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Role of obioactin on toxoplasmacidal activity within mouse peritoneal macrophages

Mhan-pyo Yang

College of Veterinary Medicine, Chungbuk National University

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마우스 복강 macrophages內의 殺톡소플라즈마 활성에 있어서 obioactin의 역활

양 만 표

충북대학교 수의과대학

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초록 : 톡소플라즈마 過免疫 牛血淸에서 유래된 면역증강제인 obioactin으로 처리한 마우스 복강 macrophages內에서의 톡소플라즈마 증식억제 활성을 검토하였다. obioactin 및 lonomycin A로 처리한 macropohages에서는 첨가농도의 증가에 따라 세포내의 톡소플라즈마 증식이 현저하게 억제되었다. 그러나 macrophages 활성물질인 muramyl dipeptide(MDP)는 톡소플라즈마의 증식억제 효과가 없었다. 이와같이 obioactin 및 lonomycin A의 첨가에 의해 macrophages內에서 톡소플라즈마의 증식이 억제되는 기전의 일부를 해명하기 위한 일환으로 활성산소 중간체 및 lysozyme 분비량을 검토하였다. obioactin과 MDP로 처리한 macrophages에서는 활성산소 중간체인 superoxide anion (O_2^-)과 hydrogen peroxide(H_2O_2)의 생산은 첨가농도에 의존해서 증가하였으나 lonomycin A 첨가군에서는 대조군과 차이가 없었다. 한편 세포내에서 분비되는 lysozyme의 양은 obioactin, lonomycin A 및 MDP를 첨가한 각각의 macrophages에서 첨가농도의 증가에 따라 무처지 대조군에 비해 감소되었다. 이러한 결과로 부터 obioactin은 macrophages를 활성화시켜 세포내에서 활성산소 중간체(O_2^- 및 H_2O_2)를 발생시켜 이것들에 의해 톡소플라마즈의 증식이 억제되는 것으로 사료되었다. 그러나 *macrophages*내에서 분비되는 *lysozyme*은 톡소플라즈마의 증식억제와는 무관하였다.

Key words : hydrogen peroxide, macrophage, obioactin, superoxide anion, *Toxoplasma*

Introduction

Toxic oxygen intermediates play an important role in antimicrobial activity of cells[1,2]. Muramyl dipeptide (MDP)- and lipopolysaccharide-treated mouse peritoneal macrophages were shown to enhance the generation of oxygen intermediates[3,4], but not to inhibit the *Toxoplasma* multiplication[5]. The enhanced generation of oxygen intermediates was not correlated with the toxoplasmacidal phenomenon in obioactin- or lonomycin A-treated kidney cells, obioactin-treated alveolar macrophages and lonomycin A-treated peritoneal macro-

phages[5]. Normal macrophages could also kill the *Toxoplasma* if they were coated with antibodies[6].

On the other hand, cells of the monocyte-macrophages series were known to play an important role in the effector phase of several phenomena of cell-mediated immunity[7]. Upon appropriate activation, macrophages can develop high cytolytic capacity, and they are associated with an enhanced ability to secrete enzymes, suppress tumor, phagocytize microorganisms[8]. Lysozyme which is found in polymorphonuclear leukocytes and macrophages during cultivation *in vitro* is known as the bacteriolytic activity of enzyme[9]. Therefore, choices were made of reactive oxygen intermediates and lysozyme as factors of toxoplasmacidal activities in this study.

However, the mechanisms of the enhanced microbicidal activity, especially toxoplasmacidal activity, of activated macrophages are poorly defined. In current study, the toxoplasmacidal activity was investigated through the participation of the reactive oxygen intermediates and the lysozyme in mouse peritoneal macrophages with obioactin, lonomycin A and MDP.

Materials and Methods

Animals : Seven to eight weeks old BALB/c female mice were used for this study. They were accessible to pellet diet and tap water *ad libitum*.

Preparation of agents : The obioactin was prepared by the same method as described previously[10]. The obioactin was dissolved in medium Tc-199(Flow Laboratories, Inc, Virginia, USA) containing 10% heat-inactivated calf serum, 100U/ml penicillin G-potassium and 100μg/ml streptomycin-sulfate and filtered in 0.45μm of millipore, referred to hereafter as Tc-199-CS. One mg of pure lonomycin A(Taisho Phar Co, Tokyo, Japan) was dissolved in 0.1 ml of 95% ethyl alcohol and adjusted to 100 ng/ml with Tc-199-CS. One mg of MDP(Choay Chimie Reactifs, Paris, France) in sterile saline solution was prepared as stock solution. The stock solution was adjusted to 50 μg/ml with Tc-199-CS.

