IMMUNOACTIVE PEPTIDES. III*. SYNTHESIS AND IMMUNOMODULATING ACTIVITY OF NOVEL FRAGMENTS OF THYMOSINS β-7–β-10

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In recent years, data have been obtained which show that small peptide fragments, liberated during limited proteolysis of immunologically active proteins and peptides, can also modulate the immune response [6]. A necessary step in the initiation of this response is the activation of lymphocytes [3]. The effect of thymic peptide hormones – thymosins β-7–β-10 – on the activation of T-lymphocytes has been little studied [7]. The aim of the present work was to synthesize and study the effects of previously undescribed synthetic fragments of thymosins β-7–β-10 – Thr-Leu-Pro-Thr (I, positions 27-30) and Asn-Thr-Leu-Pro-Thr (II, positions 26-30) – on the rosette-forming and mitogenic activities of human T-lymphocytes.

EXPERIMENTAL (CHEMISTRY)

Peptides I and II were prepared using activated p-nitrophenyl and pentafluorophenyl esters in 2+2 and 1+(2+2) schemes, respectively. All amino acids used had the L-configuration. Melting temperatures were determined on a Boetius heating block. Synthetic processes, removal of protective groups, as well as the purity of intermediates produced and final peptides were monitored by thin layer chromatography on Silufol plates in the following systems: chloroform–methanol–acetic acid, 9:1:0.5 (A); chloroform–methanol, 9:1 (B); chloroform–methanol, 5:1 (C); chloroform–methanol–25% ammonium hydroxide, 15:11:5 (D); benzene–acetone, 2:1 (E); n-butanol–acetic acid–water, 3:1:1 (F); n-butanol–acetic acid–water–ethyl acetate, 1:1:1 (G); n-butanol–pyridine–acetic acid–water, 60:10:6:12 (H); as well as by high-voltage electrophoresis on Filtrak FN-12 paper in 2% acetic acid at a field strength of about 40 V/cm. To develop chromatograms and electrophoregrams we used benzidene chloride reagent. Amino acid composition was determined with an AAA-T 339 analyzer, following acid hydrolysis in 6 N HCl at 110°C for 20 h.

N-Benzyloxycarbonyl-prolyl-threonine (III). A solution of 5.56 g (0.015 mole) of N-benzyloxycarbonyl-proline p-nitrophenyl ester in 50 ml of dioxane was added with stirring to a solution of 1.97 g (0.0165 mole) threonine in 16.5 ml 1.0 N NaOH. After stirring the reaction mixture (20°, 72 h) the dioxane was distilled off, the residue was diluted with 100 ml water, and the resulting emulsion was extracted with ether (3 × 50 ml). The aqueous layer was acidified with 2 N sulfuric acid to pH 3 and extracted with warm ethyl acetate (3 × 50 ml). The combined ethyl acetate extracts were washed with water until the washings were neutral, dried over anhydrous sodium sulfate for 2 h, evaporated to a volume of 50 ml, and the precipitate that formed during evaporation was dissolved by boiling for 15 min. After cooling the solution the precipitate was filtered, washed on the filter with ethyl acetate and hexane, and dried in air. Obtained were 4.18 g (79%) III, mp 144-147°C. R_f 0.12 (E), 0.26 (A). Analysis corresponded to the formula $C_{17}H_{22}N_2O_6$.

N-Benzyloxycarbonyl-threonyl-leucine Pentafluorophenyl Ester (IV). To a solution of 2.56 g (0.0195 mole) leucine in 19.5 ml 1.0 N NaOH was added a solution of 7.52 g (0.015 mole) N-benzyloxycarbonyl-threonine pentachlorophenyl ester in 50 ml of dioxane and 2.1 ml (0.015 mole) triethylamine. The reaction mixture was stirred (20°C, 96 h), following which

*For Communications I and II see [4].

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TABLE 1. Effect of Peptides on the Number of Rosette-forming Cells (E-RFC) in a Lymphocyte Population (in %)

Peptide concentration, μg/ml	Peptide	
	I	II
0 (control)	t 39,8±6.3	44,1±3,8
0,1	—	58,0±4,4* (7)
1,0	46,3±5,5 (5)	54,3±5,3 (7)
10	47,7±4,7 (5)	51,4±4,3 (7)
50	61,6±5,0 (5)	—
100	39,6±6,6 (5)	48,5±5,2 (7)

Note. Here and in Table 2, an asterisk indicates a significant difference from the control ($p < 0.05$). Dash) experiment not done; in parentheses) number of animals.

