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COMMUNICATION

Functional Roles of Phenylalinine[7] of Thymic Humoral Factor-γ2 in the Impaired Blastogenic Response of Uremic T-Lymphocytes

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ABSTRACT

The peptide analogs of thymic humoral factor-γ2 (THF-γ2) in which phenylalinine residue at the 7th position are replaced by phenylglycine (Phg), homophenylalanine (Hph), and 1-naphthylalanine (1-Nal) were synthesized by a solid-phase method and the immunological significance of the aromatic amino acid of this position was comparatively investigated. The in vitro restoring effect of the synthetic peptides on the impaired phytohemagglutinin (PHA) response of T-lymphocytes from uremic patients was tested. The observed activities of these peptides were in order (1-Nal[7]) thymic humoral factor [THF]-γ2 > 4-Fluoro (Phe[7]) THF-γ2 > THF-γ2. However, the other two analogs, [Phg[7]] THF-γ2 and [Hph[7]] THF-γ2, had no restoring effect even at a higher concentration.

INTRODUCTION

Cellular immunity is known to be impaired in uremic patients. This impairment has been implicated in high susceptibility to infections and an increased incidence of malignancy. The thymus plays an essential role in the development and maintenance of cellular immune competence.

Thymic humoral factor-γ2 (THF-γ2), an octapeptide essential for immune regulation, was isolated from calf thymus by Burnstein et al. (1). THF-γ2 has the amino acid sequence: H-Leu-Glu-Asp-Gly-Pro-Lys-Phe-Lys-OH and shows no sequence similarity to those of other thymic hormones.

In a previous paper (2), we demonstrated that our synthetic THF-γ2 restored the impaired blastogenic response of T-lymphocytes from uremic patients and one of our synthetic analogs. (phenylalinine [4-fluoro] (phe[4F])[7]) THF-γ2 exhibited the most potent effect. On the contrary, the synthetic (cyclohex-alanine[7]) (cHex-

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Ala[7]) THF-γ2 had no restoring effect at the same conditions. These results seem to suggest that an aromatic ring at Phe[7] of THF-γ2 plays an important role in regulating immunological activities.

To investigate the precise structural significance or contribution of Phe[7] residue of THF-γ2 in the restoring effect on impaired blastogenic response of T-lymphocytes from uremic patients, three analogs of THF-γ2 containing phenylglycine (Phg), homophenylalanine (Hph), or 1-naphthylalanine (1-Nal) instead of Phe[7] were synthesized and the immunological properties were comparatively studied by the fluorometric blast-formation test (3) of impaired uremic T-lymphocytes.

Experimental

Materials

(Boc)*tert*-butoxycarbonyl -β-naphthylalanine (Boc-1-Nal-OH), Boc-homo-phenylalanine (Boc-Hph-OH), and Boc-phenylglycine (Boc-Phg-OH) were purchased from Bachem BioScience Inc. The other Boc-amino acids, Boc-Glu (benzyl ester [Obz1]),-OH, Boc, Asp (OBzl)-OH, Boc-Leu-OH, Boc-Gly-OH, and Boc-Lys (para-chlorobenzyloxycarbonyl [Clz]) -OH, were purchased from Protein Research Inc. (Mino, Osaka, Japan) and Kokusan Chemical Works Ltd. (Kyoto, Japan). Boc-Leu-resin ester (1 mmol: Boc-Leu ester of methylated 1% divinylbenzene–crosslinked polystyrene) was purchased from Sigma Chemical Co., St. Louis, MO). Solvents were freshly distilled before using. The amino acid compositions of the hydrolysates were determined with a Hitachi type 835-50 amino acid analyzer. HPLC was conducted with a Shimadzu LC-6A apparatus coupled to a Wakopak Wakosil-II 5C18 AR column (6.0 mm × 150 mm). Fast atom bombardment–mass spectroscopy (FAB-MS) spectra were obtained on a Auto Spec Q (UQ Analytical Co., England) mass spectrometer equipped with an OPUS data processor. Purified peptides were chromatographed on silica-gel plates (Kieselgel G, Merck) and R_f^1 values refer to BuOH-pyridine-acetic acid (AcOH)-H_2O (15:10:3:12 by volume) and R_f^2 values refer to BuOH-AcOH-pyridine-H_2O (5:5:1:4 by volume).

Patient Selection

Three uremic patients who were suffering from recurrent infectious diseases were selected. Examination of the cellular immunocompetence of these patients revealed a significant decrease in blast formation by PHA. ^{3}H-Thymidine incorporation values of these patients were 11,059, 12,163, and 12,071 cpm, respectively (normal values 41,655–42,050 cpm). Venous blood was obtained from these uremic patients for the fluorometric blast-formation test. Venous blood samples from three healthy donors were used as a control. The fluorescence excitation spectrum was measured with an Oyo-Bunko Fluospec 11A fluorometer. Kits for the fluorometric blast-formation test were purchased from Japan Immunoresearch Laboratories Co., Ltd., Japan.