Mouse peritoneal macrophage monolayers : Mice were administered intraperitoneal(ip) with sterile 0.2% glycogen(Nakarai Chemicals, LTD, Tokyo, Japan)-saline solution 5 days prior to harvest of peritoneal exudate cells. Cells were obtained by peritoneal lavage with Hanks' balanced salt solution(HBSS; 137 mM NaCl, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 0.4 mM $MgSO_4$, 0.5 mM $MgCl_2$, 1.3 mM $CaCl_2$, 10 μg/ml phenol red, 5 U/ml heparin-sodium, 100 U/ml penicillin G-potassium, 100 μg/ml streptomycin-sulfate). The lavage fluid was centrifuged at 250 g for 5 minutes at 4℃. The sediments were washed with sterile 0.2% NaCl solution to lyse erythrocytes, immediately added 1.6% NaCl solution of the same amount. After the cells were resuspended in three times in HBSS, counted and then adjusted to the density of $1x10^6$ nucleated cells per ml with Tc-199-CS. One ml of aliquot of the suspension was placed in individual well of a multidish tray(FB-15-24, Limbro Chem Co USA) containing a round coverslip. To assay the lysozyme activity of macrophages, 10 ml of aliquots of the suspension were placed in vials of 25 mm diameter. The cells were incubated at 37℃ in air with 5% CO_2-humidified atmosphere. The nonadherent cells were removed after 6 hours and then supplied in fresh Tc-199-CS. The adherent cells were returned to culture for 18 hours in the same condition and thereafter used as macrophage monolayers.

Assessment of toxoplasmacidal activity : The infection of *Toxoplasma* within macrophages was determined in the same method as described previously[11]. Tachyzoites of the RH strain of *Toxoplasma gondii*(Tp) harvested from peritoneal cavity of mice 2 days after infection were suspended in Tc-199-CS. The macrophages monolayers were inoculated with $1x10^5$ tachyzoites per well, incubated for 1 hour, and rinsed with HBSS to remove the extracellular tachyzoites. The infected monolayers were reincubated for 48 hours with fresh Tc-199-CS alone, obioactin, lonomycin A or MDP. Tachyzoites multiplied within macrophages were examined by light microscopy under May-Grünwald-Giemsa stain. The infection rate was calculated by counting the number of tachyzoites in phagocytic vacuoles of 500 independent cells on one coverslip, namely, a group of cells without tachyzoites, a group of cells with 1 to 5 tachyzoite(s) and a group of cells

with more than 6 tachyzoites. *Toxoplasma* growth inhibitory factor(Toxo-GIF) as a rate of inhibition of *Toxoplasma* multiplication was calculated by the following expression.

$$\text{Toxo-GIF(\%)} = 100 \times \left(1 - \frac{\text{mean \% of macrophages with Tp in treatments}}{\text{mean \% of macrophages with Tp in control}}\right)$$

Assay of superoxide anion(O_2^-) and hydrogen peroxide(H_2O_2)

Superoxide anion : The superoxide anion was assayed by a modification of the method of Johnston et al[12]. The monolayers were placed in wells of dish, previously containing 1.6 ml of 100 μM cytochrome C (type VI, Sigma Chemical Co, St Louis, USA) in Krebs-Ringer phosphate buffer(pH 7.4) containing 5.5 mM glucose, 154 mM NaCl, 5.6 mM KCl, 1 mM $MgCl_2$ and 2.5 mM $CaCl_2$(KRPG). As the samples of zero time, 60 μl of aliquot from wells was added to 2.4 ml of ice-cold double distilled water(DDW). To use as the reaction samples of 60 minutes, 10 μl of phorbol myristate acetate solution(PMA; 10 ug/ml in dimethylsulfoxide, Sigma Chemical Co, St Louis, USA) were added to be a final concentration of 100 ng/ml. The dish containing PMA was incubated for 60 minutes with shaking at intervals of 15 minutes, and thereafter 600 μl of aliquot was added to 2.4 ml of ice-cold DDW. All the samples were run triplicate, and recorded the absorbance($\triangle$A) at λ_1 540 nm and λ_2 550 nm of wavelength and 2 mm of light path in double wavelength double beam spectrophotometer (Type 557, Hitachi Co, Tokyo, Japan). The blank samples were measured as suspension of DDW(2.47 ml) plus 600 μl of 100 uM cytochrome C in KRPG. To obtain the reactions of oxidation(RO) and reduction(RR) of cytochrome C, 100 uM of hydrogen peroxide(Sankyo Co, Tokyo, Japan) and sodium hydrosulfite(Wako Pure Chemical industries, LTD, Tokyo, Japan), respectively, were added to the blank samples. The amount of O_2^- generation was calculated according to the following formula.

