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The Study on the Biological Activity of Bovine Lactoferrin

-Growth Inhibitory Effects of Bovine Lactoferrin to *Toxoplasma gondii*-

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Tetsuya Tanaka

CONTENTS

GENERAL INTRODUCTION

1. Bovine Lactoferrin

Lactoferrin, also called lactotransferrin, is an iron binding protein present in milk, saliva, tears and mucus secretions, and in secondary granules of neutrophils.

Lactoferrin is a glycoprotein with a molecular weight of about 80,000. Bovine lactoferrin consists of 689 amino acid residues with a primary sequence 69 % identical to human lactoferrin and 64 % identical to mouse lactoferrin (26, 55), and it has four glycan chains (75). Amino acid sequence alignments indicate that, in addition to extensive sequence homology between lactoferrins of different species, the N- and C-terminal halves of the molecule exhibit about 40 % sequence identity (56).

Each lactoferrin molecule can bind two atoms of Fe tightly but reversibly. Binding of lactoferrin is dependent on the concomitant binding of anions, such as bicarbonate or carbonate, which have an essential role in holding the metal firmly (51). Thus, lactoferrin can exist in an iron-free (apo-) or iron-bound (holo-) state. The biological properties of lactoferrin are often dependent upon its iron status.

Bovine colostrum contains up to 5 mg/ml of lactoferrin and its concentration drops rapidly as lactation proceeds to a level of 0.02 to 0.35 mg/ml in mature bovine milk (66). After intramammary bacterial infections, the concentration of lactoferrin in bovine milk increases about 30-fold even though other milk proteins remain at normal concentrations (86).

Lactoferrin is also major component of human tears (about 0.4 to 1.2

mg/ml) comprising about 25 % of the total protein (3, 40). It is prominent component of uterine secretions, present at concentrations of 0.5-1.0 mg/ml (51). Lactoferrin is also present in seminal plasma, saliva, nasal secretions, bronchial mucous, hepatic bile, gastric juice (66), duodenal mucous (79), pancreatic secretions (57), amniotic fluid (61) and urine (36).

In contrast, lactoferrin is present in blood plasma at very low concentrations of 0.1-0.4 μg/ml (8). Lactoferrin is charged together lysozyme and other components of the specific granules of neutrophils when neutrophils are activated during the inflammatory response (44). Reportedly, interleukin-8 specifically induces the release of secondary granules containing lactoferrin (87). Serum lactoferrin is rapidly removed from circulation by the action of liver reticuloendothelial system (9).

Lactoferrin is a prominent antimicrobial component of mucosal surfaces prone to attack by microbial pathogens. Lactoferrin is actively secreted by neutrophils in the inflammatory response (27, 29, 31). As an anti-microbial component of colostrum and milk, lactoferrin may have a significant role for protection of neonates from infectious diseases (70).

The inhibition ability of lactoferrin to the growth of wide variety of microorganisms has been well proved *in vitro* (13). Moreover, lactoferrin is known to act synergistically with lysozyme (22) and IgA (76), and it is frequently found together with one or both of these agents in various physiological fluids.

The importance of lactoferrin in the host defense is underlined by studies indicating that patients with congenital (11) and acquired (30, 84) defects of lactoferrin production exhibit an abnormal predisposition to recurrent

infections by bacterial and fungal pathogens. Patients with acute viral illnesses such as chickenpox, measles, rubella, hepatitis or Epstein-Barr virus infection have shown to exhibit the reduction of plasma lactoferrin concentrations, although total number of neutrophil was similar to that of non infected (4).

Specific receptors for lactoferrin have been shown on the surface of various types of blood cells including macrophages, monocytes (10, 83) and lymphocytes (52). Interestingly, lactoferrin suppresses the primary antibody response of B lymphocytes to both T-dependent and T-independent antigens (20), stimulates the expression of Fc receptors on T lymphocytes (25), and induces the secretion of migration inhibitory factor from leukocyte (25). Lactoferrin stimulates the differentiation of B lymphocytes into IgA-secreting cells. It may act $CD4^-$ $CD8^-$ T cell precursors leading to the generation of helper T cell activity (50). In addition, lactoferrin inhibits calcium ionophore-induced release of histamine from rat mast cells (81). It also acts to block the classical pathway for activation of complement by inhibition of C3-convertase synthesis (39, 41).

Moreover, through interaction with receptors on the surface of monocyte-macrophage cells to lactoferrin, holo-lactoferrin suppresses the release of granulocyte-macrophage colony-stimulating factor, interleukin-1, and an acidic isoferritin-inhibitory activity factor (12, 89).

