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Restorative Effects of A Newly Synthesized Peptide, Obiopeptide-1, in Cyclophosphamide- or Carrageenan-pretreated Mice Infected with Opportunistic Bacteria

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ABSTRACT

Large numbers of cyclophosphamide- or carrageenan-pretreated immunosuppressed adult ddY mice died within 10 days after inoculation with several species of opportunistic bacteria, including *P. aeruginosa*, *K. pneumoniae*, *E. coli*, *S. aureus* and methicillin resistant *S. aureus*. When immunosuppressed mice were administered bacteria in combination with two 100 μg/mouse intramuscular doses of a newly synthesized peptide, Obiopeptide-1 (OP-1), survival rates increased significantly. At 24 and 48 hours after intraperitoneal inoculation with *P. aeruginosa*, counts of viable organisms from the livers, spleens, lungs, hearts, and kidneys of mice that were administered cyclophosphamide in combination with OP-1 were significantly lower than counts from mice that were administered cyclophosphamide alone. Bacteriocidal activity of peritoneal cells, neutrophils and monocyte-macrophages was higher in OP-1 pretreated mice than in non-OP-1 treated mice. This newly synthesized peptide, OP-1, is a potential immunomodulator which increases host resistance against bacterial infection. The peptide may also function as a nonspecific blood stimulating factor.

INTRODUCTION

During organ transplants and tumor surgery, experimental animals and clinical patients are commonly given immunosuppressive agents such as cyclophosphamide (Cyp) and carrageenan(Carra) to suppress the normal immune function. This treatment often provokes *Mycobacterium bovis* (Takahashi, & Collins, 1987), *Listeria monocytogenes* (Hugin et al, 1986), *Pseudomonas aeruginosa* (Saegusa et al., 1990; Urano, 1978; Yokota, 1984), Methicillin resistant *Staphylococcus aureus* (MRSA, Kouda et al., 1987), *Trypanosoma cruzi* (McCabe et al., 1985) and other microorganisms.

Obioactin is a native immunoregulatory substance found in lymphokines produced by the lymphocytes of *Toxoplasma*-immune cattle which has been shown to inhibit the multiplication of *Toxoplasma gondii* in macrophages and somatic cells in a variety of animal species (Suzuki et al., 1984; Fujii et al., 1992). This

immunomodulator has a molecular weight of less than 5,000 daltons.

Newly synthesized Obipeptides 1,2,3, and 4 have shown significantly higher biological activity than native Obioactin on a per weight or per mole basis (Fujii et al., 1992; Sakurai et al, 1991; Suzuki et al., 1984, 1990a,b.). Some of our synthesized Obiopeptides are similar to partial peptides of prothymosin alpha, a polypeptide with 110 amino acide residues (Fangaou et al., 1988; Watts et al., 1989). Recently, prothymosin alpha has been shown to protect mice against an opportunistic infection caused by *Candida albicans* that is particularly threatening to AIDS patients (Eschenfeld et al., 1988; Gomez et al., 1989). For these reasons, experiments in the present study were conducted to study the immunoregulatory effects of this peptide on cyclophosphamide- or carrageenan-treated immunosuppressed mice with opportunistic bacterial infections.

MATERIALS AND METHODS

Animals used: Male mice of the ddY strain that were 8-10 weeks of age were used for all experiments (Carely Co., Tokyo). Animals were maintained under standard conditions at room temperature (22 ± 1^{O}C with 60±5% humidity), fed ad lib with pellets (Clea Japan Co., Tokyo) and water. Separate groups of mice were used for the mortality test and for the tests to measure persistence of viable bacteria within internal organs.

Obiopeptide-1 (OP-1): Obiopeptide-1 , Gly-Glu-Glu-Glu-Glu-Glu, was synthesized chemically with a synthesizer (Biosearch 9600 type, Biosearch Co., USA) (Suzuki et al., 1990a, b).

Cyclophosphamide(Cyp): Cyclophosphamide monohydrate (Janssen Chemica, 2340, Beerse, Belgium) was dissolved in a physiological saline solution at a concentration of 25 mg/ml and sterilized by filtration before use.

Carrageenan (Carra): Carrageenan (Wako Pure Chemical Co., Tokyo) was mixed with a physiological saline solution in a glass mortar. A final concentration of 6 mg/ml was prepared and kept at 4^{O}C until used.