The amount of O_2^- generation(n moles/1x10^6 cells/60 minutes)=

$$100 \times \left(\frac{\triangle A \text{ after 60 minutes of incubation} - \triangle A \text{ at zero time}}{\triangle A \text{ at RR} - \triangle A \text{ at RO}}\right)$$

A coefficient, 100, indicates the concentration of cytochrome C(n moles/ml) consumed.

Hydrogen peroxide : The assay method of hydrogen peroxide(H_2O_2) employed in this experiment was determined in the same manner as described by Nakano et al[13]. The macrophage monolayers were placed in wells of multidish tray, previously holding 1.4 ml of KRPG. Then 5 μl of 25% ethyl alcohol, 100 μl of catalase solution(10 mg/ml, Sigma Chemical Co, St Louis, USA) and 15 μl of alcohol dehydrogenase solution(10 mg/ml, Boehringer Co, Mannheim, Germany) were added sequentially into each well. They were mixed and 150 μl of aliquot as blank samples from wells was added to 1.35 ml of ice-cold ethyl alcohol To prepare the samples of zero time, 50 μl of β-nicotinamide adenine dinucleotide reduced form solution(NADH, 3 mM in 1% $NaHCO_3$, Sigma Chemical Co, St Louis, USA) was added to the same wells and immediately 150 μl of aiquot was added to 1.35 ml of ice-cold ethyl alcohol in well. To stimulate the cells as H_2O_2 generator, 10 μl of PMA, at a final concentration of 100 ng/ml, was added to the wells. To use as the reaction samples of 60 minutes, the monolayers were incubated with shaking at intervals of 15 minutes. After 1 hour of incubation, 150 μl of aliquot from incubated mixtures was added to 1.35 ml of ice-cold ethyl alcohol in well. All the samples were prepared for triplicate. The fluorescence intensity (FI) of NADH was measured at 335 nm of excitation wavelength and 447 nm of emission wavelength, and 5mm and 10 mm of light path, respectively, in fluorescence spectrophotometer(Type 650-60, Hitachi Co, Tokyo, Japan). The amount of H_2O_2 generation was calculated according to the following formula.

The amount of H_2O_2 generation(n moles/1x10^6 cells/60 minutes) =

$$102.46 \times \left(\frac{\text{FI at zero time} - \text{FI after 60 minutes of incubation}}{\text{FI at zero time}}\right)$$

A coefficient, 102.46, indicates the amount of NADH(n moles) consumed for 60 minutes.

Lysozyme assay : The lysozyme activity was assayed by a modification of turbidometric method[14]. After cultivation period, the cultured media was removed

and mixed, and then 5 ml of aliquot was stored at -80℃. Macrophages were disrupted completely under 5 ml of saline solution by sonicator(Heat Systems-Ultrasonics Inc, NY USA), and then frozen at -80℃. The cultured media and the cell lysates were lyophilized (Labconco corporation, Missouri, USA) and thereafter stored at -20℃ until the assay. The substrate consisted of 6 mg of lyophilized cells of *Micrococcus lysodeikticus*(Sigma Chemical Co, St Louis, USA) and 30 ml of 0.067 M potassium phosphate buffer containing 14.04 g KH_2PO_4, 5.2 g Na_2HPO_4, 1.8 g NaCl, and 1.04 g NaN_3 in 1,000 ml of DDW, pH 6.3. Lyophilized crystalline chicken egg white lysozyme (Sigma Chemical Co, St Louis, USA) was used to establish a standard curve. The lyophilized cell lysates and cultured media were adjusted to 0.5 ml with saline solution. The reference was measured against air, and adjusted to λ_1 0 nm and λ_2 546 nm of wavelength and 10 mm of light path. The assay was performed by adding 75 μl of standard solution or test samples to 2.25 ml of substrate at 25℃ in double wavelength double beam spectrophotometer. The change in absorbance after exactly 30 seconds, 120 seconds, and 210 seconds was calculated from the standard curve by computing the mean of $\triangle A/\triangle t$(90 seconds^{-1}) and took the average of triplicate. These values were converted into μg/1x10^7 cells.

