天然免疫調整物質オビオアクチン由来合成ペプチド,Obiopeptide-1,の生物活性

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Biological Activity of Obiopeptide-1, a Synthetic Peptide Derived from the Native Immune-Regulator Obioactin

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ABSTRACT. Tachyzoites of *Toxoplasma gondii* were killed in mouse macrophage and human somatic cell monolayers by a novel synthetic peptide (Obiopeptide-1) which is a Glycil-penta-Glutaminate (GpG) derivative of native Obioactin. In view of the worldwide prevalance of this protozoan disease and the lack of effective treatments, Obiopeptide-1 may be a new and unique antimicrobial active substance of non-antibiotic chemotherapeutic agents for intracellular parasites, *T. gondii* and associated nonspecific hypoimmune responses that occur in infected hosts.—KEY WORDS: immunoregulator, macrophage activation, nonantibiotic antimicrobial agent, synthetic obiopeptide, *Toxoplasma* killing.

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It has been shown that cultivation of spleen cells from a *Toxoplasma*- hyperimmune animal in the presence of *Toxoplasma* lysate antigen (TLA) causes the release of a lymphokine or cytokine which inhibits the multiplication of *Toxoplasma* in homologous cell lines [6, 10, 11]. This lymphokine, referred to as Toxo-GIF, is a glycoprotein with a molecular weight of approximately 30,000 to 40,000 daltons which is believed to be a product of T-lymphocytes [4, 12]. Toxo-GIF inhibits multiplication of *Toxoplasma* in somatic cells as well as macrophages, but is active only against intracellular protozoa in cells of the same animal species, i.e. it does not inhibit multiplication of *Toxoplasma* in cells of different animal species [5, 6]. Because of this host specificity, Toxo-GIF is not effective against human toxoplasmosis or infections that occur in other species of animals.

We have previously described a novel polypeptide with a molecular weight of less than 5,000 daltons which inhibits the multiplication of *Toxoplasma* in a variety of animal species [13]. This polypeptide is prepared by hydrolysis of serum of *Toxoplasma*-immune animals [7–9, 13]. Serum hydrolysates which originate from *Toxoplasma*-immune cattle are generally referred to as Obioactin [13]. Obioactin has antitumor activity as well as antimicrobial activity against a variety of microorganisms and is effective as a modifier of biological responses [8, 14, 15]. Since the only current source of Obioactin is *Toxoplasma*-immune serum, it is important to determine the primary structure and active sites of this polypeptide for eventual mass production by synthetic processes.

MATERIALS AND METHODS

Source of Obioactin: Native Obioactin with a molecular weight of less than 5,000 daltons was obtained from *Toxoplasma*-immune cattle serum and hydrolyzed with proteinase, HCl and NaOH [13].

Purification: Partially purified Obioactin [13] was fractionated in a DEAE-5PW column (21.5 mm ID X 15 cm, Tosoh Co., Tokyo) by NaCl gradient ion-exchange

chromatography with a High Pressure Liquid Chromatography (HPLC) apparatus (Hitachi Co., Tokyo). A NaCl gradient ranging from 0 M to 1 M in a basic solution of 0.02 M ammonium acetate was used for the fractionation. An active fraction obtained by ion-exchange chromatoraphy was subjected to reverse-phase HPLC with an ODS-120T column (4.6 mm ID X 250 mm, Tosoh Co., Tokyo) over an acetonitrile concentration gradient of 10–100% in 0.1% trifluoroacetic acid.

Characterization of refined Obioactin: The HPLC fraction of refined Obioactin was sealed in a tube containing a constant boiling point solution of hydrochloric acid and 0.1% thioglycol. The contents of the tube were heated to 110°C for 24 hr to hydrolyse the Obioactin fraction. The amino acid composition was analyzed by the OPA method [1, 2] with a Hitachi amino acid analyzer (Model 835, Hitachi Co., Tokyo).

Peptide synthesis: Obiopeptide analogs were synthesized by the t-Boc method (Biosearch 9600 type, Biosearch Co., U.S.A.). All reagents were obtained from Biosearch Distribution, Kubota-Shoji Co., Tokyo.