Solid-Phase Peptide Synthesis

The three analogs were constructed on Boc-Leu-resin (1 mmol/g, 1%, crosslinked, 100–200 mesh) according to the coupling schedule described previously (2). The protected peptide resin was treated with anhydrous HF containing 10% anisole at 0°C for 60 min. After evaporation of excess HF under vacuum, the resulting residue was extracted with 5% AcOH. The extract was washed with ether and evaporated to dryness under vacuum. The crude peptide was applied to a Sephadex G-25 column (2.7 × 90 cm), which was eluted with 1% AcOH. The fractions corresponding to the front main peak (monitored by UV absorption measurement at 230 nm) were combined and the solvent was removed by lyophilization to produce a white fluffy powder. For further purification of each peptide, each gel-filtered sample was subjected to reversed-phase HPLC on a μBondapak C18 column (7.8 mm × 300 mm) which was eluted with a linear gradient of 10–35% acetonitrile in 0.1% trifluoroacitic acid (TFA) over 150 min at a flow rate of 3 ml/min. The eluate was monitored at 260 nm. Fractions around the main peak were checked by analytical HPLC, the pure parts were collected, and the solvents were removed by repeated lyophilization. Analytical data of the purified peptides are shown in Tables 1 and 2.

Fluorometric Blast-Formation Test

A 3-ml portion of venous blood from uremic patients was drawn into a syringe containing 25 units of heparin/ml, and mixed with 3 ml of phosphate buffered saline (PBS). Lymphocytes were isolated in a Hypaque–Ficoll gradient. Isolated lymphocytes were adjusted to 1.0×10^6/ml with PBS. The lymphocytes were cultured in 0.5 ml of Rosewell Park Memorial Institute (RPMI) 1640 (Gibco) with fetal calf serum (FCS) (Dainippon Pharmaceutical Co., Japan) in microplates. Cultures of each combination were incubated at 37°C in the presence of the peptide in a humidified atmosphere of 5% CO_2 in

Table 1

Characterization of Synthetic THF-γ2 Analogs

Peptide	Yield[a] (%)	$[\alpha]_D^{21}$ (°C) (c = 0.5, 1%, AcOH)	TLC[b] R_f^1	TLC[b] R_f^2	HPLC[c] Retention Time (min)	FAB-MS[d] (MH^+)
(1-Nal[7])THF-γ2	34	-63.9	0.37	0.52	15.48	968.13
(Phg[7])THF-γ2	36	-67.5	0.35	0.50	15.06	903.86
(Hph[7])THF-γ2	35	-65.8	0.38	0.55	15.19	932.01

[a]Final yield after deblocking and purification starting from Boc-Leu-resin.
[b]Thin layer chromatography, see the experimental section.
[c]See the experimental section.
[d]Found values were in agreement with calculated values.

Table 2

Amino Acid Analysis of Synthetic THF-γ2 Analogs[a]

Peptide	Gly	Leu	Pro	Lys	Asp	Glu	1-Nal	Phg	Hph	Average Recovery of Gly (%)
(1-Nal[7])THF-γ2	1.00	1.03	0.94	1.90	0.96	0.99	0.95			89
(Phg[7])THF-γ2	1.00	1.04	0.91	2.02	1.01	0.98		0.97		87
(Hph[7])THF-γ2	1.00	0.97	0.96	2.03	1.02	0.97			0.98	85

[a]After acid hydrolysis with 6 N HCl at 110°C for 24 hr.

air for 12 hr and phytohemagglutinin (PHA) (0.125%, 0.5 ml) was added to each well. Incubation was continued under the same conditions for 60 hr. Lymphocytes in each well were transferred to a test tube and centrifuged for 10 min at 240 × g, and an aliquot of 0.15% SDS was added to the residue and stirred for 20 min at room temperature; lymphocytes were completely destroyed and solubilized by this procedure. An ethidium bromide solution was added to the above solution and the mixture was stirred for 15 min at room temperature. The fluorescence excitation spectrum was measured by the method of Itoh and Kawai (3).

RESULTS AND DISCUSSION

The aromaticity of the amino acid in THF-γ2 seems to be crucial for the immunological activity because the replacement of the Phe[7] with cyclohexylalanine completely loses immunological effect (2). On the other hand, one of the analogs containing *para*-fluorophenylalanine at position 7 of THF-γ2 elicits 11 times the restoring activity on the impaired blastogenic response of PHA-stimulated T-lymphocytes of uremic patients (2). These results seem to suggest that evaluation of the importance of aromatic ring[7] in THF-γ2 could be a first step to obtain promising analogs for cell-mediated immunodeficiency treatment.