Statistics : All the values in this study were expressed as mean±SEM. The differences between values were determined by Student's t-test.

Results

Effects of obioactin, lonomycin A, and MDP on inhibition of *Toxoplasma* multiplication : The inhibitory effects of *Toxoplasma* multiplication were examined in peritoneal macrophages treated with different concentrations of obioactin, lonomycin A, and MDP for 48 hours, respectively(Table 1).

Table 1. Toxoplasmacidal activity in glycogen-induced mouse peritoneal macrophages treated with various concentrations of obioactin, lonomycin A, and MDP

Treatments		Mean percentages of macrophages with *Toxoplasma*(Tp)			Toxo-GIF(%)[a]
		0 Tp	1-5Tp	6〉Tp	
Obioactin (mg/ml),	0	55.2±1.7[b]	23.3±1.1	21.5±1.4	0
	0.04	74.9±3.8	14.3±1.2	10.8±2.7	44.0±8.3
	0.2	76.4±2.5	13.2±0.9	10.4±1.8	47.3±5.5
	1.0	77.4±2.7	11.5±1.1	11.1±1.7	49.6±5.9
	5.0	88.7±1.6	7.0±0.9	4.3±0.8	74.8±3.5
Lonomycin A (ng/ml),	0	52.7±1.7	25.9±1.4	21.4±1.8	0
	0.2	52.2±2.0	21.8±1.6	26.0±2.7	-1.1±4.3
	1.0	70.1±1.8	16.7±1.2	13.2±1.1	36.8±3.7
	5.0	85.6±1.3	11.1±1.4	3.3±0.4	69.6±2.8
	25	99.6±0.1	0.4±0.1	0	99.2±0.1
MDP (μg/ml)	0	49.5±2.0	24.2±1.1	26.3±1.5	0
	0.04	47.5±5.5	22.9±1.7	29.6±4.8	-4.0±8.0
	0.2	50.7±1.6	22.5±1.2	26.8±2.7	2.4±3.2
	1.0	53.0±1.8	21.9±3.1	25.1±4.0	6.9±3.6
	5.0	49.7±2.2	21.5±1.2	28.8±1.5	0.4±4.4

[a] Toxo-GIF(%) =

$$100 \times \left(1 - \frac{\text{Mean percentage of macrophages with } \textit{Toxoplasma} \text{ in treated group}}{\text{Mean percentage of macrophages with } \textit{Toxoplasma} \text{ in control group}}\right)$$

[b] Values represents mean±SEM of 6 experiments

The inhibitory effect of obioactin in *Toxoplasma* multiplication was increased in proportion to its concentration of 0.04, 0.2, 1.0, and 5.0 mg/ml and its Toxo-GIF was 44.0±8.3%, 47.3±5.5%, 49.6±5.9%, and 74.8±3.5%, respectively. The percentages of cells without *Toxoplasma* in obioactin-treated macrophages were significantly higher than that of untreated control($p<0.001$). The treatment of lowest concentration of lonomycin A, 0.2 ng/ml, was not effective in the inhibition of *Toxoplasma* multiplication. From the concentration of 1 ng/ml of lonomycin A, macrophages exhibited a good effect of dose-response, showing that the percentages of cells without *Toxoplasma* were significantly higher than that of untreated control($p<0.001$). Toxo-GIF was also distinctly augmented in parallel with the increase of the concentration of lonomycin A. Especially, 25 ng/ml of lonomycin A showed nearly 100% inhibitory effect against *Toxoplasma* proliferation. However, the *Toxoplasma* multiplication in macrophages treated with MDP was not inhibited in the range of its concentration as compared with cells without *Toxoplasma* of untreated control, and percentages of Toxo-GIF was very low.

Generations of O_2^- and H_2O_2 : As shown in Table 2, the generation of O_2^- in macrophages which were incubated with various concentrations of obioactin for 48 hours was increased from approximately twofold on 0.04 mg/ml($p<0.001$) to threefold on 5 mg/ml($p<0.001$) when compared with that of untreated control. Similarly, the H_2O_2 rised from 15.8±1.0 on 0.04 mg/ml to 21.4±1.0 n moles per million cells for 60 minutes on 5 mg/ml. These values showed significant differences($p<0.02$ to $p<0.001$) as compared with untreated control, which were 12.0±0.9 n moles per million cells for 60 minutes. The increase of generation of reactive oxygen intermediates in macrophages treated with obioactin corresponded well with the degree of intracellular *Toxoplasma* killing. Whereas in the treatment of lonomycin A, the O_2^- production was increased insignificantly until 5 ng/ml and

