IMMUNOACTIVE PEPTIDES. PART V. A THY-1 ANTIGEN 66 – 70-PENTAPEPTIDE FRAGMENT SELECTIVELY STIMULATES PHAGOCYTOSIS[1]

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Previously we have established that short peptides, entering as fragments into endogenous polypeptides, are capable of affecting the immune response and phagocytosis [1 – 6]. For example, a pronounced immuno- and phagocytosis-stimulating activity was observed [5, 9] for a 77 – 80 fragment of the immunoactive [7] Thy-1 antigen [8], which suggests that peptides possessing similar activity can be also found among the other fragments of this polypeptide. The search for Thy-1 antigen fragments possessing this type of activity was based on the knowledge that some individual amino acids entering into the peptide composition are capable of stimulating (Ala, Asn, Asp, Cys, Glu, Ser, Thr, Trp, Val [10]) or suppressing (Arg [11]) the immune response, some other amino acids (Ala, Asn, Asp, Arg, Cys, Glu, Leu, Lys, Pro, Ser, Thr, Trp, Tyr, Val) enhance phagocytosis, while a third group (His, Ile, Met, Gln, Gly, Phe) exhibit no such effects [9, 12, 13].

The purpose of this work was to synthesize a 66 – 70 fragment of the Thy-1 antigen – ProTyrIleLysVal (I) – and study its activity with respect to the immune response and phagocytosis. This peptide fragment contains three amino acids (proline, tyrosine, and lysine) activating phagocytosis and not affecting the immune response, while isoleucine is inactive in both respects and valine is active with respect to both the immune response and phagocytosis.

EXPERIMENTAL CHEMICAL PART

L-Prolyl-*L*-tyrosyl-*L*-isoleucyl-*L*-lysyl-*L*-valyl was obtained using the methods of classical polypeptide synthesis, involving sequential chain propagation (starting with the C-tail amino acid) on adding the *p*-nitrophenyl (ONp) esters of protected amino acids. Temporal protection of the α-amino groups was provided by benzyloxycarbonyl (Z) groups, the ε-amino group of lysine was protected by *tert*-butyloxycarbonyl (Boc) groups, and the phenolic hydroxyl of tyrosine was protected by a benzyl (Bzl) residue. The C-tail carboxyl group was blocked by forming benzyltrimethylammonium salts (for the synthesis of dipeptide), triethylammonium salts (for tri- and tetrapeptides), or N-ethylmorpholinium salts (for pentapeptide). In all the intermediate stages, the block was removed by catalytic hydrogenation in the presence of Pd-C. The final deblocking was effected by a mixture of trifluoroacetic acid with methylene chloride (3 : 7, v/v). The removal of solvents, high-voltage electrophoresis, amino acid composition analysis, and determination of the melting points were performed as described elsewhere [3].

Thin-layer chromatography was carried out on Silufol plates (Czech Republic) eluted in the following solvent systems (v/v): benzene – acetone, 1 : 1 (A); benzene – acetone, 2 : 1 (B); chloroform – methanol – acetic acid, 80 : 10 : 5 (C); chloroform – methanol – acetic acid – water, 30 : 24 : 4 : 6 (D); chloroform – methanol – acetic acid – ethyl acetate, 10 : 5 : 1 : 10 (E); *n*-butanol – acetic acid – water – ethyl acetate, 1 : 1 : 1 : 1 (F); and *n*-butanol – acetic acid – water, 3 : 1 : 1 (G). The chromatograms and electrophoregrams were developed using a chlorobenzidine reagent [14].

The process of condensation with activated esters was performed using the following general scheme for all the compounds synthesized. First, a salt of the given amino acid component was obtained by adding an equivalent amount of the corresponding amine to a solution of the amino component in DMF. To the resulting solution was added with stirring a solid ONp ester of the corresponding protected amino acid, after which the reaction mixture was stirred for 24 h at room temperature. Then DMF was distilled off and a 5% sodium bicarbonate solution was added to the residue until obtaining a weak alkaline reaction. The resulting suspension (or emulsion) was extracted with diethyl ether (2 × 20 ml) and ethyl acetate (2 × 20 ml). The aqueous layer was acidified

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with 1 N sulfuric acid to pH 3 – 4 and the precipitated oily product was isolated with ethyl acetate (3 × 30 ml). The combined extracts were washed with water (10 ml portions) until the washing solution exhibited a neutral reaction. Finally, the solution was dried over anhydrous sodium sulfate, concentrated by evaporation in vacuum, and recrystallized.

