after injection				
0$^+$	2960±240	13,890±1460	6930±710	3120±220
0.5	3120±270	13,410±1270	4400±230	5090±190*
5	2860±390	12,640±1812	5190±460	7430±260*
50	3820±470	12,310±1640	8650±520	9980±370*
500	2990±560	18,320±1960	12,940±310*	12,740±510*
5000	3210±480	16,400±1830	11,660±630*	—
24 h after injection				
0$^+$	2460±520	2930±370	7144±760	3630±410
0.5	3220±620	5420±590*	8917±930	6140±410*
5	2890±410	5720±514*	5986±460	9830±780*
50	5960±380*	4660±412*	5220±480	13,120±970*
500	6860±290*	10,030±810*	5560±690	17,130±1320*
5000	4120±290*	4880±370*	13,190±840*	—
Spleen				
4 h after injection				
0$^+$	14470±370	29240±560	36170±2110	23140±1580
0.5	16,700±940	24,100±320	23,360±2120	26,110±2470
5	14,820±970	28,940±640	32,113±1630	32,430±2620*
50	14,370±910	28,970±1960	29,290±1260	38910±2990*
500	14,690±860	20,680±710	28,070±1310	46,230±3420*
5000	11,980±960	26,300±260	32,790±1425	—
24 h after injection				
0$^+$	18,686±940	29,300±370	7144±760	29,230±1220
0.5	33,506±3140*	23,890±1680	38,350±1380*	36,180±1340*
5	33,180±460*	30,146±2420	42,190±2230*	43 990±2370*
50	37 790±1390*	26 190±1910	46 490±3620*	56,500±3210*
500	51,770±2460*	26,120±2110	41,150±1510*	71,090±4710*
5000	31,730±2110*	32,820±2260	49,890±2310*	—

Note. Here and in Tables 2 and 3: $^+$control; *p<0.05 compared to the control.

by 0.5 μg/ml concanavalin A was evaluated by lymphocyte blast transformation. Incorporation of ^{3}H-thymidine was analyzed using a Mark-3 counter (LKB). Production of IL-2, IL-6, and TNF by splenocytes cultured for 24 h and stimulated with 5 μg/ml concanavalin A was estimated by biological activity (U/ml) using factor-dependent CTLL-2, B9, and L929 cells, respectively.

We studied *in vitro* effects of BO on spontaneous (baseline) and induced production of IL-1, IL-2, and TNF by human peripheral blood leukocytes. To this end, heparinized blood was 6-fold

TABLE 2. *In Vivo* Effects of Preparations on Production of IL-2, IL-6, and TNF by Mouse Spleen Cells (Tests with CTLL-2, B9, and L929 Cells, Respectively, *M*±*m*, *n*=5)

Cytokine; preparation dose, μg/kg	PR	BO	Thymogen	IL-1
IL-2				
4 h after injection				
0^{+}	34.9±2.8	28.7±3.0	25.4±2.2	26.0±2.0
0.5	30.4±2.8	27.9±2.6	26.5±2.1	27.3±2.9
5	31.8±2.6	27.4±3.1	26.5±1.8	29.9±2.5
50	26.0±3.8	33.4±3.2	27.8±2.3	34.7±3.1
500	29.0±3.2	39.6±3.1*	20.5±2.6	39.8±4.6*
5000	24.6±2.9	49.9±4.1*	24.3±1.6	—
24 h after injection				
0^{+}	28.4±1.3	32.9±3.2	20.6±2.0	23.1±2.1
0.5	29.6±3.1	34.5±3.6	25.4±1.8	39.8±2.0*
5	30.2±4.3	41.5±3.7	23.8±1.9	65.7±5.0*
50	36.4±2.1	74.9±4.2*	21.5±2.0	86.1±8.5*
500	42.0±3.2*	86.1±5.6*	20.5±1.2	98.7±8.2*
5000	20.6±1.3	78.5±5.3*	20.5±2.3	—
IL-6				
4 h after injection				
0^{+}	181±29	320±33	520±42	380±45
0.5	110±23	300±26	450±47	300±29
5	130±19	290±31	420±35	290±34
50	110±18	190±24*	325±39	320±37
500	50±8*	150±23*	340±43	390±43
5000	50±9*	200±16*	480±41	—
24 h after injection				
0^{+}	160±14	160±15	100±14	180±23
0.5	150±13	150±16	160±27	210±21
5	240±28*	190±24	145±23	230±30
50	220±27	170±20	83±12	220±25
500	200±23	120±18	110±13	240±36
5000	170±19	160±15	66±19*	—
TNF				
24 h after injection				
0^{+}	4±1	5±1	4±1	4±1
0.5	4±1	5±1	4±1	4±2
5	4±1	6±1	4±1	4±1
50	4±1	4±1	5±1	6±2
500	4±1	6±1	4±1	7±2*
5000	4±1	7±2	4±1	—