TABLE 2. Effect of Peptides on Blastogenesis of Lymphocytes Induced by PHA (in counts/min)

Peptide concentration, μg/ml	Peptide	
	I	II
0 (control)	3411,3±488,9 (10)	2356,6±376,4 (9)
0,01	2294,4±467,0 (10)	4448,8±665,5* (9)
0,1	1716,2±372,3* (10)	3962,9±605,9* (9)
1,0	1851,8±309,1* (10)	3878,7±757,5 (9)
10	1901,5±258,4* (10)	4276,4±561,1* (9)

the dioxane was distilled off and the residue was diluted with 100 ml water and thoroughly extracted with ether (6 × 40 ml). The aqueous layer was acidified with 2 N H_2SO_4 to pH 3, and the abundant oil that formed was extracted with ethyl acetate (5 × 50 ml). The combined ethyl acetate extracts were washed with water until the washings were pH 7, dried with anhydrous sodium sulfate, and the solution was evaporated until the ethyl acetate had completely distilled. The remaining oil was dissolved in 25 ml ether and 150 ml of hexane was added. After standing in the refrigerator (12 h), the solution over the viscous oil that formed was poured off, the oil was again dissolved in 25 ml of ether, and 150 ml hexane was added. The reprecipitation was repeated as long as TLC in system (E) did not show complete removal of pentachlorophenol from the product. The pure oil was dried in a vacuum desiccator to constant weight. Yield was 5.17 g (94%) of dry foamy material [R_f 0.21 (E), 0.44 (A)], which was then dissolved in 100 ml dioxane. To the resulting solution was added with mixing 12.89 g (16.98 moles) of complex F [8], which was stirred (20°C, 8 h) and then left in the refrigerator 12 h; the precipitate of N,N′-dicyclohexylurea was filtered, washed on the filter with 10 ml of cold dioxane, and the filtrate was evaporated. To the residual oil was added 100 ml of hexane; this was stirred vigorously, and the bulky precipitate that formed was filtered, washed on the filter with hexane, and dried in a vacuum desiccator. Obtained was 6.62 g (88%) IV, mp 126-130°C, R_f 0.51 (A), 0.59 (E). Analysis corresponded to the formula $C_{24}H_{25}N_2O_4F_5$.

N-Benzyloxycarbonyl-threonyl-leucyl-prolyl-threonine (V). A 1.83 g portion (0.005 mole) of III was hydrogenated in methanol over palladium on charcoal. One hour after the start of the admission of hydrogen, 20 ml water was added to dissolve the hydrogenation product precipitated on the catalyst. At the end of the reaction, the catalyst was filtered, washed on the filter with 10 ml water, and the filtrate evaporated under vacuum until the methanol had completely distilled. To the residue was added 50 ml isopropyl alcohol, which was evaporated, and this was repeated twice. The solid residue was suspended in 10 ml of DMFA, and to the strongly stirred suspension was added a solution of 2.66 g (0.005 mole) IV in 10 ml DMFA and 0.63 ml (0.005 mole) N-ethylmorpholine, which was stirred until complete dissolution (20°C, 24 h), evaporated, and 20 ml of 5% sodium bicarbonate solution was added to the residue. The resulting weakly alkaline solution was thoroughly extracted with ether (5 × 30 ml), the aqueous layer acidified with 2 N H_2SO_4 to pH 3, and the oil produced was extracted with ethyl acetate (3 × 50 ml). The combined ethyl acetate extracts were washed with water until the washings were neutral, dried with anhydrous sodium sul-

fate, and evaporated. The residual oil was dissolved in 5 ml of ethyl acetate and the product was crystallized by adding 100 ml hexane. The sediment was reprecipitated from 50 ml ethyl acetate by the addition of 100 ml of diethyl ether, filtered, washed on the filter with 10 ml ether, and air-dried. Yield was 1.07 g (38%) V. R_f 0.28 (A), 0.44 (C). Analysis corresponded to the formula $C_{27}H_{40}N_4O_9 \cdot 1/2\ H_2O$.

Threonyl-leucyl-prolyl-threonine (I). A 1.29 g (0.0018 mole) portion of V was hydrogenated analogously to III. The catalyst was filtered and the filtrate was evaporated to dryness. Yield was 0.941 g (96%) VI. For purification, 0.15 g (0.00035 mole) of the material obtained was dissolved in 1 ml 0.01 N triethylammonium acetate buffer (pH 5.14) and applied to a column (250 × 10 mm) of Sephadex SP C-25. Elution was carried out with 0.01 N triethylamine acetate buffer (pH 5.14) at a rate of 4 ml in 15 min, and monitored with a UV detector (214 nm). Fractions 6-15, containing pure product, were combined and evaporated with isopropyl alcohol (5 × 50 ml). The sediment was reprecipitated from methanol with anhydrous ether. Yield was 0.101 g (67%) I, mp 144-147°C (dec). R_f 0.17 (system F), E (His) 0.52. Analysis corresponded to the formula $C_{19}H_{34}N_6O_6 \cdot CH_3COOH \cdot H_2O$. Amino acid analysis: Thr 2.10 (2); Leu 1.0 (1); Pro 1.20 (1).