N-α-Benzyloxycarbonyl-N-ε-*tert*-butyloxycarbonyllysylvaline ZLys(Boc)Val. The dipeptide was synthesized using 2.1 g (17.4 mmole) of valine, 34.8 ml of an 0.5 N trimethylbenzylammonium solution in methanol, and 6.7 g (13.4 mmole) of ZLys(Boc)ONp. The product was crystallized from a hexane – diethyl ether mixture (1 : 1) and purified by boiling in diethyl ether. Yield, 4.45 g (70%); m.p., 126.5 – 128°C; $[\alpha]_D^{20}$ + 99° (*c*, 1.0; methanol); R_f, 0.58 (A); $C_{24}H_{37}N_3O_7$.

N-α-Benzyloxycarbonylisoleucyl-N-ε-*tert*-butyloxycarbonyllysylvaline ZIleLys(Boc)Val. The above dipeptide (4.2 g, 8.75 mmole) was hydrogenated in 30 ml of ethanol for 4 h in the presence of 0.5 g of Pd-C. Then the catalyst was separated by filtration and the solvent was distilled off. To the residue was added 10 ml of DMF, 1.2 ml (8.75 mmole) of triethylamine, and 3.8 g (9.6 mmole) of ZIleONp. The target tripeptide was crystallized from diethyl ether and purified by boiling in the same solvent. Yield, 4.0 g (78%); m.p., 173 – 175°C; $[\alpha]_D^{20}$, – 29° (*c*, 1.0; methanol); R_f, 0.67 (A), 0.79 (D); $C_{30}H_{48}N_4O_8$.

N-α-Benzyloxycarbonyl-O-benzyltyrosylisoleucyl-N-ε-*tert*-butyloxycarbonyllysylvaline ZTyr(Bzl)IleLys(Boc)Val. The above tripeptide (1.8 g, 3.1 mmole) was hydrogenated in 25 ml of a methanol – acetic acid mixture (10 : 1) for 8 h in the presence of 0.2 g of Pd-C. Then the catalyst was separated by filtration and the solvent was distilled off. To the residue was added 5 ml of DMF, 0.43 ml (3.1 mmole) of triethylamine, and 1.8 g (3.4 mmole) of ZTyr(Bzl)ONp. The target product was crystallized from a hexane – diethyl ether mixture (1 : 1) and purified by sequentially boiling in ethyl acetate (doubly) and diethyl ether. Yield, 1.70 g (63%); m.p., 205°C (with decomp.); $[\alpha]_D^{20}$, –19° (*c*, 1.0; methanol); R_f, 0.53 (A), 0.45 (B), 0.70 (C); $C_{46}H_{63}N_5O_{10}$.

Tyrosylisoleucyl-N-ε-*tert*-butyloxycarbonyllysylvaline TyrIleLys(Boc)Val. The above compound (2.115 g, 2.5 mmole) was hydrogenated in 60 ml of a methanol – acetic acid – anisole mixture (1 : 4 : 1) in the presence of 0.2 g of Pd-C. Upon completion of the process (monitored by TLC in system F), the catalyst was separated by filtration and the filtrate was concentrated by evaporation in vacuum to a residual volume of 40 ml. To the residue was added 10 ml of isopropyl alcohol and the concentration continued until the solvent ceased to evaporate. This treatment was doubly repeated. The final solid residue was triturated with 50 ml of anhydrous diethyl ether, filtered, and dried in a vacuum desiccator (over P_2O_5 and KOH) to constant weight. Yield of the target tetrapeptide, 1.203 g (72%); m.p., 180 – 185°C (with decomp.); $[\alpha]_D^{20}$ + 73° (*c*, 1.0; methanol); R_f, 0.82 (F); E_{His}, 0.46; $C_{31}H_{51}N_5O_8 \cdot CH_3COOH$. Amino acid analysis: Tyr, 0.81(1); Ile, 0.97(1); Lys, 1.12(1); Val, 1.10(1).

N-α-Benzyloxycarbonylprolyltyrosylisoleucyl-N-ε-*tert*-butyloxycarbonyllysylvaline ZProTyrIleLys(Boc)Val. The pentapeptide was synthesized from the above tetrapeptide (0.636 g, 1 mmole), 0.126 ml (1 mmole) of N-ethylmorpholine, and 0.444 g (1.2 mmole) of ZProONp. The product was crystallized by adding a sodium bicarbonate solution. Upon filtration, the target compound was purified by washing with diethyl ester and reprecipitation from dioxane with diethyl ether. Yield of the target pentapeptide, 0.567 g (66%); m.p., 199 – 204°C (with decomp.); $[\alpha]_D^{20}$ + 30° (*c*, 1.0; methanol); R_f, 0.22 (B), 0.38 (E), 0.54 (A); $C_{44}H_{64}N_6O_{11} \cdot 1/2H_2O$.