Table 2. Generation of O_2^- and H_2O_2 in glycogen-induced mouse peritoneal macrophages treated with various concentrations of obioactin, lonomycin A, and MDP

Treatments		Generation of reactive oxygen intermediates[a] (n moles/1x10^6 cells/60 minutes)	
		O_2^-	H_2O_2
Untreated control[b]		1.04±0.13	12.0±0.9
Obioactin (mg/ml),	0.04	2.18±0.08	15.8±1.0
	0.2	3.10±0.23	16.5±1.8
	1.0	2.51±0.39	19.0±2.6
	5.0	3.52±0.25	21.4±1.0
Lonomycin A (ng/ml),	0.2	1.55±0.42	12.6±1.8
	1.0	1.67±0.43	10.3±1.6
	5.0	1.83±0.43	12.3±0.9
	25	1.06±0.11	13.2±0.8
MDP (μg/ml),	0.04	1.49±0.08	16.3±0.8
	0.2	1.88±0.25	15.1±0.5
	1.0	1.73±0.08	14.7±0.8
	5.0	1.80±0.21	14.5±0.4

[a] Values represent mean±SEM of 3 experiments

[b] Values in untreated control represent mean±SEM of 6 experiments

thereafter fell to the value of untreated control. In the range of 0.2 to 25 ng/ml of lonomycin A, there was no change in the H_2O_2 production. The dose-related treatment of MDP was shown by the increase of O_2 production until 0.2 μg/ml($p<0.01$) and thereafter maintained significantly greater value than that of untreated control($p<0.001$). The H_2O_2 production in cells treated with MDP was also increased and reached to a high value of 16.3±0.8 n moles per million cells for 60 minutes on 0.04 μg/ml($p<0.005$). Thereafter, the H_2O_2 production of MDP tended to decline slightly, showing significant differences from $p<0.01$ on 0.2 μg/ml to $p<0.05$ on 1 μg/ml and 5 μg/ml.

Effects of obioactin, lonomycin A, and MDP on lysozyme levels : Macrophages were treated with obioactin, lonomycin A, and MDP in the range of 0.04 to 5.0 mg/ml, 0.2 to 25 ng/ml, and 0.04 to 5.0 μg/ml, respectively, for 48 hours. At all treatments of obioactin, lonomycin A, and MDP, there were remarkable decreases($p<0.05$ to $p<0.001$) in the released lysozyme levels when compared with value of untreated control(Table 3). Both the released lysozyme levels and the total lysozyme levels were decreased in dose-dependent fashion by *in vitro* treatment with obioactin, lonomycin A, and MDP. However, the intracellular lysozyme levels seemed to be considerably less hyporesponsive for the suppressive effect as compared with those of released lysozyme or a constant value regardless of increasing the concentrations. The decrease of total lysozyme levels equaled to the reduction of released lysozyme levels in each concentration of all treatments. The net suppression was caused by released lysozyme levels than by total lysozyme levels.

Table 3. Lysozyme levels in glycogen-induced mouse peritoneal macrophages treated wiht various concentrations of obioactin, lonomycin A, and MDP

Treatments		Lysozyme levels(μg/1x10^7 cells)[a]		
		Inside levels	Released levels	Total levels[c]
Untreated control[b]		2.07±0.15	7.68±0.44	9.75±0.40
Obioactin (mg/ml),	0.04	2.12±0.17	6.03±0.42	8.15±0.29
	0.2	1.92±0.28	5.23±0.52	7.15±0.38
	1.0	1.64±0.35	5.26±0.50	6.90±0.20
	5.0	1.79±0.05	4.64±0.19	6.44±0.16
Lonomycin A (ng/ml),	0.2	2.55±0.37	7.40±0.72	9.95±0.70
	1.0	2.17±0.13	6.45±0.34	8.62±0.21
	5.0	1.89±0.06	5.80±0.31	7.69±0.27
	25	1.74±0.16	5.32±0.12	7.06±0.09
MDP (μg/ml),	0.04	1.87±0.13	6.42±0.17	8.29±0.06
	0.2	1.79±0.18	6.00±0.14	7.79±0.20
	1.0	1.46±0.03	4.21±0.26	5.67±0.28
	5.0	1.57±0.06	3.38±0.30	4.95±0.28

[a] Values represent mean±SEM of 3 experiments

[b] Values in untreated control represent mean±SEM of 6 experiments

[c] Inside levels plus released levels

Discussion

Obioactin is obtained from *Toxoplasma* immune bovine serum by hydrolyzation[10], and it is one of the immunopotentiators as a natural immunoregulator protein in the biological response modifiers(BRM). This substance, approximately 3,000-5,000 in molecular weight, possesses the antimicrobial activity of bacteria and viruses in homologous and heterologous cells[15]. A recent study revealed that *Toxoplasma* lysate antigen, derived from *Toxoplasma* tachyzoites, augmented the cytotoxicity of homologous spleen cells against lymphoma cells[16].

