AN *IN VITRO* STUDY ON THE TOXOPLASMACIDAL ACTIVITY OF LONOMYCIN A IN HOST CELLS

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Lonomycin A at various concentrations was tested for its inhibitory effect on *Toxoplasma* multiplication in host cells cultured *in vitro*. Results indicated that lonomycin A at a concentration of 0.01 μg per ml in TC-199 medium demonstrated a high degree of antitoxoplasma activity with complete inhibition of *Toxoplasma* multiplication in the host cells. Lymphokines, a supernatant produced from spleen cells of mice infected chronically with *Toxoplasma gondii*, inhibited *Toxoplasma* multiplication in mice macrophage and kidney cell monolayers. However, lonomycin A inhibited completely *Toxoplasma* multiplication in non-specific cells, *i.e.* not only in mice macrophages and kidney cells but also in cells of human and other animal species.

Toxoplasma gondii multiplies in macrophages from normal mice under culture conditions *in vitro* leading eventually to the rupture of the cells and liberation of the organisms. As reported in the authors' preceding *Toxoplasma* studies[1~5], non-immune macrophages infected with *T. gondii in vitro* can possess an ability to inhibit the intracellular multiplication of the organisms either by cocultivation with immune lymphocytes or by addition of *Toxoplasma*-immune lymphokines (LKs), a supernatant fluid obtained from immune lymphocytes cultured with specific *Toxoplasma* lysate antigen[1,2,4,5]. However, this Toxo-immune LKs shows a strict species-specificity, which inhibits *Toxoplasma* multiplication only in murine macrophages, not in guinea pig macrophages or canine monocytes[2].

At the moment, treatment of acute human toxoplasmosis generally consists of the combination of antibiotics and sulfa drugs. However, there is no effective drug of therapeutic value so far for the treatment of chronic toxoplasmosis.

Lonomycin A[6~9], a new polyether antibiotic which possesses activity against Gram-positive bacteria and some species of filamentous fungi, is also effective against the avian species of *Eimeria* in broiler chickens.[10]

In the present paper, the authors are interested in determining whether lonomycin A can inhibit *Toxoplasma* multiplication in macrophages, comparable to that of Toxo-immune LKs in mice macrophages, and kidney cells as well as in macrophages of other animal origin.

Materials and Methods

Animals and *Toxoplasma* strain used

Inbred Balb/c mice, guinea pigs, cats and beagles were used in the experiment. Mice weigh-

ing 20~30 gms were inoculated intraperitoneally (i.p.) with 100 tachyzoites of the S-273 strain of *T. gondii*. They were challenged with 1,000 tachyzoites of the same strain *via* the same route 4 weeks after the first inoculation. To obtain mice which will serve as donors of immune lymphocytes, the same species of animals was further challenged with 100 tachyzoites of the virulent S-273 strain 4 weeks later.

Preparation of *Toxoplasma* lysate antigen (TLA)

TLA was prepared according to the method described by IGARASHI *et al.*[1] Tachyzoites of the RH strain were collected from the peritoneal cavity of 2-day infected mice and washed 3 times with HANKS' balanced salt solution (HBSS). After washing, ten-fold sterile double distilled water was added and the suspension was sonicated with an ultrasonic vibrator (100 w, Kubota sonicator, Model 200, Tokyo) for 3 minutes and kept at 4°C for 24 hours. The extracted lysate was centrifuged at 10,000 rpm for 1 hour and the supernatant mixed with an equal volume of sterile 1.7% NaCl. The total protein content was estimated by the LOWRY method, using bovine serum albumin fraction V as the standard.

Preparation of spleen cells and lymphokines (LKs)

Spleen cells were separated from spleen suspensions of normal and *Toxoplasma*-infected mice by means of a slight modification of the CONRAY-FICOLL method.[11] Cells were washed twice with heparinized HBSS (5 units heparin/ml) by centrifugation. The resulting sediments were resuspended in TC-199 medium containing 10% heat-inactivated calf serum (CS) and antibiotics (100 I.U./ml penicillin G and 100 mcg/ml dihydrostreptomycin) and adjusted to a concentration of approximately 1×10^7 cells/ml. The cells were cultured at 37°C for 48 hours in multidish trays containing round coverslip in a humidified atmosphere containing 5% CO_2 together with 50 mcg/ml TLA. Cultured supernatants were then pooled by centrifugation at 3,000 rpm for 30 minutes at 4°C, filtered through millipore membrane filters (0.3 μ type PH, Millipore Co., Boston) and stored at −80°C until used.