Prolyltyrosylisoleucylvaline ProTyrIleLysVal (I). The above pentapeptide (0.469 g, 0.55 mmole) was hydrogenated for 4 h in the presence of Pd-C. The hydrogenated product (0.319 g, yield, 81%) was crystallized from diethyl ether, dried, and dissolved in a mixture of trifluoroacetic acid with methylene chloride (3 : 7, v/v). The solution was allowed to stand for 1 h at room temperature and concentrated by evaporation to a residual volume of 1 ml. Product I was precipitated by adding 100 ml of anhydrous diethyl ether: yield, 0.366 g (97%); m.p., 168 – 179°C (with decomp.); $[\alpha]_D^{20}$ + 65° (*c*, 1.0; methanol); R_f, 0.12 (G); $C_{31}H_{50}N_6O_7 \cdot 2CF_3COOH$. Amino acid analysis: Pro, 1.03(1); Tyr, 0.89(1); Ile, 0.96(1); Lys, 1.07(1); Val, 1.04(1).

The data of elemental analyses agree with the results of analytical calculations using the corresponding empirical formulas.

EXPERIMENTAL BIOLOGICAL PART

Biological tests were performed on a group of 256 male CBA-5 mice aged 5 weeks and weighing 14 – 16 g.

Pentapeptide I and the reference compound tafcine ThrLysProArg (II) [15, 16] were dissolved in an apyrogenic

TABLE 1. Effect of Peptides I and II on Thymus-Dependent and Thymus-Independent Immune Response

Peptide	Dose, g-mole / mouse	Number of anti-IgM-AFC per 10^6 splenic caryocites in mice immunized with	
		goat erythrocytes	Vi-antigen
PYIKV I	10^{-11}	7.9 ± 1.6 (28)	...
	10^{-9}	5.7 ± 0.9 (28)	6.2 ± 0.6 (10)
	2×10^{-8}	5.2 ± 1.2 (10)	4.8 ± 0.6 (10)
TKPR II	10^{-11}	7.7 ± 0.4 (12)	...
	10^{-9}	17.8 ± 1.3* (12)	6.8 ± 0.5 (12)
	2×10^{-8}	21.5 ± 1.3* (12)	7.2 ± 0.3 (12)
Control (APS)	–	8.0 ± 1.2 (28)	6.8 ± 1.1 (22)

* $p < 0.05$ against control (APS = apyrogenic physiological solution; values in parentheses indicate the number of test animals).

TABLE 2. Phagocytosis-Stimulating Activity of Peptides I and II

Peptide	*in vitro*			*in vivo*		
	Concentration, g-mole / ml	Phagocyte index	Phagocyte number	Dose, g-mole / mouse	Phagocyte index	Phagocyte number
PYIKV I	10^{-12}	15.3 ± 2.4	1.6 ± 0.22	10^{-12}	17.4 ± 0.5	1.9 ± 0.1
	10^{-11}	40.5 ± 3.4*	1.7 ± 0.1	10^{-11}	44.2 ± 2.1*	1.9 ± 0.1
	10^{-9}	38.3 ± 0.4*	1.9 ± 0.1	10^{-9}	51.0 ± 2.2*	1.9 ± 0.1
TKPR II	10^{-12}	18.0 ± 1.7	1.7 ± 0.1	10^{-12}	18.5 ± 0.5	1.8 ± 0.1
	10^{-11}	41.2 ± 2.6*	2.3 ± 0.1*	10^{-11}	45.5 ± 1.5*	2.1 ± 0.1
	10^{-9}	45.8 ± 2.3*	2.3 ± 0.3	10^{-9}	45.6 ± 1.9*	1.9 ± 0.1
Control*	0	19.2 ± 0.4	1.8 ± 0.1	0	22.7 ± 1.9	1.8 ± 0.1

* $p < 0.01$ against control (cells in Hank's solution).

physiological solution (Polfa, Poland) and subcutaneously injected to the test mice over a period of 5 days. Animals in the control group were injected with the same dose of the plain apyrogenic physiological solution (APS).