Preparation of bacterial suspensions: Standard agar cultures of *Pseudomonas aeruginosa* (IFO 12689), *Klebsiella pneumoniae* (PCI 602, ATCC 10031) *Escherichia coli* (K-12), *Staphylococcus aureus* (209P, IFO 12732, ATCC 6538P), and Methicillin resistant *Staphylococcus aureus* (MRSA, strain Nos. 910308-508, 910309-502, 910309-504, 910311-520, 910312-521, 910313-591) were cultivated more than 3 times (BBL, Becton Dickinson Microbiology Systems, USA). The 6 strains of MRSA were kindly shared by Dr. M. Kouda, Department of Central Clinical Lab., Tokyo Metropolitan Police Hospital, Tokyo. Bacteria were cultured on a standard agar plate 2 days prior to preparation of a suspension. A loop of bacteria was collected with a Stickmate 10 (Ono Pharmaceutical Co., Tokyo), and suspended in 10 ml of a physiological saline solution. The suspension was standardized with a Hitachi double beam spectrophotometer at 600 nm to give a constant turbidity and used as a bacterial stock suspension. The concentration of bacteria was determined precisely by preparing serial 10-fold dilutions of the suspension according to the plate dilution method.

Tests of infectivity: Mice were inoculated intraperitoneally (ip) with 0.1 mg of Cyp solution per 10 g body weight 4 days prior to receiving injections of bacteria. Final dosage of Cyp was 250 mg/kg. Other experimental mice were inoculated ip with 0.1 ml of Carra solution per 10 g of body weight 2 days prior

to receiving infection of MRSA. Final dosage of Carra was 60 mg/kg. Mice were inoculated ip with 1 ml of bacterial suspension. This experimental scheme followed the same method as Yokota (1984).

Treatment of Inoculated mice: Obiopeptide-1 was injected intramuscularly (im) in the femoral area of mice that has been treated with Cyp or Carra. Dosage of 100 μg/mouse were administered 7 days and 1 day prior to intraperitoneal inoculation with bacteria.

Counts of viable bacteria: Mice were killed at specified times after inoculation (ai) with bacteria and injected ip with 3 ml of Hanks balanced salt solution (HBSS). The abdomens were massaged and 1.5 ml of peritoneal exudate was collected with a heparinized syringe from each mouse. Animals were then laparotomized and blood was collected from the inferior vena cava. Livers, spleens, kidneys, stomachs, lungs and hearts were excised, weighed and cut in half. One half of each organ was fixed in 10 % phosphate buffered formalin (pH7.0) for histological examination. Remaining halves were weighed and used to estimate numbers of viable bacteria. Serial 10-fold dilutions were prepared of peritoneal exudates and blood with sterile physiological saline. Other organs were homogenized in 10 ml of sterilized saline with a glass homogenizer. Serial 10-fold dilutions were prepared from homogenates with sterile physiological saline. One ml aliquots of the dilutions were placed in Petri dishes and mixed with warm 40^{O}C agar. After the agar solidified, the dishes were incubated at 37^{O}C for 2 days. The number of bacterial colonies were counted in each dish and used to estimate numbers of viable bacteria in each organ.

Preparation of spleen lymphoide cells: Spleens were removed under sterile conditions from mice that had been killed by cervical dislocation. The organs were chopped into fine pieces at ice-cold temperatures, ground between glass slides, and suspended in HBSS. The tissue suspension was filtered through a # 40 stainless steel mesh to remove large pieces of tissue and centrifuged at 800 x g for 10 min at 4^{O}C. Red blood cells were hemolyzed with 0.83% ammonium chloride at 37^{O}C. Non-hemolyzed cells were filtered through a glass fiber column to eliminate filamenteous tissue and adherent cells and centrifuged. The pellet was resuspended in HBSS and applied to a Conray-ficoll density gradient for collection of the lymphocyte rich fraction (Suzuki et al., 1984). The lymphocyte fraction was washed twice by centrifugation at 800 x g for 7 min at 4^{O}C, once with HBSS and once with RPMI 1640. These cell suspensions were analyzed by flow cytometry (Cell Sorter FACS, Showadenko Co., Tokyo).