The results of this study showed a good example that macrophages treated with obioactin could not only inhibit the intracellular *Toxoplasma* proliferation but also generate clearly O_2^- and H_2O_2. It was reported that the ability to inhibit the intracellular parasite might be derived from a Toxo-GIF-like active substances which were obtained from antigen-exposed lymphocytes of immune mice[17]. The importance of oxygen-dependent mechanisms to macrophage microbicidal activity has been emphasized by finding that the killing capacity of *Toxoplasma* by activated macrophages can be inhibited by superoxide dismutase, catalase and agents that remove hydroxyl radical(· OH) or singlet oxygen($^1O_2$)[18]. According to Suzuki et al[10], mouse kidney cells activated by obioactin inhibited the intracellular *Toxoplasma*, but there was no change in O_2^- and H_2O_2 generation observed up to 72 hours of incubation. The enhancement of reactive oxygen intermediates was not associated with the intracellular *toxoplasma* killing in obioactin-treated alveolar macrophages of mouse[5]. Therefore, it was implyed that *Toxoplasma* multiplication within mouse peritoneal macrophages treated with obioactin was inhibited by the oxygen-dependent toxoplasmacidal mechanism and that it may be different from the toxoplasmacidal activities of mouse kidney cells and alveolar macrophages.

Lonomycin A, an antibiotic characterized as a cation ionophore[19], showed significantly an inhibitory effect against the intracellular *Toxoplasma*, whereas it did not generate the reactive oxygen intermediates in peritoneal macrophages. This result suggested that the toxoplasmacidal activity by treatment of lonomycin A may be different from oxygen-dependent mechanism in obioactin-treated peritoneal macrophages and that oxygen-indepedent toxoplasmacidal mechanism may be, therefore, existent in macrophages activated by lonomycin A.

It was of interest that the macrophages treated with 25 ng/ml of lonomycin A revealed nearly complete inhibition of *Toxoplasma* multiplication, without cytotoxicity in the cytoplasm. In the observation of cytotoxicity, the macrophages treated with lonomycin A of below 100 ng/ml were not accompanied by any symptom of side effects in cytoplasm[20].

MDP treatment in peritoneal macrophages never increased the toxoplasmacidal activity. On the contrary, O_2^- and H_2O_2 generation was greatr than that of untreated control. Like the report of Murray and Cohn[21] who observed the correlation between the amount of PMA-stimulated H_2O_2 generation and the toxoplasmacidal activity of mouse macrophages, the failure of MDP to enhance the activity of human macrophages against *Toxoplasma* correlated with the failure of MDP to enhance the macrophage O_2^- generation in response to phagocytic stimuli[22,23]. However, injection of MDP *per se* effectively and rapidly acitvated the macrophages in the recipient animals[24]. This means that either the production of reactive oxygen intermediates in MDP-induced mouse peritoneal macrophages or MDP *per se* acted on macrophages may serve, perhaps ultimately, as potentially a means of enhancing host defence against infection and cancer[25].

Lysozyme is a cell-specific marker for mononuclear phagocytes and polymorphonuclear leukocytes which contained preformed enzymes, since it is absent in lymphocytes, plasma cells, basophils, mast cells or marrow reticuloendothelial cells[26]. Before this study, it was postulated that lysozyme may be an enzyme of macrophages responsible for a factor of oxygen-independent toxoplasmacidal mechanism. Because that the monocytes and macrophages which contained their lysozymes may play a significant role in host defence against various bacterial infection and tumor cells was reported by several observations[27-29]. No evidences are, at present, suggested that lysozyme acts as an enzyme on antiparasite agents, especially killing of intracellular *Toxoplasma*. The effect of dose-response on lysozyme

levels showed that *in vitro* treatments of obioactin, lonomycin A and MDP reduced the levels of total as well as released lysozymes. Whereas the intracellular lysozyme levels were relatively constant or considerably less hyporesponsive for dose-response. Warfel and Franklin[30] reported that lipopolysaccharide and interferon, as a kind of BMR, which were shown to enhance the macrophage functions, suppressed the released and intracellular lysozyme levels in untreated-resident peritoneal macrophages. They expressed this fact the phenomenon of down-regulation. Such agents which were capable of enhancing some macrophage activities might concomitantly down-regulate other functions by some mediators. In these results, lysozyme was, anyway, shown by the negative response against toxoplasmacidal activities of obioactin and lonomycin A. In judgment from these observations, it is unlikely that lysozyme within cells or released from cells has a direct effect on toxoplasmacidal activities in macrophages treated with obioactin and lonomycin A.