The test mice treated with the preparations studied were divided into two groups. The first group was immunized (single intravenous injection) either with a thymus-dependent antigen (goat erythrocytes, 2×10^6 cells) or with a thymus-independent Vi-antigen (0.001 μg). On the fourth day upon immunization, the animals were decapitated. The spleen of each mouse was studied by the local hemolysis technique in agarose (Sigma, USA) [17] to determine the amount of anti-IgM antibody-forming cells (AFC). In determining the anti-IgM-AFC with respect to the Vi-antigen, the latter (20 μg/ml) was loaded by the goat erythrocytes [18]. To remove the unbound antigen, the erythrocytes were washed with a physiological solution (the procedure was repeated not less than eight times).

Animals in the second group were used to evaluate the phagocyte activity of peritoneal neutrophiles under both *in vitro* and *in vivo* conditions. In the *in vitro* experiment, the cells of a peritoneal exudate were incubated with compounds I and II ($10^{-12} - 10^{-9}$ g-mole / ml) for 15 min at 37°C. The exudate was obtained (for both *in vitro* and *in vivo* experiments) 2.5 h after intraperitoneal injection of the test mice with a sterile 10% peptone solution. By this time, the exudate accumulated a maximum (97 – 98%) amount of neutrophiles. The exudate cells were isolated by thoroughly washing the abdominal cavity with a sterile Hank's solution and used in a final concentration of 12.5×10^6 cells / ml. The test microbe was a daily-grown culture of *Staphylococcus aureus*. A suspension of test microbes (250×10^6 cells / ml) was added to the peritoneal exudate cells (*in vitro*, upon treating these cells with peptides) and the mixture was incubated for 15 min at 37°C. Then the suspension was centrifuged for 7 min at 1200 rpm and the smears of deposit were studied by staining according to Romanovskii to determine the phagocyte index (percentage of neutrophiles involved in the phagocytosis) and the phagocyte number (amount of microbial cells per phagocyte) [19]. Each experiment was repeated at least doubly or triply, the total neutrophile count reaching 900 – 10,000.

RESULTS AND DISCUSSION

It was established that pentapeptide I changed neither the amount of anti-IgM-AFC for the goat erythrocytes nor the level of thymus-independent immune response to the Vi-antigen. The reference compound tafcine increased the number of anti-IgM-AFC for the goat erythrocytes, while also not affecting the immune response to the Vi-antigen (Table 1). At the same time, the phagocytosis-stimulating activity of compound I was virtually the same (both *in vitro* and *in vivo*) as that of tafcine (II). For a peptide concentration of 10^{-11} or 10^{-9} g-mole / ml and the doses 10^{-11} and 10^{-9} g-mole / mouse, the phagocyte index doubled (Table 2). Under the *in vitro* conditions, only compound II (at a concentration of 10^{-11} g-mole / ml) increased the phagocyte number.

Before analyzing the experimental results, it should be noted that the biologically active thymus peptide fragments affect both the specific and nonspecific characteristics of resistance. In particular, the tetrapeptide tymantine (ThrThrLysAsp) [5] at a dose of 10^{-9} g-mole / mouse increased the anti-IgM-AFC production for the goat erythrocytes [12] and simultaneously increased the phagocyte index *in vivo* [19]. Similarly to tymantine, the pentapeptide tymopentine (ArgLysAspValTyr) [20] also enhanced both processes [21]. All the peptides, favoring (like tafcine) an increase in the phagocyte index, stimulated the thymus-dependent immune response while not affecting the thymus-independent response. In contrast to the above peptides, the Thy-1 antigen fragment (ProTyrIleLysVal) synthesized in this work stimulates the phagocyte activity of neutrophiles, while modifying the levels of neither thymus-dependent nor thymus-independent immune responses. For this reason, we have called this fragment "stifagine."

The selectivity of the stifagine action upon the phagocyte index cannot be explained by the predominant content of the phagocytosis-stimulating amino acids (Pro, Tyr, Lys, Val) in this molecule, since the tetrapeptide fragment ProTyrIleLys of the Thy-1 antigen (containing three phagocytosis-stimulating amino acids – Pro, Tyr, Lys) is incapable of enhancing phagocytosis [9].

The ambiguity of action upon the phagocytosis and immune response observed for various Thy-1 antigen fragments indicates that the immunological activity of the whole Thy-1 antigen molecule differs from the properties of its fragments. This fact allows us to search for new immunomodulants among the fragments of endogenous polypeptides. The results obtained in this work agree with our earlier [12] hypothesis that there are two systems of immunogenesis regulation, one related to peptides and the second to amino acids.

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