Pine Resin and Biopin Ointment: Effects of Water-Soluble Fractions on Cytokine Production by Peripheral Blood Neutrophils

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We studied the effects of water-soluble fractions of pine resin and Biopin-10 and Biopin-20 ointments used for the therapy of burns, wounds, and purulent and inflammatory diseases on spontaneous and induced synthesis of tumor necrosis factor and interleukins 1, 2, and 8 by human peripheral blood neutrophils *in vitro*. These preparations contain various bioactive substances producing opposite and dose-dependent immunomodulatory effects. Our results indicate that the composition and doses of preparations should be selected to achieve the maximum therapeutic effect.

Key Words: *pine resin; Biopin ointment; chromatographic fractionation; immunomodulatory effect*

Pine resin (PR) is widely used in traditional medicine and now serves as the major component of some medicinal preparations (ointments). Biopin ointment (BO) contains 10 and 20% PR (BO-10 and BO-20, respectively) and is effective during the therapy of burns, wounds, and purulent and inflammatory diseases of the skin and subcutaneous fat [1].

Our previous studies showed that PR and BO-10 exhibit pronounced immunomodulatory activity and affect *in vivo* and *in vitro* production of cytokines [3]. The aim of the present study was identification of bioactive components of PR and BO. These studies are of considerable importance for optimization of the composition of preparations and selection of effective therapeutic doses.

Extensive thermal and mechanical damages to the skin and subcutaneous tissues are associated with immunodeficiency. The degree of this immunodeficiency depends on the severity of injuries and is associated with impaired cytokine synthesis and qualitative and quantitative changes in the system of cell-mediated immunity [4,5]. Proliferation and maturation of immune cells are induced by mitotic factors. Various inflammatory mediators, including interleukins (IL-1, IL-2, and IL-8) and tumor necrosis factor (TNF) produced by macrophages and T lymphocytes, play an important role in these processes.

Here we studied the effects of fractions obtained after chromatographic separation of initial water-soluble fractions from PR, BO-10, and BO-20 on cytokine production by peripheral blood neutrophils.

MATERIALS AND METHODS

Water-soluble fractions were obtained from PR and BO. PR, BO-10, and BO-20 (20 g) were dissolved in 150 ml hexane. An equivalent volume of acetonitrile-isopropanol (3:1) mixture was added, agitated, and the light (hexane) fraction was removed. Water (165 ml) was added to the heavy fraction (150 ml), agitated, and centrifuged at 3000*g* for 2 min. The light (floating) fraction was removed. The volume of the

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TABLE 1. Effect of PR on Cytokine Synthesis by Peripheral Blood Neutrophils (*M*±*m*, *n*=4)

Parameter, dose, μg/ml		Fraction								
		2	3	4	5	6	7	8	9	10
IL-1, 10^{-1} pg/ml	0	277±17				210±26				
	0.001	220±28	260±27	90±8*	340±36	80±10*	62±7*	162±28	150±27	110±10*
	0.01	220±24	240±30	120±15*	250±37	110±13*	80±9*	140±31	90±8*	110±12*
	0.1	260±29	210±37	150±18*	320±35	110±11*	200±23	80±7*	160±18	220±23
	1	195±40	260±28	160±16*	250±31	110±10*	190±21	24±8*	100±11*	270±30
	10	240±32	280±34	160±17*	240±27	54±9*	180±24	160±29	140±19	200±24
IL-2, units/ml	0	27.7±0.7								
	0.001	31.2±0.7	26.1±0.4	26.8±1.2	34.2±2.2	29.1±1.3	32.7±2.4	26.5±2.1	36.2±4.2	25.6±1.2
	0.01	29.8±0.6	22.2±1.2	27.8±0.8	27.2±0.7	29.1±0.8	31.2±1.5	55.0±4.9*	36.6±1.9	28.4±2.1
	0.1	32.4±0.9	29.8±0.4	34.2±1.9	31.2±1.3	30.5±1.3	29.1±1.2	59.0±4.5*	30.5±0.9	29.1±2.6
	1	27.5±0.4	23.1±0.7	31.2±0.8	30.5±0.9	32.2±2.2	31.2±1.6	56.4±4.2*	30.5±1.2	31.6±2.2
	10	25.9±0.8	6.5±0.4*	19.4±0.6*	26.5±0.8	28.4±1.1	27.8±0.9	57.7±2.9*	64.8±3.9*	31.9±2.3
IL-8, 10^{-1} pg/ml	0	440±20				395±45				
	0.001	450±49	380±39	410±40	400±47	490±41	340±39	390±34	380±32	460±34
	0.01	440±39	390±42	380±39	450±47	530±40*	380±36	390±31	400±43	460±35
	0.1	495±47	440±47	460±41	450±44	535±43*	480±47	405±36	450±49	535±42*
	1	500±48	450±51	460±36	480±39	540±41*	500±38*	400±41	500±41*	540±43*
	10	510±53	480±46	500±52	540±46*	540±45*	500±41*	460±42	500±45*	600±48*