RESULTS

Protective effects of OP-I on Cyp-treated immunosuppressed mice that were administered bacteria:

When untreated control mice (P-G-1) were inoculated ip with 8x 10^{6} *P. aeruginosa*, all individuals survived 10 days ai and had an overall increase in body weight of 12.3% (Table 1). None of the 10 Cyp-treated mice (P-G-2) survived to 10 days ai. By 8 days ai, their body weights had an overall decrease of 23.3%. Seven of the 10 mice that were treated with Cyp and OP-1 survived 10 days ai, but had overall decline in body weight of 12.8%.Similar results were obtained when mice were inoculated ip with 6x 10^{6} *K. pneumoniae*. All untreated control mice survived 10 days ai with an overall weight gain of 12.3%. Only one of the 10 Cyp-treated mice survived to 10 days ai. Mice in this group had an overall weight loss of 20.8%. An overall decrease in body weight of 22.3% occurred in mice that were injected with both Cyp

Number of Bacxteria		Administration (Days) of OP-1 and Cyp				Days after inoculation							Percent survival
		-8	-7	-4	-1	0	1	2	3	4	6	10	(%)
P.aeruginosa($8x10^6$)													
P-G-1	OP-1		-	-	-								
	Cyp		-	-	-								
	B.W. (g)	34.2							37.0			38.4	
	(change %)	(0)							(+8.2)			(+12.3)	
	Survival of mice					10	10	10	10	10	10	10	100
P-G-2	OP-1		-	-	-								
	Cyp		-	+	-								
	B.W. (g)	34.8							33.4		26.7		
	(change %)	(0)							(-4.0)		(-23.3)		
	Survival of mice					10	6	5	1	1	1	1	0
P-G-3	OP-1		+	-	+								
	Cyp		-	+	-								
	B.W. (g)	34.5							33.7			28.0	
	(change %)	(0)							(-4.0)			(-12.8)	
	Survival of mice					10	7	7	7	7	7	7	70
K.pneumoniae($6x10^6$)													
K-G-1	OP-1		-	-	-								
	Cyp		-	-	-								
	B.W. (g)	34.2							37.0			38.4	
	(change %)	(0)							(+8.2)			(+12.3)	
	Survival of mice					10	10	10	10	10	10	10	100
K-G-2	OP-1		-	-	-								
	Cyp		-	+	-								
	B.W. (g)	34.6							32.6			27.1	
	(change %)	(0)							(-5.8)			(-20.8)	
	Survival of mice					10	8	6	1	1	1	1	10
K-G-3	OP-1		+	-	+								
	Cyp		-	+	-								
	B.W. (g)	34.5							33.0			26.8	
	(change %)	(0)							(-4.4)			(-22.3)	
	Survival of mice					10	10	8	7	6	6	6	60

Notes: Approximately 6-8x 10^6 *P. aeruginosa* or *K. pncemoniae* were inoculated intraperiloneally (ip) into a mouse: Cyclophosphamidc(Cyp, 250mg/kg) was injected ip, and Obiopeptide-l(OP-l, 100 μg/mouse) was injected intramuscularly. G-l, 10 mice were not treated with OP-1 and Cyp; G-2, 10 mice were treated with Cyp alone 4 days before inoculation of bacteria; and G-3, 10 mice were treated with Cyp 4 days before inoculation of bacteria and with OP-1 7 and 1 days before inoculation of bacteria.

and OP-1. Only 6 of the 10 mice in this last group survived until 10 days ai.

Estimates were made of viable *P. aeruginosa* in organs of mice that were administered Cyp alone (G-Cyp) or Cyp in combination with OP-1 (G-Cyp+OP-1) (Table 2). Large numbers of bacteria were initially present in peritoneal exudates and peripheral blood of mice in group G-Cyp between 3 and 24 hrs ai and then tended to decrease. Bacteria showed a clear tendency to shift from the liver to the kidney between 3 and 48 hrs ai. Numbers of bacteria in group G-Cyp+OP-1 were substantially lower at 3 and 24 hrs ai than those in group G-Cyp. By 48 hrs ai, the most notable differences between the 2 groups in

Table 2. Average Number of Viable *P. aeruginosa* in the Organs of Mice that were Administered Cyclophosphamide Alone or in Combination with Obiopeptide-1

Hours after inoculation	Average numbers of viable organisms after inoculation							
	Peritoneal fluids	Peripheral blood	Liver	Spleen	Lungs	Heart	Kidney	Stomach
3 hrs								
G-Cyp*	$464x10^3$	8200	850	200	550	85	25	0
G-Cyp+OP-1**	$173x10^3$	4550	725	220	650	25	0	0
24 hrs								
G-Cyp	$680x10^3$	1365	0	305	105	945	1350	2
G-Cyp+OP-1	$143x10^3$	1210	0	10	25	800	1080	3
48 hrs								
G-Cyp	$146x10^3$	290	10	15	$160x10^3$	2875	$107x10^5$	3
G-Cyp+OP-1	10	0	2	0	1	0	1	0
216 hrs								
G-Cyp	0	0	65	40	0	0	20	215
G-Cyp+OP-1	0	0	35	8	0	0	7	0