Lactoferrin damages the outer membrane of Gram-negative bacteria causing the loss of lipopolysaccharide (21, 22). It results in enhancing the susceptibility to lysozyme and antibiotics such as rifampcin, in manner similar to polycationic membrane-active agents, such as poly-L-lysine and

the peptide antibiotic polymyxin B (21, 22). Recent studies have demonstrated that antimicrobial peptides which is derived from lactoferrin and has more potent activity than that of lactoferrin are generated upon enzymatic cleavage of this protein (82). This work has led to identification of the structural region responsible for the membrane-disruptive properties of lactoferrin and its lethal effect against various microorganisms (5).

2. *Toxoplasma gondii*

Toxoplasma gondii (*T. gondii*), an obligate intracellular parasite, which has been assigned to the phylum Apicomplexa and class Sporozoa. *T. gondii* infects a wide variety of birds and mammals. Acute toxoplasmosis in these hosts is characterized by intracellular growth of the rapidly dividing stage termed tachyzoite. Some of the zoites become encysted, particularly in muscle and brain, in a latent infection termed bradyzoite.

In humans it cause serious, and potentially fatal disease in immunocompromiced patients [including with malignant, organ transplants and the acquired immune deficiency syndrome] (49) as well as newborn infants (53).

Cell-mediated immunity has shown to be critical for defense against *T. gondii*. Macrophages and T lymphocytes have been demonstrated to be major contributors to the defense (67). In the present decade numerous studies have been investigated that many cytokines mediate the cellular immune responses to *T. gondii*. The effective defense system which *T. gondii* induces, has been shown to be associated with the development of $CD4^+$ and $CD8^+$ T cell displaying a type 1 cytokine expression profile (24,

77). In particular, interferon gamma (IFN-γ) has been demonstrated to play a major role in both acquired immunity to acute infection and the control of parasite growth in chronically infected hosts (24, 77). Recent studies have documented that *T. gondii* triggered type 1 cell-mediated immune response to events occurring during the first few days after parasite entry into the host. Furthermore, the interaction of tachyzoites and macrophages has been shown to lead the production of cytokines (IL-12, TNF-α and IL-1β), which induce the synthesis of IFN-γ by both T cell and natural killer (NK) cells.

3. Aims of the Present Study

Although lactoferrin was first isolated and investigated for more than 30 years, the function and biological role remain unclear.

Particularly, the biological role of lactoferrin thus remains an enigma. Further work needs to concentrate on providing *in vivo* evidence for hypotheses suggested by *in vitro* data. The author studied the biological activity of bovine lactoferrin in *T. gondii* infection to reveal some unclear points. These experiments were carried out as follows.

1. Growth inhibitory effects of bovine lactoferrin to *T. gondii* parasites in murine somatic cells.

2. The mechanism of growth inhibitory effects of bovine lactoferrin to *T. gondii* parasites in mouse macrophages.

3. Protective effect of bovine lactoferrin in mice infected with *T. gondii* parasites.

4. Parasiticidial effects of lactoferricin and C-lobe in bovine lactoferrin to *T. gondii* parasites.

5. Protective effect of bovine lactoferricin in mice infected with *T. gondii* parasites.

Chapter 1

Growth Inhibitory Effects of Bovine Lactoferrin to *Toxoplasma gondii* Parasites in Murine Somatic Cells

1. INTRODUCTION

Lactoferrin (LF), a cationic, iron-binding protein which is produced and secreted by mammary glands and neutrophils, is known to have broad spectrum antimicrobial properties (6, 13, 21). It has been shown to have activation properties of natural killer cytotoxicity in tumor cells (34, 45, 62) and induction of phagocytic activity and killing of amastigotes, an intracellular parasitic form of *Trypanosoma cruzi* (*T. cruzi*) in macrophages (46, 85).

Toxoplasma gondii (*T. gondii*), an intracellular parasitic protozoan, can also penetrate in mammalian and avian somatic cells. In the host cells, the parasites of *T. gondii* are enclosed in parasitophorous vacuoles allowing them to evade the fusion of lysosomes, resulting in its growth even in macrophages (37). Thus, the intracellular parasitism of *T. gondii* is different from that of *T. cruzi*.

In the present study, the author focused his interests to determine the possibility of LF inducing activation properties of macrophages and examined the effects of LF on the development of *T. gondii* parasites in mouse peritoneal macrophages and mouse embryonal cells.