After extraction and Tosoh-column HPLC, the identification of the peptides was determined with a Type CCP & 8010 HPLC unit (Tosoh Co., Tokyo) that employed a TSKgel ODS-80TM column (21.5 mm X 300 mm) and the purity was checked by an analytical TSKgel ODS-80TM column (4.6 mm X 250 mm). HPLC profiles of these peptides indicated that they had a purity of more than 95%. To confirm that synthesis progressed to completion, an amino-terminus sequence analysis was performed on all peptides with an Applied Biosystems 470-A gas phase protein sequence apparatus (Applied Biosystems Co., USA). PTH-derivatized amino acids, generated from the sequencer, were analyzed with the Tosoh HPLC CCP & 8010 super system (Tosoh Co., Tokyo).

Preparation of Obiopeptide: A stock solution of Obiopeptide was prepared by dissolving 10 mg of synthetic polypeptide in 1 m*l* of 100 mM $NaHCO_3$ (pH 6.4). Aliquots from the stock solution were diluted with TC-199 medium containing 10% calf serum (Medium) just prior to use to give final concentrations of 500, 100, 10 and 1 μg/m*l*. A solution of Medium was used alone as a control.

Preparation of macrophage and somatic cell monolayers: Two milliliters of sterile 0.2% glycogen saline solution were injected intraperitoneally (i.p.) into adult mice (BALB/c male mice, 8–10 weeks age). Five days later, peritoneal exudate containing elicited macrophages were harbested by washing the peritoneal cavities of the mice with heparinized HBSS. The cell suspension was centrifuged at 200 X G for 5 min and the sediment was suspended in Medium at a concentration of 1×10^6 nucleated cells per m*l*. One milliliter of this suspension was placed in a multidish tray (FB-16–24-TC, Linbro Chemical Co., Inc.) containing round coverslips and incubated in a humidified 5% CO_2 incubator at 37°C for 4 hr. Cells that did not adhere to the coverslips were removed by two rinses with Medium. The coverslips were then reincubated overnight with the same medium in a CO_2 incubator. Thereafter, the cell cultures were rinsed with Medium and used 24 and 48 hr after preparation for assays of *Toxoplasma* growth inhibition.

Assessment of antiprotozoal activity: Tachyzoites of the RH strain of *T. gondii* were obtained from the peritoneal cavity of mice 2 days after inoculation (ai). Tachyzoites were cultured in 1 m*l* aliquots of various concentrations of Obiopeptide at 37°C in a 5% CO_2 incubator. Each aliquot contained approximately 5×10^6 tachyzoites. At intervals of 1, 2 and 3 hrs after exposure to

Obiopeptide, 0.2 m*l* aliquots were removed from each culture, stained with 1 drop of 0.2% trypan blue and examined. Two counts of the number of dead, stained tachyzoites per 100 tachyzoites were made and averaged to calculate average mortality (%).

Obiopeptide solution was added in 1.5 m*l* aliquots to individual wells of multidish trays that contained mouse macrophage or somatic cell monolayers. One hour prior to the addition of Obiopeptide, the monolayers were infected with approximately 5×10^4 tachyzoites per well, giving an approximate ratio of one tachyzoite per 20 mononuclear cells. One hr ai, the monolayers were washed thoroughly to remove excess parasites and then incubated at 37°C for 24 and 48 hrs in a CO_2 incubator. Obiopeptide solution (0.05 mg/m*l*) was also added in 1.5 m*l* aliquots to multidish trays containing cultures of guinea pig macrophages, canine monocyte-macrophages, or human heart cells that had been infected with 5×10^4 tachyzoites per dish one hr earlier. These cultures were also incubated at 37°C for 24 and 48 hrs in a 5% CO_2 atmosphere.