Notes: All averages were calculated from 3 mice independently in each group.
*: G-Cyp, mice were treated with Cyclophosphamide (Cyp) alone 4 days before intraperitoneal inoculation with $2x\ 10^8$ *P. aeruginosa*.
**: G-Cyp+OP-l, mice were treated with Cyp 4 days before inoculation of bacteria and with Obiopeptide-l (OP-l) 7 and 1 days before inoculation with *P. aeruginosa*.

numbers of viable bacteria were in peritoneal exudates, lungs, hearts and kidneys with OP-1 treated animals having significantly fewer bacteria.

When Cyp-treated and Cyp+OP-1 treated mice were inoculated ip with $5.5x\ 10^6$ *E. coli* or $5.4x\ 10^9$ *S. aureus*, survival rates were 50% and 60%, or 60% and 70%, respectively (Table 3). Mice receiving Cyp+OP-1 combinations had slightly higher rates of survival. However, rates of survival of groups treated with Cyp and Cyp+OP-1 were similar after treatment with different strains of MRSA. Survival was 20% and 20%, respectively, after administration of strains 2 and 3, 20% and 40% after administration of strains 4 and 5, and 20% and 0% after administration of strains 6 and 7.

***Protective effects of OP-1 on carrageenan pretreated mice against MRSA infection*:**

When untreated mice (G-1-1-1) were inoculated ip with $1.6x\ 10^9$ of the No.2 strain, 4 of 5 mice survived until 5 days ai (Table 4). Three of the 5 Carra-treated mice (G-1-1-2) survived until 5 days ai. All of the 5 mice that were treated with Carra+OP-1 (G-1-1-3) survived until 5 days ai.

After inoculation with $1.2x10^9$ of the No.3 strain, one of 5 mice in G-2-2-2 survived, and 4 of 5 Carra+OP-1 treated mice (G-2-2-3) survived, respectively. When 5 Carra-treated mice were inoculated with $8x10^8$ of the No.3 strain (G-2-3-2), 3 of 5 mice survived, and all of 5 Carra+OP-1 treated mice (G-2-3-3) survived until 5 days ai.

After inoculation of $1.1x\ 10^{10}$ organisms of MRSA, one of 5 Carra-treated mice (G-3-2-2) survived, and 3 of 5 Carra+OP-1 treated mice (G-3-2-3) survived. When 5 Carra-treated mice were inoculated with $2.2x\ 10^9$ of the No.4 strain (G-3-3-2), 3 of 5 mice survived, and all of the 5 Carra+OP-1 treated mice survived.

Table 3. Survival Rates of Mice Inoculated with *E. coli, S. aureus* and MRSA after Administration of Cyclophosphamide Alone or in Combination with Obiopeptide-1

Number of Bacteria	Number of Mice examined	Survival of mice after bacterial inoculation (Days)						Percent Survival
		0	1	2	3	4	5	(%)
E.coli(5.5x10^{6}CFU/mouse)								
Cyp*	10	10	8	5	5	5	5	50
Cyp+OP-1**	10	10	10	7	6	6	6	60
S.aureus(5.4x10^{6}CFU/mouse)								
Cyp	10	10	8	6	6	6	6	60
Cyp+OP-1	10	10	9	7	7	7	7	70
MRSA***								
No.2(521)(3.2x10^{6}CFU/mouse)								
Cyp	5	5	1	1	1	1	1	20
Cyp+OP-1	5	5	1	1	1	1	1	20
No.3(502)(7.5x10^{6}CFU/mouse)								
Cyp	5	5	3	1	1	1	1	20
Cyp+OP-1	5	5	3	1	1	1	1	20
No.4(520)(5.1x10^{6}CFU/mouse)								
Cyp	5	5	1	1	1	1	1	20
Cyp+OP-1	5	5	2	2	2	2	2	40
No.5(508)(3.2x10^{6}CFU/mouse)								
Cyp	5	5	3	1	1	1	1	20
Cyp+OP-1	5	5	4	2	2	2	2	40
No.6(504)(8.8x10^{6}CFU/mouse)								
Cyp	5	5	1	1	1	1	1	20
Cyp+OP-1	5	0	0	0	0	0	0	0
No.7(591)(6.5x10^{6}CFU/mouse)								
Cyp	5	5	1	1	1	1	1	20
Cyp+OP-1	5	0	0	0	0	0	0	0

*: Treated with Cyclophosphamide (Cyp) 4 days before bacterial inoculation.
**: Treated with Cyp in Combination with Obiopcplide-l (OP-l) 7 and 1 days before bacterial inoculation.
***: Methicillin Resistant *Staphylococcus aureus*.