Note. Here and in Table 2 and 3: *p<0.05 compared to the control (dose 0).

heavy fraction was adjusted to 500 ml with 40% acetonitrile. Thus obtained initial fraction 0 (10 ml) was applied onto a Dynomax-60A C_{18} column (21.5×100 mm) and eluted with a linear gradient of acetonitrile in water (40-100%) at a flow rate of 5 ml/min and 30°C. Elution profiles (UV detection at 206 nm) and fractionation of preparations were described elsewhere [2].

The effects of water-soluble fractions of PR and BO on spontaneous (baseline) and induced production of IL-1, IL-2, IL-8, and TNF by neutrophils were studied. Freshly isolated heparinized human blood cells were diluted 6-fold with RPMI-1640 medium and cultured in microplates for 18 h in the absence and presence of inductors of cytokine synthesis. Water-soluble fractions of PR and BO were added in doses of 0.00001 (only BO-20), 0.0001 (only BO), 0.001, 0.01, 0.1, 1, 10, and 100 μg/ml (only BO) at the start of culturing. Prodigiozan served as the inductor for IL-1, IL-8, and TNF. IL-2 synthesis was induced with phytohemagglutinin and phorbol myristate acetate. The contents of IL-1, IL-8, and TNF (pg/ml) were measured by solid-phase enzyme immunoassay using monoclonal antibodies against human cytokines. The concentration of IL-2 (units/ml) was estimated by its biological activity using CTLL-2 cells.

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

PR fractions in the studied doses did not induce cytokine production by blood neutrophils and had no effect on induced TNF synthesis. All fractions inhibited induced IL-1 synthesis (Table 1): fractions 4 and 6 in all doses significantly suppressed IL-1 synthesis, while fractions 7-10 inhibited this process in 2 of 5 doses. Our previous studies showed that fractions 2-9 increase the oxidation-reduction potential of neutrophils [2]. In the maximum doses fractions 3-4 inhibited, while fractions 8-9 stimulated induced IL-2 synthesis. Fraction 8 in various doses stimulated induced IL-2 synthesis. All fractions in high doses stimulate induced IL-8 synthesis. Fractions 5-7 and 9-10 were most potent.

None of BO-10 fractions modulated IL-2 synthesis. Spontaneous synthesis of TNF was induced by fractions 7-8 in low doses and fraction 0 in the maximum dose. Only fraction 0 in the highest dose stimulated induced TNF synthesis (Table 2). IL-1 synthesis was induced by fraction 0 in all doses, but not by fractions 1-8. Fraction 0 did not affect, while fractions 2-5 suppressed induced IL-1 synthesis. The inhibitory effect was typical of fractions 3-4. Fractions 7-8 in low doses stimulated induced IL-1 synthesis (Table 3).

TABLE 2. Effect of Fraction 0 from BO-10 and BO-20 on Cytokine Production by Peripheral Blood Neutrophils (*M*±*m*, *n*=6)

Cytokine, dose, μg/ml		BO-10		BO-20	
		spontaneous	inductor	spontaneous	inductor
IL-1, 10^{-1} pg/ml	0	0	220±20	145±24	260±22
	0.001	50±4*	170±16	84±14	230±19
	0.01	130±11*	210±21	80±11	180±24
	0.1	110±10*	210±19	110±9	200±18
	1	70±5*	220±18	64±29	160±11*
	10	120±6*	280±21	100±9	144±9*
IL-2, units/ml	0	0	128.9±10.1	0	162.4±18.2
	0.001	0	110.8±8.9	—	—
	0.01	0	120.4±11.5	0	123.2±20.6
	0.1	0	107.2±9.0	0	127.3±16.4
	1	0	117.0±11.3	0	135.1±18.7
	10	0	117.6±10.8	0	83.2±7.8*
TNF, 10^{-1} pg/ml	0	0	18±2	0	90±7
	0.01	0	12±2	0	80±7
	0.1	0	26±4	0	100±8
	1	0	14±2	0	44±4*
	10	0	17±2	0	35±4*
	100	12±2*	42±3*	0	54±6*

TABLE 3. Effect of Fractions of BO-10 and BO-20 on Cytokine Production by Peripheral Blood Neutrophils (*M*±*m*, *n*=4)