Coverslips were fixed and stained with May-Grüenwald Giemsa double stain at regular time intervals and intracellular parasites in all cultures were counted by phase contrast microscopy. *Toxoplasma* can be readily identified by its characteristic morphology within cytoplasmic vacuoles of infected cells. Infection rates were calculated by counting the number of parasites in phagocytic vacuoles of 1,000 individual cells on each coverslip. The number of tachyzoites per individual cell was recorded as O Tp for cells devoid of parasites, 1–5 Tp for cells containing 1–5 organisms, and 6 Tp for cells containing 6 or more organisms. Experiments were repeated at least 3 times.

RESULTS

Gel filtration and ion-exchange chromatography: Obioactin prepared as described by Suzuki *et al.* [13] was fractionated by NaCl gradient ion-exchange chromatogra-

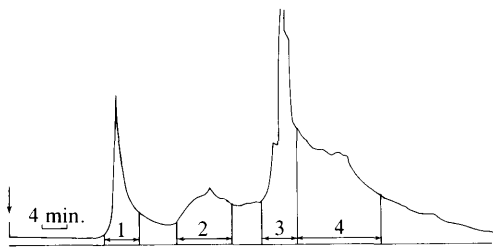

Fig. 1. HPLC elution pattern from a DEAE-5PW column after application of Obioactin.

Note: A typical trace for Obioactin (total 100 mg). Operating conditions are as described in Materials and Methods. (Flow rate, 3.0 m*l*/min; Eluate, 0.02 M CH_3COOH_4, O to 1 M NaCl, linear gradient to 2 hrs; wave-length, 280 nm)

Table 1. Inhibition of *Toxoplasma* by DEAE-5 PW fractions

Sample	Percentage of cells containing *Toxoplasma* (%)			Toxo-GIF	
	0 Tp	1 to 5 Tp	≧6 Tp/cell	Activity (%)	Cytotoxicity
Control (Tc-199)	62.8± 7.5	22.6±4.9	14.6±3.9	–	–
Fr. 1	48.6± 9.3	29.0±3.8	22.4±8.8	−38.2	–
Fr. 2	66.8±15.7	17.8±8.6	15.4±8.8	10.8	–
Fr. 3	81.4±11.4	11.8±4.8	6.8±7.4	50.0	–
Fr. 4	68.8±10.8	17.4±5.9	13.6±5.6	16.1	–

Note: Results are the means (± standard deviation) from 5 independent experiments. Operating conditions are described in Materials and Methods. See Fig. 1.

phy (Fig. 1). Each fraction was lyophilized, desalted by gel filtration through Sephadex G15 (Pharmacia Co., Sweden) and then re-lyophilized. Each powdered fraction was dissolved in Medium to a final concentration of 5 mg/m*l* and evaluated for Toxo-GIF activity with mouse peritoneal macrophages. Toxo-GIF activity was −38.2% for fraction 1, 10.8% for fraction 2, 50.0% for fraction 3 and 16.1% for fraction 4 (Table 1). Because of its higher Toxo-GIF activity, the 3rd fraction was collected and refined by reverse-phase liquid chromatography with an ODS-120 T column as described earlier (Fig. 2). Each fraction was concentrated under reduced pressure and lyophilized. These sub-fractions were then tested for Toxo-GIF activity (Table 2). Activities were 97.6% in fraction 3-I, 96.8% in fraction 3-II, 97.2% in fraction 3-III, and 99.2% in fraction 3-IV. Fraction 3-IV was selected for further characterization because of its high activity.

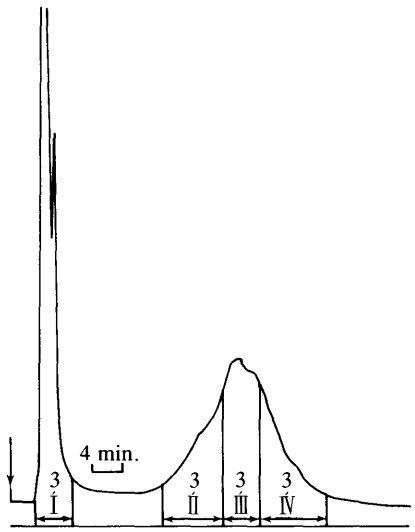

Fig. 2. HPLC-separation of fraction-3 (Fig. 1) with an ODS-120 T column by reverse phase chromatography.