When Carra-treated mice were inoculated with either 8x 10^8 of the No.5 strain (G-4-2-2) or 1.4x 10^9 of the No.6 strain (G-5-2-2), 3 of the 5 mice in the former group and none of the 5 mice in the latter group survived to 5 days ai. Among Carra+OP-1 treated mice in G-4-2-3 and G-5-2-3, all mice in the former and 4 of the 5 mice in the latter group survived.

When mice treated with either Carra alone (G-6-2-2 or G-6-3-2) or Carra+OP-1 (G6-2-3 or G-6-3-3) and inoculated with either 1.3x 10^9 or 2.8x 10^8 of the No.7 strain, one of the 5 in G-6-2-2 and G-6-3-2 survived until 5 days ai, while 2 of the 5 in G-6-2-3 and 3 of the 5 in G-6-3-3 survived until 5 days ai, respectively.

Bacteriocidal activities of peritoneal cells:

Numbers of *P. aeruginosa* from peritoneal exudates of groups 1,2,3, and 4 were compared at 0.5, 1 and 3 hrs after bacterial inoculation (Table 5). The numbers of organisms in non-treated control mice from group 1 and OP-1 treated mice from group 2 were 269x 10^4 and 148x $l0^4$ at 0.5hr, 121x $10^4$and 18x 10^4 at 1 hr ai. Numbers of mouse peritoneal cells at 5 hrs after ip injection of 0.5% glycogen solution were 20.3x 10^2 in group 1 and 34.0x 10^2 in group 2. A higher percentage of neutrophils was found in group 2.

Table 4. Changes in Survival Rates of Mice Inoculated with *Staphylococcus aureus* (MRSA strains) after Administration of Carrageenan Alone or in Combination with Obiopeptide-1

No. of strains	Inoculated number of the strain	Administration of OP-1 and Carrageenan			Survival of mice after bacteria inoculation						No. of survival /no. examined (%)	Percent survival
		-7	-2	-1(days)	0	1	2	3	4	5		
No.2(521)												
G-1-1-1	$1.6x10^9$											
	OP-1	-	-	-	5	4	4	4	4	4	4/5	80
	Carra	-	-	-								
G-1-1-2	$1.6x10^9$											
	OP-1	-	-	-	5	3	3	3	3	3	3/5	60
	Carra	-	+	-								
G-1-1-3	$1.6x10^9$											
	OP-1	+	-	+	5	5	5	5	5	5	5/5	100
	Carra	-	+	-								
No.3 (502)												
G-2-2-2	$1.2x10^9$											
	OP-1	-	-	-	5	5	3	3	3	1	1/5	20
	Carra	-	+	-								
G-2-2-3	$1.2x10^9$											
	OP-1	+	-	+	5	5	4	4	4	4	4/5	80
	Carra	-	+	-								
G-2-3-2	$8.0x10^8$											
	OP-1	-	-	-	5	4	4	3	3	3	3/5	60
	Carra	-	+	-								
G-2-3-3	$8.0x10^8$											
	OP-1	+	-	+	5	5	5	5	5	5	5/5	100
	Carra	-	+	-								
No.4(520)												
G-3-2-2	$1.1x10^{10}$											
	OP-1	-	-	-	5	4	1	1	1	1	1/5	20
	Carra	-	+	-								
G-3-2-3	$1.1x10^{10}$											
	OP-1	+	-	+	5	3	3	3	3	3	3/5	60
	Carra	-	+	-								
G-3-3-2	$2.2x10^9$											
	OP-1	-	-	-	5	3	3	3	3	3	3/5	60
	Carra	-	+	-								
G-3-3-3	$2.2x10^9$											
	OP-1	+	-	+	5	5	5	5	5	5	5/5	100
	Carra	-	+	-								
No.5(508)												
G-4-2-2	$8.0x10^8$											
	OP-1	-	-	-	5	5	5	5	4	3	3/5	60
	Carra	-	+	-								
G-4-2-3	$8.0x10^8$											
	OP-1	+	-	+	5	5	5	5	5	5	5/5	100
	Carra	-	+	-								
No.6(504)												
G-5-2-2	$1.4x10^9$											
	OP-1	-	-	-	5	4	3	1	0	0	0/5	0
	Carra	-	+	-								
G-5-2-3	$1.4x10^9$											
	OP-1	+	-	+	5	4	4	4	4	4	4/5	80
	Carra	-	+	-								
No.7(591)												
G-6-2-2	$1.4x10^9$											
	OP-1	-	-	-	5	1	1	1	1	1	1/5	20
	Carra	-	+	-								
G-6-2-3	$1.4x10^9$											
	OP-1	+	-	+	5	2	2	2	2	2	2/5	40
	Carra	-	+	-								
G-6-3-2	$2.8x10^8$											
	OP-1	-	-	-	5	2	2	1	1	1	1/5	20
	Carra	-	+	-								
G-6-3-3	$2.8x10^8$											
	OP-1	+	-	+	5	5	4	4	3	3	3/5	60
	Carrag	-	+	-								