N-Benzyloxycarbonyl-asparaginyl-threonyl-leucyl-prolyl-threonine (VI). To a solution of 0.161 g (0.0037 mole) I in 5 ml DMFA was added a solution of 0.215 g (0.0055 mole) N-benzyloxycarbonyl-asparagine p-nitrophenyl ester in 5 ml DMFA and 0.094 ml (0.0074 mmole) N-ethylmorpholine. This was stirred at 20° for 48 h, the DMFA was distilled, the residue dissolved in 25 ml of 5% sodium bicarbonate, and the resulting solution extracted with ether (3 × 15 ml). The aqueous layer was acidified with 2 N H_2SO_4 to pH 3 and the oil produced was extracted with ethyl acetate (4 × 25 ml). The combined extracts were washed with a saturated solution of NaCl until the washings were pH 7, dried with anhydrous sodium sulfate, evaporated to a volume of 1 ml, and 30 ml ether was added dropwise and the mixture left in the refrigerator for 12 h. The precipitate was filtered, washed on the filter with ether, and air-dried. Yield was 0.089 g (35%) VI, mp 114-117°C. R_f 0.34 (A), 0.36 (B), 0.54 (G). Analysis corresponded to the formula $C_{23}H_{40}N_6O_9 \cdot H_2O$.

Asparaginyl-threonyl-leucyl-prolyl-threonine (II). A 0.079 g (0.00012 mole) portion of VI was hydrogenated analogously to III. The catalyst was filtered, and the filtrate was evaporated to a volume of 0.5 ml and 25 ml of anhydrous ether added. The precipitate was filtered, washed on the filter with ether, and air-dried. Yield was 0.05 g (79%) II, mp 169-172°C (dec). R_f 0.15 (H), 0.77 (D). Analysis corresponded to the formula $C_{23}H_{40}N_6O_9$. Amino acid analysis: Asp 0.91 (1); Thr 2.03 (2); Leu 1.07 (1); Pro 1.00 (1).

EXPERIMENTAL (BIOLOGY)

The immunologic activity of the synthetic peptides was determined in vitro by the method of spontaneous rosette formation [9]. Lymphocytes were isolated from heparinized peripheral blood from patients with bronchopulmonary pathology by centrifugation on a one-step gradient of Verografin-Ficoll with a density of 1.077 g/cm^3. The isolated cells were resuspended in medium 199 to a final concentration of $2.5 \cdot 10^6$ cells/ml, and incubated for 30 min at 37°C with different concentrations of the peptides studied (from 0.1 to 100 μg/ml). Lymphocytes incubated under the same conditions without peptides served as the control. The number of rosette-forming cells in 200-400 lymphocytes was calculated, expressing the results in percent. A lymphocyte linked to three or more sheep erythrocytes was considered to be a rosette-former. The results obtained are presented in Table 1.

The mitogenic activity of the peptides was studied in short-term cultures of lymphocytes from peripheral blood of the same patients, and was measured by the method of blast transformation of lymphocytes by phytohemagglutinin (PHA) [2]. Cells at a concentration of $5 \cdot 10^5$ cells/ml were cultured in round polystyrene planchettes (Medpolymer, Leningrad) in a volume of 0.2 ml for 48 h at 37°C, with 5% CO_2, in RPMI-1640 medium with the addition of 10% fetal calf serum, 100 μg/ml gentamicin, and 2 mM L-glutamine. Synthetic peptides were added to the culture medium in a concentration of 0.01 to 10 μg/ml at the same time as the mitogen (30 μg/ml). Lymphocytes treated with PHA in the absence of synthetic peptides served as the control. ^{3}H-Thymidine (1 mCi in 0.2 ml) was added to the well 4 h before the end of the incubation period. The amount of ^{3}H-thymidine incorporated by the lyphocytes was determined with a Rack-beta liquid scintillation counter (LKB, Sweden), expressing the final results as counts/min per sample. The data obtained are presented in Table 2.

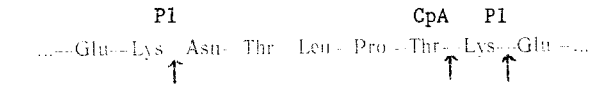
Statistical treatment of the experimental results was carried out using Student's criterion [5].