Note: A typical trace of the eluation pattern after application of 10 mg of fraction 3 from Fig. 1. Operations are described in Materials and Methods. (Flow rate, 1.0 m*l*/min; Eluate, initial stage-acetonitrile (10%) +0.1% TFA (90%) to final stage-acetonitrile (100%), linear gradient to 1 hr; wave length, 230 nm)

Composition and amino acid sequence: Refined Obioactin in fraction 3-IV was hydrolysed in a constant boiling point tube as described earlier and analyzed with a Hitachi amino acid analyzer. The fraction contained 30% Asx (total of Asn + Asp), 43% Glx (total of Glu + Gln), 7% Gly, 6% Ala, 5% Val and 3% Pro. Predominant amino acids in fractions 3-I, 3-II and 3-III were also Asx, Glx, Gly, Val, Lys, and Ala. Pro and Leu were also detected as minor components.

The N-terminal amino acids of fraction 3-IV as determined by the Edman degradation method included Glu, Asp, Gly and

Table 2. Inhibition of *Toxoplasma* by ODS-120 T fractions

Sample	Percentage of cells containing *Toxoplasma* (%)			Toxo-GIF	
	0 Tp	1 to 5 Tp	≧6 Tp/cell	Activity (%)	Cytotoxicity
Control (Tc-199)	49.8±16.3	26.8±7.1	23.4±9.3	−	−
Fr. 3-I	98.8± 1.1	1.2±1.1	0	97.6	−
Fr. 3-II	98.4± 1.5	1.6±1.5	0	96.8	−
Fr. 3-III	98.6± 2.1	1.2±1.6	0	97.2	−
Fr. 3-IV	99.6± 0.5	0.4±0.5	0	99.2	−

Note: See Table 1 and Fig. 2. Each eluated fraction, Fr. 3-I to Fr. 3-IV, was concentrated under reduced pressure, and then lyophilized. Each powdered fraction was reconstituted to a concentration of 0.5 mg/m*l* and then evaluated for Toxo-GIF activity.

Table 3. Arrangement of amino acids in refined Obioactin, Frac. 3-IV by ODS 120 T fractionation

Amino Acid No.	(n mol)									
	Glu	Asp	Gly	Ala	Val	Pro	Tyr	Leu	Ile	Les
1	2.6	1.2	1.0	0.8	0.4	0.3	0.2	0.1	0.1	0.1
2	2.1	0.7	0.8	0.3	0.4	0.2	–	0.2	–	–
3	1.2	0.5	–	0.2	0.3	0.3	–	0.2	–	–
4	0.5	0.3	–	–	0.1	0.1	–	0.1	–	–
5	0.3	0.2	–	–	–	–	–	–	–	–
6	0.2	0.1	–	–	–	–	–	–	–	–
7	0.1	0.1	–	–	–	–	–	–	–	–

Note: Operating conditions are described in Materials and Methods. (≧0.1 n mol)

Table 4. Inhibition of *Toxoplasma* by synthetic peptides

Sample (0.5 mg/m*l*)	Percentage of cells containing *Toxoplasma* (%)			Toxo-GIF Activity (%)	Cytotoxicity
	0 Tp	1 to 5 Tp	≧6 Tp/cell		
10%CS-Tc-199 (Control)	83.6±3.6	7.6±1.8	8.8±3.5	–	–
Glu-Glu-Glu-Glu-Glu	89.4±8.5	5.2±4.9	5.4±3.9	35.4	–
Gly-Glu-Glu-Glu-Glu-Glu	96.6±3.4	2.6±2.6	0.8±0.8	79.3	–
Ala-Glu-Glu-Glu-Glu-Glu	76.0±20.5	10.8±7.6	13.0±12.9	−46.3	–
Asp-Asp-Asp-Asp-Asp	90.0±8.5	3.8±2.4	6.2±6.4	39.0	–
Gly-Asp-Asp-Asp-Asp-Asp	83.2±7.5	9.8±4.6	7.0±3.7	−2.4	–
Asp-Asp-Asp-Asp-Asp-Asp	90.0±6.0	5.8±3.1	4.2±3.3	39.0	–

Note: Each of the synthetic peptides was dissolved in Medium+10%CS to a concentration of 0.5 mg/m*l*. Toxo-GIF activity was measured using mouse peritoneal macrophages.