A study implies that resident alveolar macrophages may possess a powerful, oxygen-independent toxoplasmacidal mechanism that is not present in resident murine, alveolar and peritoneal macrophages[31]. Further studies are needed to determine the exact oxygen-independent mechanism responsible for the killing *Toxoplasma* by lonomycin A.

Summary

The present study was undertaken to examine the effects of obioactin, lonomycin A, and MDP on toxoplasmacidal activities in glycogen-induced mouse peritoneal macrophages. The killing effect of obioactin on *Toxoplasma* multiplication was increased significantly in proportion to its concentrations. O_2^- generation in obioactin-treated macrophages was also increased from twofold to threefold when compared wtih that of untreated control. Similarly, H_2O_2 continued to rise in parallel with increase of the concentration of obioactin. Lonomycin A-treated macrophages also exhibited a good effect of dose-response on toxoplasmacidal activities. However, O_2^- and H_2O_2 were not generated significantly in lonomycin A-treated macrophages. Macrophages treated with muramyl dipeptide (MDP) were not found to inhibit the proliferation of *Toxoplasma* but showed the enhancement of O_2^- and H_2O_2 generation. The released lysozyme levels from macrophages into cultured media were decreased in dose-dependent fashion by *in vitro* treatment of obioactin, lonomycin A, and MDP. The intracellular lysozyme levels appeared to be a constant value regardless of increasing the concentrations of obioactin, lonomycin A, and MDP.

Therefore, these results suggest that *Toxoplasma* multiplication within macrophages treated with obioactin was inhibited by the generation of O_2^- and H_2O_2 and that lysozyme *per se* within or released from macrophages had no effect on toxoplasmacidal activity.

References

1. Haidaris CG, Bonventre PF. A role for oxygen-dependent mechanisms in killing of *Leishmania donovani* tissue forms by activated macrophages. *J Immunol* 1982; 129: 850-855.
2. Murray HW, Rubin BY, Rothermel CD. Killing of intracellular *Leishmania donovani* by lymphokine-stimulated human mononuclear phagocytes: evidence that interferon-γ is the activating lymphokine. *J Clin Invest* 1983; 72: 1506-1510.
3. Pabst MJ, Johnston Jr RB. Increased production of superoxide anion by macrophage exposed *in vitro* to muramyl dipeptide or lipopolysaccharide. *J Exp Med* 1980; 151: 101-114.
4. Leclerc C, Chedid L. Immunomodulation of macrophages by muramyl peptides: recent finding concerning somnogenic activity of muramyl dipeptides and monokine. *Int J Tissue React* 1984; 6: 213-218.
5. Saito A, Sakurai H, Saito T, et al. Correlation between released of reactive oxygen intermediates and inhibition of *Toxoplasma* multiplication in mouse peritoneal and alveolar macrophages and kidney cells after *in vitro* incubation with obioactin, lonomycin A, muramyl dipeptide, lipopolysaccharide of *Toxoplasma* lysate antigen. *Zbl Bakt*