TABLE 3. *In Vitro* Effects of BO on Cytokine Production by Human Peripheral Blood Leukocytes ($M\pm m$, n=5)

Dose, μg/kg	IL-1, pg/ml		TNF, pg/ml		IL-2, U/ml	
	baseline	induced	baseline	induced	baseline	induced
0+	0	2200±200	0	180±20	0	128.9±10.1
0.001	500±40*	1700±160	—	—	0	110.8±8.9
0.01	1300±110*	2100±210	0	120±20	0	120.4±11.5
0.1	1100±100*	2100±190	0	260±40	0	107.2±9.0
1.0	700±50*	2200±180	0	140±20	0	117.0±11.3
10	1200±60*	2800±210	0	170±20	0	117.6±10.8
100	—	—	120±15*	420±30*	—	—

diluted with medium RPMI-1640 and cultured in multiwell plates for 18 h in the presence or absent of inductors of cytokine synthesis. The water-soluble fraction of BO in doses of 0.001, 0.1, 1, 10, and 100 μg/ml was added at the beginning of culturing. Prodigiozan was used as the inductor for IL-1 and TNF. Phytohemagglutinin and phorbol myristate acetate served as inductors for IL-2. The contents of IL-1 and TNF were measured by enzyme-linked immunosorbent assay using monoclonal antibodies against human cytokines. IL-2 concentration was estimated using CTLL-2 cells. We performed 5 parallel measurements for 1 control and 5 experimental groups.

The observed mean and dispersion were calculated for each group. The differences between control and experimental groups were evaluated by Student's t test (probability 0.95).

RESULTS

Intraperitoneal administration of PR and BO had no effect on differentiation of T lymphocyte precursors, while thymogen and IL-1 in different concentrations modulated this process ($p<0.05$).

Proliferation of thymic and splenic lymphocytes remained unchanged 4 h after administration of PR and BO (Table 1). Intensification of cell proliferation in the thymus (PR and BO) and spleen (PR) was observed 24 h after intraperitoneal injection of preparations. PR and BO in a dose of 500 μg/kg were most effective. Thus, BO in various doses and PR in high concentration stimulated cell proliferation in the thymus. As differentiated from BO, PR in various doses stimulated proliferation of lymphocytes in the spleen. Potent effects of IL-1 are associated with its biological role in the organism.

Stimulation of IL-2 production by BO and PR in various doses was observed 24 h after treatment (Table 2). These preparations in a dose of 500 μg/kg produced the most pronounced stimulatory effects. IL-6 synthesis was suppressed for a short time after intraperitoneal injection of preparations, but returned to normal 24 h after treatment (Table 2). In this period BO and PR in intermediate doses tended to stimulate IL-6 production. This was probably a gradually decreasing response of the system to inhibitory factors. BO in high doses stimulated TNF synthesis (Table 2). BO *in vitro* stimulated spontaneous production of IL-1; in high doses the preparation promoted TNF secretion (Table 3).

Our results show that preparations from PR possess pronounced immunomodulatory activity and hold much promise for the therapy of burns, wounds, and purulent-and-inflammatory diseases.

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