Six strains (No.2-No.7) of Staphylococcus aureus were isolated clinically as the MRSA from the human patients at the Police hospital, Tokyo. Approximately $2.8x10^8$- $lx10^{10}$ MRSA were inoculated ip into each mouse. Carrageenan (Carra., 60mg/kg as 6 mg/ml of saline solution) was injected ip 2 days, and Obiopeptide-l (OP-l, 100μg/mouse) was injected im 7 days and 1 day before bacterial inoculation, respectively.

Table 5. Bacteriocidal Activities of Peritoneal Cells Collected from Mice Injected with 0.5% Glycogen Solution 5 hours before Bacterial Inoculation.

Group	Administration (Days) of OP-1 and Cyp			Average number($1x10^4$) of viable organisms in peritoneal exudates after inoculation			Number of peritoneal cells	Differentiation of peritoneal exudate cells just before inoculation				
	-7	-4	-1	0.5	1	3 (hrs)	($x10^2$)	Eos.	Neut.	Baso.	Lymph.	Mono.(%)
Group 1												
OP-1	-	-	-	269±100	121±40		20.3±9.7	1.7±1.2	24.7±15.1	0	72.0±14.0	2.3±0.9
Cyp	-	-	-									
Group 2												
OP-1	+	-	+	148±48	18±3		34.0±5.0	1.7±1.7	54.3±23.5	0	43.3±24.4	0.7±0.9
Cyp	-	-	-									
Group 3												
OP-1	-	-	-			46.6±12	8.0±2.0	0	2.5±0.5	0	97.5±0.5	0
Cyp	-	+	-									
Group 4												
OP-1	+	-	+			17.3±5	7.6±2.0	0	11.4±7.5	0	88.5±7.5	0
Cyp	-	+	-									
Significant difference between	Group 1 and 2			P<0.001	P<0.001							
	Group 3 and 4					P<0.05						

Note: Mean ± SD values were calculated from 10 mice in Group 1 and 2. Each mouse was injected intraperitoneally with 2.0 ml of 0.5% glycogen solution 5 hours before inoculation with *P. aeruginosa*. Collection of peritpncal exudates was done by injection of 5 ml of Hank's balanced salt solution.

Table 6. Bacteriocidal Activities of Peritoneal Cells Collected from Mice Injected with 0.2% Glycogen Solution 5 days before Bacterial Inoculation.

Group	Administration (Days) of OP-1 and Cyp			Average number($1x10^4$) of viable organisms in peritoneal exudates after inoculation			Number of peritoneal cells	Differentiation of peritoneal exudate cells just before inoculation				
	-7	-4	-1	1	4	24(hrs)	($x10^2$)	Eos.	Neut.	Baso.	Lymph.	Mono.(%)
Group 1												
OP-1	-	-	-	1072±115	468±176		20.0±11.0	0.3±0.5	0	0	95.0±2.9	4.7±2.6
Cyp	-	-	-									
Group 2												
OP-1	+	-	+	538±213	323±153		37.0±23.7	1.0±1.4	15.3±17.4	0	80.0±19.1	3.7±1.2
Cyp	-	-	-									
Group 3												
OP-1	-	-	-			68±56	9.5±5.0	0.3±0.8	1.8±4.3	0	95.8±2.4	2.5±1.1
Cyp	-	+	-									
Group 4												
OP-1	+	-	+			14±12	10.5±3.9	0.5±0.5	4.0±1.4	0	93.5±2.9	2.2±2.3
Cyp	-	+	-									
Significant difference between	Group 1 and 2			P<0.002	P<0.5							
	Group 3 and 4					P<0.5						