Hyg A 1987; 264: 446-454.
6. Sibley LD, Weidner E, Krahenbul JL. Phagosome acidification blocked by intracellular *Toxoplasma gondii*. *Nature* 1985; 315: 416-419.
7. North RJ. The concept of activated macrophage. *J Immunol* 1978; 121: 806-809.
8. Karnovsky ML, Lazdins JK. Biochemical criteria for activated macrophages. *J Immunol* 1978; 121: 809-816.
9. Fleming A. On remarkable bacteriologic element found in tissues and secretions. *Proc R Soc Ser B* 1933; 93: 306-317.
10. Suzuki N, Izumo A, Sakurai H, et al. Toxoplasmacidal activity of obioactin derived from hydrolyzed *Toxoplasma* immune bovine serum in heterologous cell cultures. *Zbl Bakt Hyg A* 1984; 256: 356-366.
11. Sakurai H, Igarashi I, Saito A, et al. Effects of neonatal spleen cell products on the multiplication of *Toxoplasma* in rat peritoneal macrophages. *J Immunol* 1982; 131: 1527-1530.
12. Johnston Jr RB, Godzik CA, Cohn ZA. Increased superoxide anion production by immunologically activated and chemically elicited macrophages. *J Exp Med* 1978; 148: 115-127.
13. Nakano M, Tsutsumi Y, Danowski TS. Enzymatic determination of small quantities of hydrogen peroxide. *Proc Soc Exp Biol Med* 1968; 129: 960-963.
14. Weisner B. Lysozyme(muramidase). In: Bergmeyer A, ed. *In methods of enzymatic analysis*. 3rd ed. Florida, Basel: Verlag Chemie, 1984; 189-195.
15. Osaki H, Furuya M, Nagasawa H, et al. Antimicrobial activity of obioactin. *Zbl Bakt Hyg A* 1984; 256: 328-334.
16. Yang MP, Goitsuka R, Ono K, et al. Effect of *Toxoplasma* lysate antigen(TLA) on feline cytotoxicity against FeLV positive lymphoma cells. *Jpn J Vet Sci* 1990; 52: 735-742.
17. Shirahata T, Shimizu K, Noda S, et al. Studies on production of biologically active substance which inhibits the intracellular multiplication of *Toxoplasma* within mouse macrophages. *Z Parasitenk* 1977; 53: 31-40.
18. Murray HW, Juangbhanich CW, Nathan CF, et al. Macrophage oxygen-dependent antimicrobial activity: Ⅱ. the role of oxygen intermediates. *J Exp Med* 1979; 150: 950-964.
19. Mitani M, Otake N. Studies on the ionophorous antibiotics: XVI. the ionophore-mediated calcium transport and concomitant osmotic swelling of mitochondria. *J Antibiotics* 1978; 31: 888-893.
20. Miyagami T, Takei Y, Matsumoto Y, et al. An *in vitro* study on toxoplasmacidal activity of lonomycin A in host cells. *J Antibiotics* 1981; 34: 218-223.
21. Murray HW, Cohn AZ. Macrophage oxygen-dependent antimicrobial activity: Ⅲ. enhanced oxidative metabolism as an expression of macrophage activation. *J Exp Med* 1980; 152: 1596-1609.
22. Wilson CB, Tsai V, Remington JS. Failure to trigger the oxidative metabolic burst by normal macrophages: possible mechanism for survival of intracellular pathogens. *J Exp Med* 1980; 151: 328-346.
23. Wilson CB, Bohnsack J, Weaver WM. Effects of muramyl dipeptide on superoxide anion release and on anti-microbial activity of human macrophages. *Clin Exp Immunol* 1982; 49: 371-376.
24. Cummings NP, Pabst MJ, Johnston Jr RB. Activation of macrophages for enhanced release of superoxide anion and greater killing of *Candida albicans* by injection of muramyl dipeptide. *J Exp Med* 1980; 152: 1659-1669.
25. Fidler IJ, Schroit AJ. Synergism between lymphokines and muramyl dipeptide encapsulated in liposomes: *in situ* activation of macrophages and therapy of spontaneous cancer metastases. *J Immunol* 1984; 133: 515-518.
26. Osserman EF, Lawlor DP. Serum and urinary lysozyme(muramidase) in monocytic and monomyelocytic leukemia. *J Exp Med* 1966; 124: 921-952.
27. Feldman BF, Madewell BR, Miller RB. Serum lysozyme(muramidase) activity in dogs with neoplastic disease. *Am J Vet Res* 1980; 42: 1319-1321.
28. Ezekowitz RAB, Austyn J, Stahl PD, et al. Surface properties of Bacillus Calmette-Guerin-ac-

tivated mouse macrophages: reduced expression of mannose-specific endocytosis, Fc receptor, and antigen F4/80 accompanies induction of Ia. *J Exp Med* 1981; 154: 60-76.

29. Cohen AB, Chenoweth DE, Hugli TE. The release of elastase, myeloperoxidase, and lysozyme from human alveolar macrophages. *Am Rev Respir Dis* 1982; 126: 241-247.
30. Warfel AH, Franklin DZ. Down-regulation of macrophage lysozyme by lipopolysaccharide and interferon. *J Immunol* 1986; 137: 651-655.
31. Catterall JR, Sharma SD, Remington JS. Oxygen-independent killing by alveolar macrophages. *J Exp Med* 1980; 163: 1113-1131.