RESULTS AND DISCUSSION

As can be seen from the data in Table 1, treatment of lymphocytes with both peptides alters their ability to form spontaneous rosettes: both peptides studied markedly stimulate the process of E-rosette formation, tetrapeptide I at a concentration of 50 μg/ml and pentapeptide II at 0.1 μg/ml. With increasing concentration (from 1 to 50 μg/ml), compound I increases the activation of lymphocyte E-receptors; however, at 100 μg/ml, the number of E-RFC in the population of lymphocytes is decreased to a value slightly below the controls. It should be noted that the elongation of peptide I by one amino acid at the N-terminal (Asn) changes its effect on the ability of T-cells to form rosettes. In this case, the maximal effect is seen at the minimum (0.1 μg/ml) concentration of pentapeptide II, but with increasing concentration of the peptide in the incubation medium the number of E-RFC gradually decreases. However, even at the highest concentrations of peptide II studied, the number of E-RFC is above the control value.

The results of studying the proliferative response of human lymphocytes to PHA stimulation in the presence of the peptides at different concentrations, given in Table 2, show that peptides I and II have disparate effects on lymphocyte blastogenesis. Tetrapeptide I lowers the incorporation of ^{3}H-thymidine by cultures of human lymphocytes at all concentrations studied. In concentrations of 0.1-10 μg/ml, peptide I significantly suppresses the PHA-induced proliferation of lymphocytes by 50 and 44%, respectively. Peptide II, in contrast, stimulates PHA-induced lymphocyte proliferation at all concentrations studied; only at 1 μg/ml was the difference between the experimental and the control not significant. The maximal stimulatory effect on cellular proliferation (by 89%) is seen at a concentration of 0.01 μg/ml. It is noteworthy that adding just one amino acid to the N-terminal of peptide I reverses the nature of its effect on the proliferative response of T-lymphocytes. Previously, when studying the analgesic activity of both peptides, we noted a considerable increase in the Naloxone-dependent anesthetic effect upon adding a residue of asparagine to tetrapeptide I [1].

Our data indicates that the activation of peripheral T-lymphocytes may involve short fragments of thymosins β-7–β-10. It is important to note that it is quite possible that immunopeptides are liberated in the organism in the course of limited proteolysis of thymosin molecules. Thus, pentapeptide II can be generated from the starting molecule by the action of a few enzymes, e.g. plasmin (Pl) and carboxypeptidase A (CpA) (arrows designate bonds hydrolyzed):



In the course of a computer search [1], we found that tetrapeptide I, which corresponds to fragment 27-30 of thymosins β-7–β-10, is also part of the amino acid sequences of legumine of peas (residues 190-193), B-gordeine of barley (residues 239-242), γ-gliadin of wheat (residues 190-193), and nerve growth factor (residues 29-32 and 112-115). Hence, peptide fragments with pronounced immunoregulatory properties may arise in vivo as the result of limited proteolysis not only of endogenous immunoactive oligo- and polypeptides, but also of other exo- and endogenous proteins which carry out quite different functions in the organism.

Thus, a study of the biologic activity of novel synthetic fragments of thymosins β-7–β-10 showed that peptides I and II have pronounced immunoregulatory properties, which justifies further study to determine the possibility of creating new drugs, based on them, to correct various pathogenic states in which the pathogenesis of the disease is the result of disturbances in the functioning of the immune system.

LITERATURE CITED

1. V. P. Ivanova, E. I. Sorochinskaya, T. K. Lozhkina, and V. V. Anokhina, Ukr. Biokhim. Zh., 60, No. 5, 3-9 (1988).
2. E. P. Kiseleva, A. S. Tsveibakh, E. I. Gol'dman, et al., Immunologiya, No. 1, 76-78 (1985).
3. V. A. Lyashenko, V. A. Drozhennikov, and I. M. Molotkovskaya, Mechanisms of Activation of Immunocompetent Cells [in Russian], Moscow (1988).

4. V. F. Martynov, V. V. Anokhina, G. A. Belokrylov, et al., Zh. Obshch. Khim., 59, No. 10, 2369-2377 (1989); V. V. Anokhina, E. I. Sorochinskaya, V. P. Golubovich, et al., ibid., 60, No. 1, 203-211 (1990).
5. V. Yu. Urbakh, Statistical Analysis in Biological and Medical Investigations [in Russian], Moscow (1975).
6. G. I. Chipens, E. A. Korneva, S. N. Sklyarova, et al., Interaction of the Immune and Central Nervous Systems: Searching for the Carriers of Afferent Signals. Preprint. Riga (1987).
7. T. Abiko and H. Sekino, J. Appl. Biochem., 5, No. 6 446-452 (1983); T. Abiko, and H. Sakino, Chem. Pharm. Bull., 32, No. 1, 228-236 (1984).
8. J. Kovacs, L. Kisfaludy, and M. Q. Ceprini, J. Am. Chem. Soc., 89, No. 1, 183-184 (1967).
9. M. Tondall, G. Holm, and H. Wigzell, J. Exp. Med., 136, No. 1, 207-215 (1972).