Note: Mean ± SD values were calculated from 10 mice in Group 1 and 2. Each mouse was injected intraperitoneally with 2.0 ml of 0.2% glycogen solution 5 days before inoculation with *P. aeruginosa*. Collection of peritoneal exudates was done by injection of 5 ml of Hank's balanced salt solution.

Numbers of *P. aeruginosa* from peritoneal exudates of Cyp-treated mice from group 3, and Cyp+OP-1 treated mice from group 4 were compared at 3 hrs ai. Numbers of organisms were 46.4x 10^4 in group 3 and 17.3x 10^4 in group 4 at 3 hrs ai. Numbers of neutrophils from the exudate cells in group 4 were larger than those in group 3. Numbers of viable bacteria in peritoneal exudates from OP-1 treated mice in groups 2 and 4 were significantly lower than those in groups 1 and 3 at 0.5, 1 and 3 hrs ai.

Numbers of bacteria in peritoneal exudates in mice injected with 0.2 % glycogen solution 5 days before

inoculation is presented in Table 6. Numbers of organisms in non-treated mice (group 1) and OP-1 treated mice (group 2) were 1072x 10^4 and 538x 10^4 at 1 hr, and 468x 10^4 and 323x 10^4 at 4 hrs ai, respectively. Numbers of organisms in Cyp-treated mice (group 3) and Cyp+OP-1 treated mice (group 4) were 68x10^4 and 14x 10^4 at 24 hrs ai. Numbers of peritoneal exudate cells and neutrophils in OP-1 treated mice in groups 2 and 4 were larger than those from non-OP-1 treated groups 1 and 3.

DISCUSSION

Yokota (1984) and Ogata (1983) reported a correlation between increased susceptibility to opportunistic pathogens and reduced leukocytes counts in mice that were administered Cyp or Carra. In these experiments, marked reduction of body weight and total numbers of spleen mononuclear cells were not noted until 4 to 6 days after administration of a single 250 mg/kg dose of Cyp. These values recovered in time to normal values or values that were higher than normal. Most mice that were administered Cyp died within 5 days after they were inoculated with bacteria. Accordingly, the authors suggested that reduced resistance to infection may be related mainly to a reduction of leukocytes and macrophages.

Obiopeptide (Suzuki et al., 1990) has an activity as an immunomodulator and is capable of increasing resistance to the growth of tumor cells (Sakurai et al., 1991). In the present study, doses of 100 μg /mouse were capable of increasing resistance to bacterial infection, especially against *P. aeruginosa* and *K. pneumoniae*, and reducing mortality. This was demonstrated by the almost complete elimination of live bacteria from organs of mice that were administered Cyp and OP-1 within 2 days ai. It is still not known whether the antibacterial mechanism of OP-1 stems from increases in the activity of macrophages and neutrophils. Nevertheless, increases in the numbers and the functional activation of the cells in OP-1 treated mice is important evidence suggesting that the hosts showed a strong resistance against bacterial infection.

In recent years, immunosuppressive agents such as Cyp have been used routinely worldwide after organ transplantation or for leukemia therapy and treatment of some tumors. Agents that can prevent opportunistic infections in immunosuppressed individuals are urgently needed. Results of our study demonstrate that OP-1 is capable of moderating opportunistic bacterial infections in immunosuppressed animals and has potential as a nontoxic immunomodulator. Further studies on the mode of action of Obiopeptide and application of this immunomodulator in conjunction with antibiotics and other chemotherapeutic agents may make it possible to develop treatments for infectious diseases that are currently intractable.

REFERENCES

Eschenfeld W. H. Marrow, R. E. Krug, M. S. and Berger L. S. 1989. Isolation and partial sequencing of human Prothymosin alpha gene family. *J. Biol. Chemist 264*: 7546-7555.

Fanganou-Lazarides.M.,Clinton, M., Goodall, G. J. and Horecker, B. L. 1988. Prothymosin alpha and Parathymosin: amino acide sequences deduced from cloned rat spleen cDNAs. *Arch. Biochem. Biophy. 263*: 305-310.