Preparation of lonomycin A

The stock sample of pure lonomycin A was used.[6] Lonomycin A (1.0 mg) was dissolved in 0.1 ml 99.5% (v/v%) ethyl alcohol and the solution added to 1.9 ml TC-199 medium containing 10% CS; this stock solution contained of 1,000 mcg/ml. Aliquots from the stock solution were diluted with TC-199 medium (10% CS added) just prior to use to give final concentrations of 500, 100, 10, 1, 0.1, 0.01, 0.001 and 0.0001 mcg/ml. Similarly, solutions of TC-199 medium containing 10% CS alone or 2% ethyl alcohol were used as the control.

Macrophage and kidney cell monolayer preparations

Adult mice, guinea pigs and kittens were injected i.p. with sterile 0.2% glycogen-saline solution. Five days later, peritoneal exudates containing macrophages were harvested by washing the peritoneal cavity with HBSS. The cells were centrifuged at 1,200 rpm for 5 minutes. The sediments were suspended in TC-199 medium containing 10% CS and antibiotics and adjusted to a concentration of 1×10^7 nucleated cells/ml. One ml of each cell suspension was placed on a multidish tray (FB-15-24, Linbro Chem. Co., U.S.A.) containing a round coverslip and incubated at 37°C for 6 hours in a 5% CO_2 incubator. Cells which did not adhere to the coverslips were removed by rinsing twice with TC-199 medium (10% CS and antibiotics added) and reincubated with the same medium overnight in a CO_2 incubator. Finally, the cell cultures were rinsed with the same medium and used as macrophage monolayers for assay of antiprotozoal activity. Similarly, canine monocyte macrophage monolayers were prepared according to the method described by ISHIMINE *et al.*[12]

Mice kidney cell monolayers were prepared by removal of fat and fibrosa from the kidney and placed in a petri dish containing antibiotics (100 I.U./ml penicillin G and 100 mcg/ml dihydrostreptomycin) in HBSS for 1 hour at 4°C. The organs were then minced with a pair of scissors and homogenized between two slide glasses. The homogenized tissues were transferred to a conical flask containing a Teflon-coated magnet, 40 ml of 0.25% trypsin solution was added and the homogenate kept at 37°C for 40 minutes under stirring, then it was filtered through a glass fiber column and centrifuged at 1,500 rpm for 10 minutes. The pellet was washed with heparinized HBSS 3 times by centrifugation at 1,500 rpm for 10 minutes and the suspension of the mononucleated cells was adjusted to ap-

proximately 5×10^5 cells/ml with TC-199 medium containing 10% CS and antibiotics. One ml was dispensed in a multidish tray containing round coverslips, rinsed with TC-199 medium to remove non-adhering cells, and incubated with fresh medium at 37°C for 5 days in a humidified CO_2 incubator. Likewise, the human heart cell monolyers (Girardi heart cells, 03-085, Dai-Nihon Seiyaku Co., Osaka) were prepared by the same method as used for mouse kidney cells.

Assesment of antiprotozoal activity.

Tachyzoites of the RH strain used were obtained from the peritoneal cavity of mice 2 days after inoculation. Approximately 5×10^4 tachyzoites per ml of TC-199 medium (10% CS added) were cultured with 1.5 ml lonomycin A at various concentrations prepared previously at 37°C in a 5% CO_2 incubator. Dead tachyzoites were counted at regular intervals of 1, 2 and 3 hours 0.2 ml from each culture was stained with 1 drop of 0.2% trypan blue stain and examined. The number of stained tachyzoites per 100 tachyzoites was determined twice to give an average mortality (%).