2. MATERIALS AND METHODS

Preparation of lactoferrin

Bovine lactoferrin was prepared from bovine milk whey by the method of Law and Reiter (43). The amount of Fe^{2+} ion in the LF was 14.5 mg/100 g protein. Apo- and holo-lactoferrin were prepared by the method of Shimazaki and Hosokawa (73).

Experimental animals

Seven weeks old male and female ICR mice were used throughout the experiments.

Preparation of mouse peritoneal macrophages

Mouse peritoneal macrophages (MPMs) were harvested from the peritoneal cavity of mice inoculated with phosphate bufferd saline (PBS) containing 0.2 % glycogen on the 5th day post inoculation (p.i.). They were centrifuged at 800 ×g for 10 min and were suspended in Dulbecco's modified essential medium (D-MEM) containing 10 % fetal bovine serum (FBS). In the 24 wells tissue culture microplate (Corning IWAKI Inc. Osaka, Japan), 10^5 cells/well of macrophages suspension was incubated at 37 ℃ for 2 h, then were washed thoroughly to remove non-adherent cells, and were further incubated overnight at 37 ℃ in D-MEM containing 10 % FBS.

Preparation of mouse embryo cells

Mouse embryo cells (MECs) were prepared as described elsewhere (63).

Preparation of parasites

The RH strain parasites of *T. gondii* were harvested under anestesia from the peritoneal cavity of mice on the 3rd day p.i. The parasites were washed by centrifugation at 1,200 ×g for 10 min in PBS 3 times, and then were suspended in D-MEM containing 1 % bovine serum albumin (D-MEM-BSA).

Effect of lactoferrin to the parasites

The growth activity of the parasites in the host cells were performed by the measurement of ^{3}H-uracil incorporation assay (32, 71). Briefly, MPM and MEC monolayers were incubated with 1.0 ml of 10^{5} parasites suspension at 37 ℃ for 2 and 6 h, respectively. After removing free parasites by several washing in D-MEM, the monolayers were further incubated with either D-MEM-BSA alone, or supplemented with 100, 500 and 1,000 μg/ml of LF for 24 h. One ml of D-MEM-BSA containing 0.146 MBp of ^{3}H-uracil was added to the culture and incubated at 37 ℃ for last 6 h. After washing thoroughly in PBS to remove free ^{3}H-uracil, the monolayers were treated with 0.5 ml of 1 % SDS for 10 min at room temperature and precipitated with 1 ml of 25 % TCA at 4 ℃ overnight. The precipitates were collected on glass filters, dried at room temperature, and counted for radioactivity in a liquid scintillation spectrometer. To determine the role of Fe^{2+} ion on the inhibitory effect of LF to the parasites, some of monolayers were

supplemented with 1,000 μg/ml of apo-LF, holo-LF or transferrin (TF).

To confirm the effect of LF, the development of intracellular parasites was examined by microscopic observation. Co-culture of MPMs or MECs with the parasites on cover slips were supplemented with the same dose of LF in the culture media at 37 ℃ for varying period of time. After incubation, the cover slips were washed several times with PBS, stained with Giemsa and monitored microscopically to determine the percentage of infected cells per 500 total cells in each group.

Statistical analysis

All experiments were done in triplicate and repeated at least twice. Data from each experiment was evaluated using Student's *t*-test. A value of $p<0.05$ was considered statistically significant.

3. RESULTS

Lactoferrin did not produce any damage of the host cells, because there are no morphologic change of the incubated cells. The viability of cells was >98 % by trypan blue staining. Parasites pretreated with LF sustained penetration activity of host cells and grew in them, indicating that LF had no parasiticidial effects to *T. gondii* directory. The viability of parasites was >98 % by trypan blue staining.

To determine the putative anti-*Toxoplasma* effect of LF for MPM, ^{3}H-uracil incorporation was examined in infected MPMs during 3 h intervals (Fig. 1). Higher incorporation of ^{3}H-uracil was shown between 0-3, 9-12, 15-18 and 21-24 h post incubation, guessing that the nucleic acids synthesis

