Effects of Collagenolytic Protease Preparations from Invertebrates on the Immune System

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We studied the effects of an active ingredient of new enzyme preparations containing collagenolytic proteases from various hydrobionts (Collagenase from hydrobionts, Polycollagenase-K, and Fermenkol). These substances in therapeutic doses did not affect the humoral immune response estimated by hemagglutination and local hemolysis in gel and caused no local irritation and allergic reactions after long-term application to the skin.

Key Words: *enzyme preparations; collagenolytic proteases; humoral immunity; irritation; allergic reaction*

Serine proteases (EC 3.4.21 [14]) or collagenases (EC 3.4.24 [14]) are active ingredients structure-degrading enzyme preparations [1,2,7]. These specific proteases display high activity in relation only to certain polypeptide substrates [10,11] and are inefficient against complex structural proteins. New enzyme preparations such as Collagenase from Hydrobionts, Polycollagenase K, Fermenkol (cosmetic polycollagenase), Collasorb and Colladiosorb (wound-draining sorbents) are based on natural or synthetic synergistic nonspecific collagenolytic proteases from invertebrates (CLPI) [3-5,13] catalyzing hydrolysis of various polypeptide substrates (*e.g.*, native or partially denatured collagen, elastin, casein, fibrin, and hemoglobin) with the formation of individual amino acids. These substances are much more potent in degrading mesh and massive multilayer structures compared to other enzyme preparations. Here we evaluated the effects of a highly purified (more than 99% protein) natural CLPI complex isolated from the hepatopancreas of king crab *Paralithodes camtschatica* on the immune system [3, 4]. Analogous data were obtained for preparations containing CLPI of other composition and origin (calculation per the content of the active ingredient).

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MATERIALS AND METHODS

The effects of CLPI on humoral immunity were studied on 117 (CBA×57Bl)F1 mice kept under standard conditions. After immunization, experimental mice were daily treated with CLPI for 5 days. CLPI in doses of 5 and 50 mg/kg was dissolved in water and applied with a pipette to a 4-cm^2 area of depilated skin on the back. CLPI in doses of 0.1 and 1.0 mg/kg was injected intraperitoneally in isotonic NaCl ($^1/_{1000}$ and $^1/_{100}$ of LD_{50}, respectively [8]). The reference drug levamisole in a daily dose of 25 mg/kg was injected intraperitoneally for 3 days. Control mice received an equivalent volume of the solvent. Reaction of hemagglutination was conducted at the peak immune response 7 days after immunization with 1% sheep erythrocyte suspension (500 μl intraperitoneally). Blood serum was titrated by serial 1:1 dilutions with isotonic NaCl (180 μl), 1% suspension of sheep erythrocytes was added (50 μl/well), and after 2 h the maximum dilution producing distinct reaction was evaluated at 24°C [6]. Local hemolysis in gel (preliminary immunization with sheep erythrocytes, 10^8 intraperitoneally) was conducted after culturing of mouse splenocytes with an excess of sheep erythrocytes. The content of antibody-producing cells in the spleen was estimated individually from the number of hemolysis

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zones [12]. The means and variances were calculated for each group, and then differences between the control and experimental groups were tested by means of Student's t test for independent samples with equal variances at a significance level of $p<0.05$.

Local irritating and allergic effects of CLPI were studied on 30 outbred rats weighing 150-180 g and 30 guinea pigs weighing 200-250 g. Water solution of CLPI in a daily dose of 150 μg/kg (3 therapeutic doses) was applied with a pipette to a 4-cm^2 area of depilated skin on the side for 14 days (20 rats and 20 guinea pigs). Control animals (10 rats and 10 guinea pigs) received an equivalent volume of water. The duration of treatment was 14 days, because the assumed period of therapy with CLPI-containing preparations in medical practice is 3-5 days. Skin reaction was daily assessed by the Suvorov scale [9]. Ten days after the end of treatment, CLPI in the same daily dose was repeatedly applied to guinea pigs on the opposite side of the body for 20 days to test its probable sensitizing effects.

RESULTS

CLPI injected intraperitoneally in doses of 0.1 and 1.0 mg/kg and applied on the skin in a dose of 5 mg/kg did not affect the humoral immune response (Table 1). Cutaneous application of CLPI in a concentration 1000-fold surpassing the therapeutic dose (50 mg/kg) suppressed the immune reaction ($p<0.05$).

This effect can be explained as follows: fibroblasts, proliferating and migrating cells producing collagen, are the source of interleukins 1 and 6, granulocyte-macrophage colony-stimulating factor, and interferon-β. It was shown that interleukin-1 stimulates synthesis of type III collagen by enhancing expression of procollagen genes, while tumor necrosis factor inhibits production of collagen by fibroblasts. In addition, activated fibroblasts secrete collagenase during wound healing (specific granules of neutrophils contain collagenase active in relation to type I collagen). Hence, fibroblasts are undoubtedly involved in the immune defense, and changes in fibroblast activity modulate immune reactions. Apart from fibronectin, glycosaminoglycans, proteoglycans, and elastin, collagen is the major component of the extracellular matrix. The connective tissue contains primarily type I and III collagen fibrils bound through fibronectin to plasma membrane proteins of fibroblasts (integrins). Partial degradation of collagen fibrils by CLPI (exogenous collagenolytic factor) leads to the release of fibronectin fragments activating macrophage chemotaxis. Macrophages secrete plasminogen activator, which breaks contacts between fibroblasts and components of the extracellular matrix. This probably changes the usual flat shape of fibroblasts, inhibits their proliferation and cytokine production, and attenuates the immune response.

TABLE 1. Effects of CLPI on Humoral Immune Response in Mice Estimated by Reaction of Hemagglutination ($M\pm m$)

Route of administration	Series	$\log_2$ N*
Cutaneously	Control (n=8)	4.7±0.3
	CLPI, mg/kg	
	5 (n=8)	4.0±0.5
	50 (n=9)	1.9±0.5+
Intraperitoneally	Control (n=10)	4.5±0.5
	CLPI, mg/kg	
	0.1 (n=8)	4.8±0.4
	1.0 (n=6)	5.3±0.7
	Levamisole, 25 mg/kg (n=8)	6.8±0.5+

Note. *N: maximum dilution producing distinct reaction. Here and in Table 2: +$p<0.05$ compared to the control.

TABLE 2. Effects of CLPI on Antibody Production in the Spleen of Mice Estimated by Reaction of Local Hemolysis in Gel ($M\pm m$, n=15)

Series	Content of antibody-producing cells, ×10^3
Control	12.7±1.7
CLPI	
5 mg/kg cutaneously, 5 days	14.3±1.1
1 mg/kg intraperitoneally, 5 days	13.2±2.5
Levamisole, 25 mg/kg intraperitoneally, 3 days	21.5±0.3+

Stimulation of the humoral immune response after intraperitoneal injection of 1 mg/kg CLPI is probably related to changes in the activity of peritoneal macrophages.

The data on antibody production in mouse spleen also indicated that CLPI produced no effects on the primary immune response (Table 2).

CLPI produced no nonallergic contact dermatitis in rats and guinea pigs. Only minor erythema in 2 of 20 guinea pigs was noted 6-7 days after CLPI application, which persisted to the end of treatment. Repeated daily application of CLPI to the skin caused no sensitization in guinea pigs.

In conclusion, long-term cutaneous application of CLPI in therapeutic doses did not affect the humoral

immune response and caused no local irritation and allergic reactions.

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