Parameter; preparation, dose, mg/ml		Fraction							
		1	2	3	4	5	6	7	8
IL-1, 10^{-1} pg/ml									
Spontaneous synthesis									
BO-20	0	0	0	0	0	0	0	0	0
	1	10±3*	0	0	0	0	15±3*	0	0
	10	40±6*	36±5*	0	0	17±4*	74±9*	0	0
Induced synthesis									
BO-10	0	44±7	150±17	150±17	100±12	80±9	140±18	140±8	120±14
	0.0001	33±9	90±12	50±7*	44±6*	60±8	90±11	490±37*	510±45*
	0.001	34±7	87±14	70±8*	17±3*	30±3*	80±9	410±23*	320±26*
	0.01	53±7	130±16	50±9*	35±5*	50±6	100±13	230±24	120±11
	0.1	24±9	90±11	91±9*	28±4*	39±4*	90±8	220±18	144±17
	1	24±8	60±10*	74±12*	36±6*	40±5*	170±8	230±29	200±26
	10	24±9	74±11*	0*	110±16	60±9	240±28	220±19	180±16
BO-20	0	44±8	56±8	72±8	56±10	54±9	90±11	40±8	80±9
	0.001	46±7	70±12	60±7	61±8	55±8	64±9	70±9	80±11
	0.01	60±8	75±11	80±10	60±9	82±9	54±8	36±7	80±10
	0.1	38±7	54±9	72±9	46±7	76±10	82±8	40±7	64±8
	1	50±6	74±10	74±8	73±8	84±11	80±9	60±9	84±9
	10	70±9	120±10*	110±13	64±7	46±7	130±12	30±6	84±10
TNF (spontaneous synthesis), 10^{-1} pg/ml									
BO-10	0	0	0	0	0	0	0	0	0
	0.0001	0	0	0	0	0	0	100±13*	90±7*
	0.001	0	0	0	0	0	0	90±11*	18±2*
	0.01	0	0	0	0	0	0	26±3*	0
	0.1	0	0	0	0	0	0	020±3*	0
IL-2 (induced synthesis), units/ml									
BO-20	0			89.1±6.9					
	0.0001	92.6±7.1	94.1±7.9	85.8±7.7	74.7±9.0	78.2±6.7	99.1±9.5	71.3±7.9	93.1±9.8
	0.001	94.4±6.5	94.1±7.6	98.2±8.6	89.8±9.2	89.6±6.6	89.8±9.1	71.4±8.2	74.7±7.6
	0.01	98.4±7.0	94.1±8.3	78.2±8.4	109.0±11.4	93.0±8.6	94.1±8.4	68.1±7.6	85.7±8.2
	0.1	93.6±6.4	108.0±9.7	65.0±9.9	108.0±9.5	89.6±7.2	74.8±8.1	98.4±9.7	94.0±8.9
	1	186.2±9.9*	68.1±8.4	89.8±10.1	108.0±9.9	88.9±5.4	73.2±6.8	78.2±8.0	95.0±8.8
	10	163.5±9.2*	89.8±10.1	94.4±8.2	124.0±12.8	85.8±9.6	74.6±8.3	68.1±7.4	74.8±6.7

It should be emphasized that fraction 3 in a dose of 10 mg/ml completely blocked IL-1 synthesis (Table 3), while in doses of 0.001-0.1 μg/ml this fraction significantly enhanced 3 parameters of phagocytosis [2].

Fraction 0 from BO-20 did not affect spontaneous synthesis of IL-2 and TNF (Table 2), slightly inhibited spontaneous IL-1 synthesis, and suppressed induced synthesis of IL-1, IL-2, and TNF (in high doses, Table 2). Fractions 1-2 and 5-6 in the maximum doses induced IL-1 synthesis. In high doses fractions 1-4 and 6 stimulated, while fractions 5 and 7 inhibited induced IL-1 synthesis. Fractions 1-8 had no effect on spontaneous IL-2 synthesis. Induced IL-2 synthesis was stimulated by fractions 1 and 4 in high doses and inhibited by fractions 6 and 7 in the maximum doses (Table 3). Fraction 1 in doses of 1 and 10 μg/ml stimulated synthesis of IL-1 and IL-2 and in doses of 0.001-0.1 μg/ml significantly inhibited phagocytosis [2].

Our results show that preparations of PR, BO-10, and BO-20 contain various biologically active substances that produce opposite and dose-dependent immunomodulatory effects. These data indicate that the composition and doses of preparations should be carefully selected to achieve maximum therapeutic effect.

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