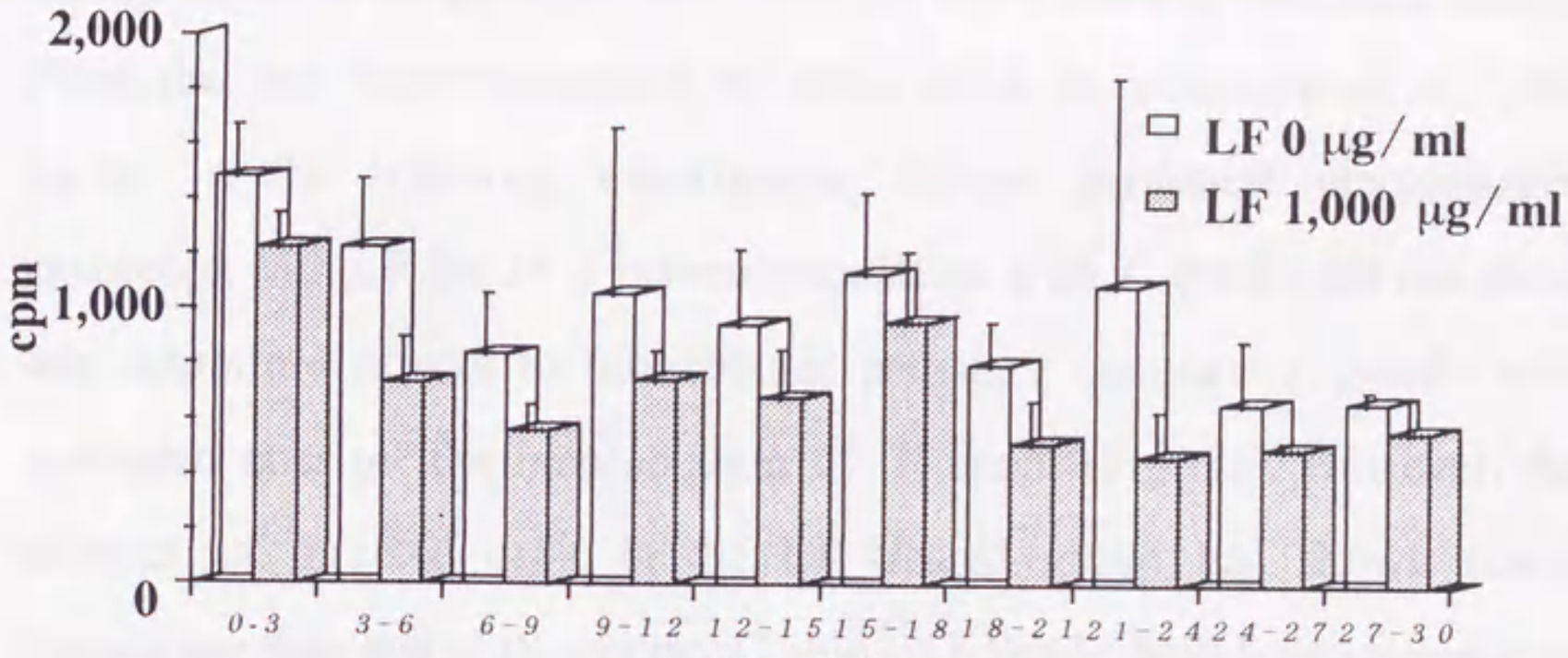


Fig.1. Effect of LF to the growth of *T. gondii* parasites. Incorporation of ^{3}H-uracil in macrophages inoculated with *T. gondii* was examined at 3 h intervals for 30 h of incubation. Each value is the mean ± S. D. of triplicate measurement in a typical experiment (replicated twice). Control: Cultured D-MEM-BSA alone.

of the parasites would be approximately 6 h interval. Whereas, supplement with 1,000 μg/ml LF suppressed ^{3}H-uracil incorporation, especially between 21-24 h post incubation. The growth inhibitory effect was also evaluated at different concentrations of LF in the culture up to 30 h post incubation. As shown in Fig. 2, the growth inhibitory effect was shown in a dose dependent manner. At concentration of 1,000 μg/ml, ^{3}H-uracil incorporation of the parasites was 27.8 % in comparison to untreated control. From this, the author examined the effect of LF at concentration of 1,000 μg/ml in the following experiments. Mouse peritoneal macrophages incubated with LF for 24 h before inoculation with *T. gondii* did not show any inhibitory effects to intracellular parasites, compare *T. gondii* with untreated ones on the incorporation of ^{3}H-uracil (Fig. 3a). However, the number of infected cells in the LF treated group had shown lower percentage than that of the control (Table 1A). While, MPMs incubated with LF for 24 h after inoculation with *T. gondii* LF treated group inhibited the growth of intracellular parasites as well that of the simultaneous incubation group (Fig. 3b and 3c). The number of infected cells in the LF treated group had tendency to show lower percentage than that of the control (Table 1B and 1C). Thus, MPMs produced an inhibitory effect to the intracellular parasites in the presence of LF in the culture, and required the existence of LF in the culture to maintain the activity. This suggests one possibility that MPMs incubated with LF may have suppressed the growth activity of the parasites, but not killed the parasites. Such inhibitory effect was also observed in the group of MPMs treated with apo-LF or holo-LF. Oppositely, MECs preincubated with LF for 24 h showed the inhibitory effect as well