Aliquots of 1.5 ml of lonomycin A solution, Toxo-immune LKs (66%), TC-199 medium+ethyl alcohol and TC-199 medium alone were added to a multidish tray in which mice macrophages or kidney cell monolayers had been formed. One hour prior to the addition of lonomycin A solutions, the monolayers were infected with approximately 5×10^4 tachyzoites of the RH strain per dish with an approximate ratio of one tachyzoite to 20 mononuclear cells. One hour after infection, the monolayers were washed thoroughly to remove excess parasites. The cultures were then incubated at 37°C for 24 and 48 hours in a CO_2 incubator. Similarly, 1.5 ml of 0.01 mcg/ml of lonomycin A was added to multidish trays in which guinea pig macrophages, canine monocyte-macrophages, feline macrophages or human heart cells had been formed and infected with 5×10^4 tachyzoites of the RH strain per dish one hour preceding the addition of the drug. Incubation was carried out at 37°C for 24 and 48 hours with 5% CO_2 in the atmosphere.

The intracellular parasites in all cell cultures were counted under phase contrast microscopy at regular time intervals. Since *Toxoplasma* can be readily identified by its morphological appearance within cytoplasmic vacuoles, the cultures were stained with May-Grunwald-Giemsa double stain. Infection rate of the cells was calculated by counting the number of parasites in the phagocytic vacuoles of 1,000 individual cells on each coverslip and recorded as 0 Tp for cells devoid of any organism, 1~5 Tp for cells containing 1 to 5 organisms, and 6 Tp for cells containing 6 or more organisms. To obtain the mean infection rate, experiments were repeated at least 3 to 5 times.

Results

As shown in Table 1, when *Toxoplasma* tachyzoites suspended in TC-199 medium containing 10% CS were inoculated with various concentrations of lonomycin A for 1, 2 and 3 hours at 37°C in a humidified atmosphere with 5% CO_2, an increase in the percentage of dead organisms for increasing concentration of the drug was observed. After 3 hours of incubation, 100% mortality was observed at 500 mcg/ml, 75% at 0.1 mcg/ml and 24% at 0.0001 mcg/ml, respectively. These data indicated that lonomycin A is strongly inhibitory against *Toxoplasma* tachyzoites even at low concentration.

A complete inhibitory effect on the multiplication of the organisms in mice macrophage

Table 1. Average percentage of dead *Toxoplasma* tachyzoites after the addition of varying concentration of lonomycin A.

Concentration of lonomycin A (mcg/ml TC-199 medium*)	Incubation time		
	1 hour	2 hours	3 hours
500	100	100	100
100	44	55	90
10	43	51	88
1	20	42	81
0.1	14	18	75
0.01	6	8	39
0.001	3	14	24
0.0001	2	9	24
TC-199 Med. + Ethyl alcohol	0	2	7
TC-199 Med. alone	0	0	5

* 10% calf serum was added to the medium.

monolayers was shown at all concentrations of lonomycin A after 24 and 48 hours incubation as shown in Table 2. However, at 100 mcg/ml, strong cytotoxicity was observed and the cells appeared to be ruptured. Presence of some degenerated vacuoles was observed at 10 mcg/ml. At a concentration of 0.1 mcg/ml it showed only a slight cytotoxicity, and almost no toxic symptom was observed below 0.1 μg/ml.

For comparison, the inhibitory effect of Toxo-immune LKs is given in Table 2; it is far less active than lonomycin A.

Effect of lonomycin A on the multiplication of *Toxoplasma* tachyzoites in mice kidney monolayers was compared with that of Toxo-immune LKs in Table 3. Complete inhibition of the multiplication

Table 2. Inhibitory effect of Toxo-immune LKs and various concentration of lonomycin A on *Toxoplasma* multiplication in mice macrophage monolayers after incubation for 24 and 48 hours.

Culture components	No. of macrophages containing parasite (mean %) and degrees of cytotoxicity**							
	24 hours				48 hours			
	0 Tp	1~5 Tp	≥6 Tp	Cyto.**	0 Tp	1~5 Tp	≥6 Tp	Cyto.**
Lonomycin A (mcg/ml)								
100	100	0	0	##	100	0	0	##
10	100	0	0	+	100	0	0	#
1	100	0	0	−	100	0	0	+
0.1	100	0	0	−	100	0	0	−
0.01	100	0	0	−	100	0	0	−
0.001	100	0	0	−	100	0	0	−
0.0001	100	0	0	−	100	0	0	−
Toxo-immune LKs	98.8±0.3	0.9±0.2	0.3±0.1	−	97.2±7.7	2.4±2.0	0.4±0.9	−
Control*	83.0±4.3	11.8±2.9	5.2±1.9	−	48.2±9.0	37.4±7.1	14.4±3.9	−

* TC-199 medium containing 10 % CS with or without ethyl alcohol.