Table 5. Dose dependency of glycil-penta-glutaminate (GpG) in the inhibition of *Toxoplasma*

Concentration of GpG (mg/m*l*)	Percentage of cells containing *Toxoplasma* (%)			Toxo-GIF Activity (%)	Cytotoxicity
	0 Tp	1 to 5 Tp	≧6 Tp/cell		
0	84.0±1.6	26.8±7.1	5.0±2.3	–	–
1.000	99.8±0.4	0.2±0.4	0	98.8	±~–
0.500	99.4±0.5	0.6±0.5	0	96.3	–
0.250	93.4±6.3	4.2±4.1	2.4±2.4	58.8	–
0.100	90.8±6.4	5.2±3.4	4.0±3.4	42.5	–
0.050	88.4±7.6	7.0±4.5	4.6±4.2	27.5	–
0.025	82.6±10.8	9.8±6.3	7.6±4.8	− 8.8	–

Note: See Table 4. Operating conditions are described in Materials and Methods.

Ala as major constituents of the first cycle (first residue) and Val, Pro, and Tyr as minor constituents. Glu and Asp were major constituents of the second cycle. The second cycle also contained Ala, Val, and Pro. Glu and Asp were the major constituents of the third to seventh cycles (Table 3). Similar results were obtained from analysis of fractions 3-I, 3-II and 3-III. None of the four fractions obtained by reverse phase chromatography could be refined further by additional chromatography.

Synthesis and biological activity of peptides: Oligopeptides that were estimated to be the smallest constitutive units of Obioactin were synthesized as described earlier [1,

3]. Each of the peptides was dissolved in Medium to give a final concentration of 0.5 mg/m*l* and evaluated for Toxo-GIF activity with mouse peritoneal macrophages (Table 4). Toxo-GIF activities of the respective peptides were 35.4% for penta-Glutaminate (pG), 79.3% for Glycil-penta-Glutaminate (GpG), −46.3% for Alanyl-penta-Glutaminate (ApG), 39.9% for penta-Asparaginate (pA), −2.4% for Glycil-penta-Asparaginate (GpA) and 39.3% for Alanyl-penta-Asparaginate (ApA). None of the peptides were cytotoxic at concentrations of 0.5 mg/m*l*.

The dose dependency of GpG was examined because of its high Toxo-GIF activity (Table 5). This peptide showed Toxo-GIF activity at concentrations above 0.05 mg/m*l* with saturation at a concentration of 0.5 mg/m*l*. Shrinkage and exfoliation of cultured cells were observed at a concentration of 1.0 mg/m*l*. Significant Toxo-GIF activity was detected at GpG concentrations of 0.25 mg/m*l*. By contrast, the Toxo-GIF activity of native Obioactin is not appreciable at a concentration of below 5 mg/m*l*.

GpG exhibited Toxo-GIF activity in canine monocytes, human cardiac muscle cells and mouse macrophages (Table 6), indicating that this peptide did not exhibit the host restriction that is characteristic of native Obioactin. Combinations of GpG and other synthesized peptides were also examined for possible synergistic effects in stimulating Toxo-GIF activity (Table 7). Synergistic effects were not observed, even when 0.5 mg/m*l* concentrations of various peptides were added to 0.5 mg/m*l* concentrations of GpG.

Table 6. Inhibition of *Toxoplasma* by glycil-penta-glutaminate (GpG) in heterologous cells

Cell	Concentration of GpG (mg/m*l*)	Percentage of cells containing *Toxoplasma* (%)			Toxo-GIF Activity (%)	Cytotoxicity
		0 Tp	1 to 5 Tp	≧6 Tp/cell		
Mouse	0	76.6±9.2	16.5±5.1	7.2±4.3	–	–
Macrophages	0.05	88.4±7.6	7.0±4.5	4.6±4.2	50.4	–
Dog	0	40.1±15.5	28.5±6.8	31.3±8.2	–	–
Monocytes	0.05	80.5±6.4	12.8±5.4	6.7±3.3	67.4	–
Human	0	54.4±18.3	23.5±9.3	22.1±9.7	–	–
Cardiac Muscle	0.05	80.7±6.1	10.6±3.4	8.7±2.1	57.7	–

Note: Operating conditions are described in Materials and Methods.