The substitution of Phe[7] in THF-γ2 with its analogs Phg, Hph, or 1-Nal was undertaken. The immunological effects of the synthetic THF-γ2, [Phe(4F)[7]]THF-γ2, and its new three analogs were examined by the JIMRO (Japan Immunoresearch Laboratories Ltd., Japan) fluorometric blast-formation test (3).

Responses of T-lymphocytes to mitogenic stimulation were significantly lower in uremic patients than were those of normal persons. The in vitro effect of the synthetic peptides on the impaired PHA response of T-lymphocytes from uremic patients is shown in Tables 3 and 4.

One of the analogs, [1-Nal[7]]THF-γ2, which has a naphthyl group instead of phenyl group, exhibited stronger restoring effect than those of THF-γ2 and [Phe(4F)[7]THF-γ2. The other two analogs containing Phg or Hph in place of Phe at the seventh position of THF-γ2, [Phg[7]]THF-γ2 and [Hph[7]]THF-γ2, exhibited no restoring effect even at higher concetration.

These findings show that the space of one CH_2 between peptide skeleton and aromatic nucleus is necessary at least in order to activate the receptors on T-lymphocytes of uremic patients and indicate that the

Table 3

Effects of Synthetic THF-γ2 and Its Analogs on the Impaired PHA Stimulation of Uremic T-Lymphocytes

Peptide	Dose (μg/ml)	SI[a,b]
–[c]	–	279.6 ± 50.1
–[d]	–	118.4 ± 49.3[g]
THF-γ2[d,e]	0.1	208.7 ± 48.7[h]
(Phe[4F][7])THF-γ2[d,e]	0.010	209.3 ± 49.6[h]
(1-Nal[7])THF-γ2[d,e]	0.008	207.5 ± 50.3[h]
(Phg[7])THF-γ2[d,e]	10.0	116.2 ± 51.3
(Hph[7])THF-γ2[d,e]	10.0	115.6 ± 50.2
H-Gly-Gly-Lys-OH[d,e,f]	10.0	117.2 ± 51.1

[a]Each value represents the mean ± SD of triplicate measurements.
[b]SI (stimulation index) was calculated according to the following formula: SI = $(I_2 - I_0)/(I_1 - I_0) \times 100$, where I_2 is mean fluorescence intensity of PHA-activated lymphocytes, I_1 is mean fluorescence intensity of PHA-nonactivated lymphocytes, and I_0 is mean fluorescence intensity of ethidium bromide.
[c]Normal venous lymphocytes.
[d]Uremic patient's lymphocytes.
[e]Incubation was carried out at 37°C in a humidified atmosphere of 5% CO_2 in air for 12 hr in the presence of each peptide.
[f]Control: purchased from the Peptide Institute Inc., Osaka, Japan.
[g]$p < 0.05$, when compared with the normal subject using Student's t test.
[h]$p < 0.01$, when compared with the uremic patients using Student's t test.

existence of an aromatic ring and the distance between an aromatic ring and a peptide backbone are both important for immunological activities. Extention of methylene group (Hph) seems to be the cause of losing immunological activity and one of the synthetic analogs containing Phg has fewer rotational degrees of freedom than those which contain Phe. As a result, the substitution of Phe with Phg was not favorable for immunological activity. Among the analogs, [1-Nal[7]]THF-γ2 showed a stronger restoring effect on the impaired blastogenic response of uremic T-lymphocytes than that of [Phe(4F)[7]]THF-γ2. This result exhibited that not only the 4-fluorophenyl ring, but also more bulky naphthyl ring could bind with the receptor more strongly than that of THF-γ2. Furthermore, those results provided us with some ideas to develop potent and useful immune potentiating analogs for cell-mediated immunodeficiency treatment.

Table 4

Relative Potencies of THF-γ2 and Its Analogs on the Impaired PHA Stimulation of T-Lymphocytes of Uremic Patients

Peptide	Relative Potency (Molar Basis)
THF-γ2	1.00
(Phe[4F][7])THF-γ2	11.08
(1-Nal[7])THF-γ2	12.03
(Phg[7])THF-γ2	–
(Hph[7])THF-γ2	–

REFERENCES

1. Y. Burnstein, V. Buchner, M. Pecht, and N. Trainin, Biochemistry, 27, 4066–4071 (1988).
2. T. Abiko and H. Sekino, Biotechnol. Appl. Biochem., 19, 355–360 (1994).
3. Y. Itoh and T. Kawai, Rinsho Kensa, 27, 928–931 (1983).