** Degrees of cytotoxicity graded as follows: (##) strong cytotoxicity; (#), presence of some degenerated vacuoles in the cytoplasm; (+), rounding of cells; and (−), normal.

Table 3. Inhibitory effect of Toxo-immune lymphokines and various concentrations of lonomycin A on *Toxoplasma* multiplication in mice kidney cell monolayers after 24 and 48 hours incubation.

Culture component	Mean percentage of kidney cells containing intracellular parasites					
	24 hours			48 hours		
	0 Tp	1~5 Tp	≥6 Tp	0 Tp	1~5 Tp	≥6 Tp/cell
Lonomycin A (mcg/ml)						
1	100	0	0	100	0	0
0.1	100	0	0	100	0	0
0.01	100	0	0	100	0	0
0.001	98.2±0.3	0.6±0.2	1.2±0.9	81.8±2.1	7.6±1.3	10.6±0.4
0.0001	98.2±0.1	0.4±0.1	1.4±0.7	81.2±1.6	8.0±1.5	10.8±2.1
Toxo-immune LKs	99.1±0.2	0.8±0.2	0.1±0.1	96.7±1.1	2.6±0.9	0.7±0.3
Non-lonomycin A	98.4±0.4	0.7±0.3	0.9±0.2	87.1±2.5	5.5±1.1	7.4±1.1

Table 4. Inhibitory effect of lonomycin A on *Toxoplasma* multiplication in normal guinea pig macrophages, canine monocyte-macrophages, feline macrophages and human heart cells after 24 and 48 hours incubation.

Source of cells	Lonomycin A (0.01 mcg/ml)	Mean percentage of macrophages with intracellular *Toxoplasma*					
		24 hours			48 hours		
		0 Tp	1~5 Tp	≥6 Tp	0 Tp	1~5 Tp	≥6 Tp/cell
Guinea pig macrophages	With	99.9±0.1	0.1±0.1	0	98.9± 0.1	1.1±0.4	0
	Without	96.8±0.8	2.6±0.9	0.6±0.9	88.5± 2.5	5.5±1.1	6.0±1.1
Canine macrophages	With	99.1±0.1	0.9±0.3	0	99.7± 0.3	0.3±0.1	0
	Without	92.0±2.0	5.4±1.1	2.6±1.9	76.8± 7.4	14.4±4.9	8.8±3.9
Feline macrophages	With	99.6±0.5	0.4±0.5	0	98.8± 1.1	1.2±1.1	0
	Without	96.2±2.6	2.6±1.9	1.2±0.8	64.2± 9.5	22.0±5.8	13.8±4.1
Human heart cells	With	99.9±0.2	0.1±0.2	0	100	0	0
	Without	91.5±3.3	3.5±2.1	5.0±2.9	69.4±10.7	13.2±4.4	17.4±8.3

was observed at a concentration of 0.01 mcg/ml without any symptom of side effect. It is of interest that, even at the low concentrations of 0.001 mcg/ml and 0.0001 mcg/ml, the antibiotic still showed a significant inhibitory effect, comparable with that of Toxo-immune LKs.

Comparison of the relative inhibitory effect of lonomycin A on *Toxoplasma* multiplication in the macrophage monolayers derived from various origins such as guinea pig, dog, cat and human heart cells, is presented in Table 4. A remarkable inhibitory effect was observed especially after 48 hours incubation.

Discussion

It is well known that the peritoneal exudated macrophages obtained from *Toxoplasma*-immune mice have an ability to kill intracellular parasites,[1~5,13~16] but this immuno-activity is strictly species-specific.

Lonomycin A, an antibiotic characterized as a cation selective ionophore,[17,18] showed an inhibitory effect at significantly lower concentrations (0.01~0.001 mcg/ml) against intracellular multiplication of the parasite in macrophage monolayers derived from various animal origins. It is noticeable that these results strongly suggest a possible use of lonomycin A for the treatment of toxoplasmosis. Further studies are under progress.

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