Fujii, Y., Maki, Y., Sakurai, H., Igarashi, I., Omata, Y., Saito, A. and Suzuki, N. 1992. Growth inhibitory effect of a newly synthesized peptide, Obiopeptide-1, on mice bearing methylcholanthrene induced murine tumors. *Jpn. J. Vet. Sci. 54*: 351-353.

Gomez-Marquez, J., Segade, F., Dosil, M., Pichel, J. G., Bustelo, X. R., and Freire, M. 1989. The expression of Prothymosin alpha gene in T-lymphocytes and leukemic lymphoide cells is tied to lymphocyte proliferation. *J. Biol. Chem. 264*: 8451 –8454

Hugin, A. W., Cerny, A., Wran, M., Hengarter, H. and Zinkernagel, R. M. 1986. Effect of cyclosporin A on immunity to *Listeria monocytogenes*. *Infect. Immunit 52*: 12-17.

Kouda, M., Kobayashi, J., Kumagai, I., Uemura, C., and Mathuzaki, H. 1987. Detection rate of *Staphylococcus aureus* isolated from blood cultures and evaluation of in vitro activity of various antibiotics against methicillin-resistant *S. aureus* isolated from blood cultures. *J. Jpn. Assoc. Infect. Dis. 61*: 1230-1238 (in Japanese).

MaCabe, A. E., Remington, J. S., and Araujo, F. G. 1985. In vivo and in vitro effects of cyclosporin A on *Trypanosoma cruzi*. *Am. J. Trop. Mod. Hyg. 34*: 861 -865.

Nozawa, T. 1986. Phagocyte cells and defense system against microbiology. *Jpn. J. Bact. 41*: 783-795 (in Japanese).

Ogata, N., 1983. Analysis of protective mechanisms against infection by *Pseudomonas aeruginosa*. *Med. J. Fukuoka 74*: 335-350.

Saegusa. J., Ueda, K. and Fujiwara, K, 1979. Fatal infection with *Pseudomonas aeruginosa* in mice treated with cyclophosphamide and *Propionibacterium acnes*. *Exp. Anim. 28*: 61-64,

Sakurai, H., Fujii, Y., Maki, Y., Igarashi, I., Omata, Y., Saito, A., Ono, K., and Suzuki, N. 1991. In Vitro and In Vivo studies of growth inhibitory effect of a newly synthesized peptide, Obiopeptide-1, on mice-bearing methycholanthrene-induced murine tumors. *J. Vet. Med. Sci. 53*: 823-831.

Suzuki, N., Izumo, A., Sakurai, H., Saito, A., Miura, H., and Osaki, H. 1984. Toxoplasmacidal activity of Obioactin derived from hydrolyzed Toxoplasma immune bovine serum in heterologous cell culture. *Zbl. Bakt. Hyg. A. 256*: 356-366.

Suzuki, N. 1987. Obioactin as a native immunoregulatory factor in the biological response modifiers. pp 76-90. *In*: Progress in Veterinary Science 1987. Izawa. H. & Shimizu, Y. (Eds), Kindai Shuppan, Tokyo (in Japanese).

Suzuki, N., Sakurai, H., Saito, A., Igarashi, I., Omata, Y., and Osaki, H. 1990. Biological activity of Obiopeptide-1, a synthetic peptide derived from the native immune-regulator Obioactin. *Jpn. J. Vet. Sci. 52*: 907-914.

Suzuki, N., Fujii, Y., Maki, Y., Sakurai, H., Igarashi, I. and Saito, A. 1990. In vitro cytocidal effect of novel synthetic Obiopeptides 1, 2, and 3 on *Toxoplasma gondii*. *Allergy & Immunol. 9*: 159-158.

Takashima, T. and Collins, F. M.1987. Immunosuppressive effect of cyclosporin A on *Mycobacterium bovis* infections in mice. *Infect. Immunt 55*: 1701-1706.

Urano, T. and Maejima, K. 1978. Provocation of pseudomoniasis with cyclophosphamide in mice. *Lab. Animals 12*:159-161

Watts, J. D., Gary, P. D. and Crane-Robinson, C. 1989. Prothymosin alpha is a nuclear protein, *FEBS letter 245*:17-20.

Yokota, Y. 1984. Host defense mechanism in experimentally immunocompromised animals and restorative effect of immunopotentiators. *Jpn. J. Bact. 39*: 29-46 (in Japanese).