Table 7. Synergistic effect of synthetic peptides

Sample		Percentage of cells containing *Toxoplasma* (%)			Toxo-GIF Activity (%)	Cytotoxicity
A (0.5 mg/m*l*)	B (0.5 mg/m*l*)	0 Tp	1 to 5 Tp	≧6 Tp/cell		
Control	(Tc-199)	76.6±9.2	16.2±5.1	7.2±4.3	–	–
GpG	GpG	100	0	0	100	±
GpG	–	100	0	0	100	–
GpG	pA	95.6±2.3	4.0±1.9	0.4±0.5	81.2	+
GpG	pG	98.0±1.0	2.0±1.0	0	91.5	±
GpG	GpA	91.4±4.7	7.8±4.8	0.8±0.4	63.2	±
GpG	ApA	91.4±5.7	8.6±5.2	0.2±0.4	61.5	–
GpG	ApG	100	0	0	100	++

Note: See Materials and Methods.

DISCUSSION

Results of this study indicate that active fractions of Obioactin have an N-terminal containing Glu, Asp, Gly, or Ala and biologically active regions that contain mainly 4 to 5 molecules of Glu or Asp. The high biological activity of GpG indicates that it is the major active unit of the native Obioactin molecule. GpG has a molecular weight of about 720. Penta-Glutaminate, ApA and pA also exhibit Toxo-GIF activity and may have potential uses in inhibiting the intracellular multiplication of *Toxoplasma* and promoting host immunoregulation. The biological activity of GpG was 10 to 20 times higher than that of native Obioactin on a per weight basis, and 40 to 140 times higher on a per mol basis.

Refined Obioactin is probably a mixture of peptides that resemble each other and have heterologous N-terminals. It is not clear why low molecular weight oligopeptides can be separated from native Obioactin which has a major molecular weight of approximately 3,000. It is believed, though, that active regions of Toxo-GIF and other natural lymphokines are composed of 10 to 20 active units of low molecular weight that are weakly bound to each other to form a high molecular weight aggregate. During the refining operation, these dissociate into several units with different molecular weights. This is one reason why it is difficult or rather impossible to purify native Obioactin as a single primary structure [4, 6, 13].

Our objective has been to refine Obioactin and identify the sequence of amino acids in this immunoregulator that is capable of enhancing cell functions. We synthesized the oligopeptide GpG, termed Obiopeptide-1, which has significantly higher biological activity than native Obioactin on a per weight or per mol basis. This peptide does not exhibit species specificity and has important potential applications in the treatment of protozoan infections and modulation of hypoimmune responses in infected hosts.

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要　約

天然免疫調整物質オビオアクチン由来合成ペプチド，Obiopeptide-1，の生物活性：鈴木直義・桜井治久・斎藤篤志・五十嵐郁男・小俣吉孝・尾崎文雄(帯広畜産大学獣医学科家畜生理学教室・原虫病細胞免疫研究室)——トキソプラズマ原虫栄養型虫体はマウスマクロファージ及びヒト体細胞単層培養細胞内において合成ペプチド(Obiopeptide-1)添加によって著しく増殖抑制され，多くは死滅する．本ペプチドは1 Glycil と 5 Glutaminate よりなり，天然オビオアクチンの有する細胞内殺トキソプラズマ原虫活性単位の1つとして合成された．世界の動物および人に認められるトキソプラズマ感染症に対する有効な治療薬のない現在，Obiopeptide-1は新規の抗微生物活性を有する非抗生物質治療補助剤として細胞内寄生原虫および一般感染症に随伴する宿主免疫能の低下に対する有効な免疫調整作用物質になり得るかも知れない．