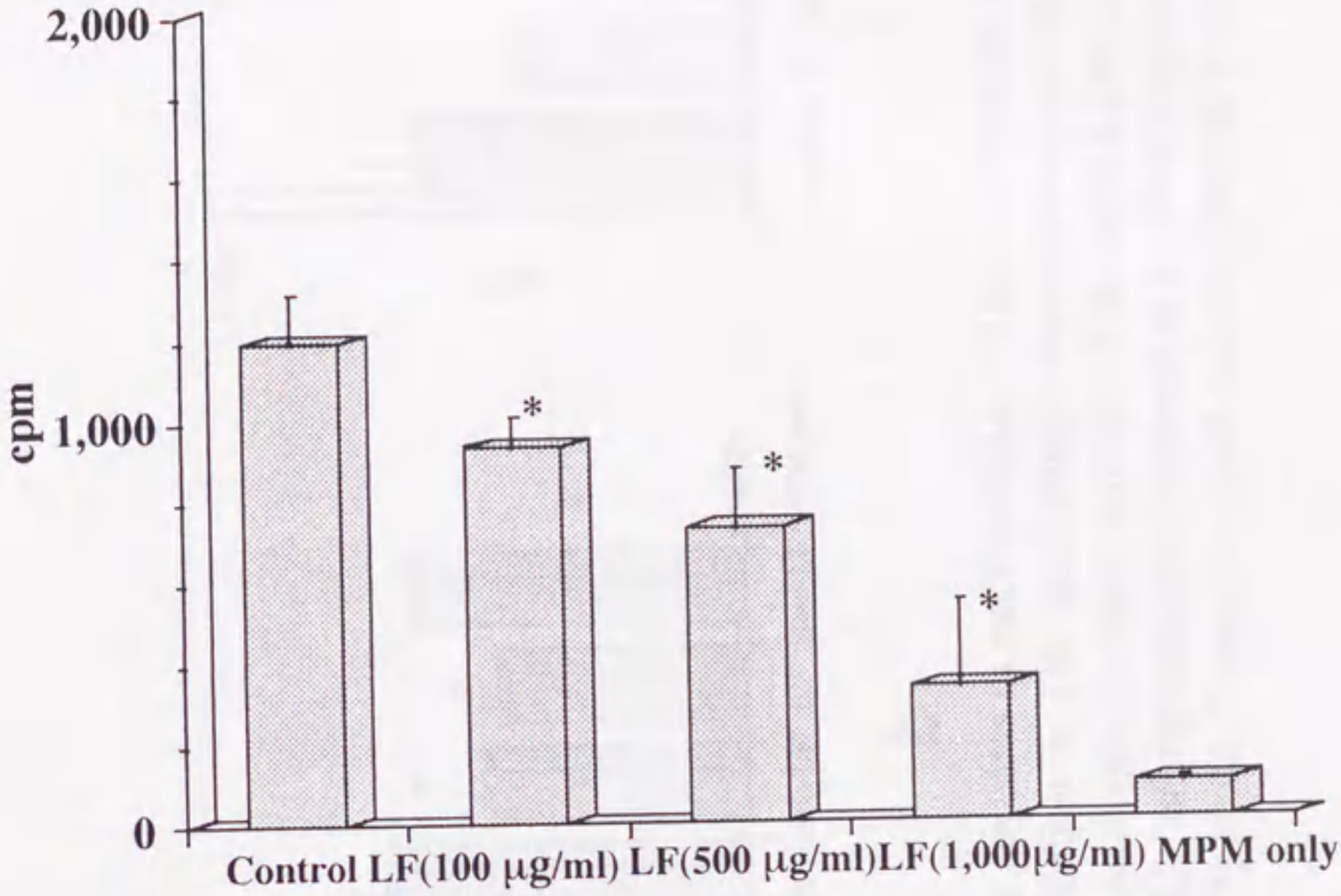


Fig. 2. Effect of various concentration of LF to the growth of intracellular parasites in macrophages. Incorporation of ^{3}H-uracil in macrophages inoculated with *T. gondii* was examined for last 6 h before harvesting. Each value is the mean $\pm$ S. D. of triplicate measurement in a typical experiment (replicated twice). Control: Cultured D-MEM-BSA alone. *$p<0.05$, Control vs. LF (Student's *t*-test).

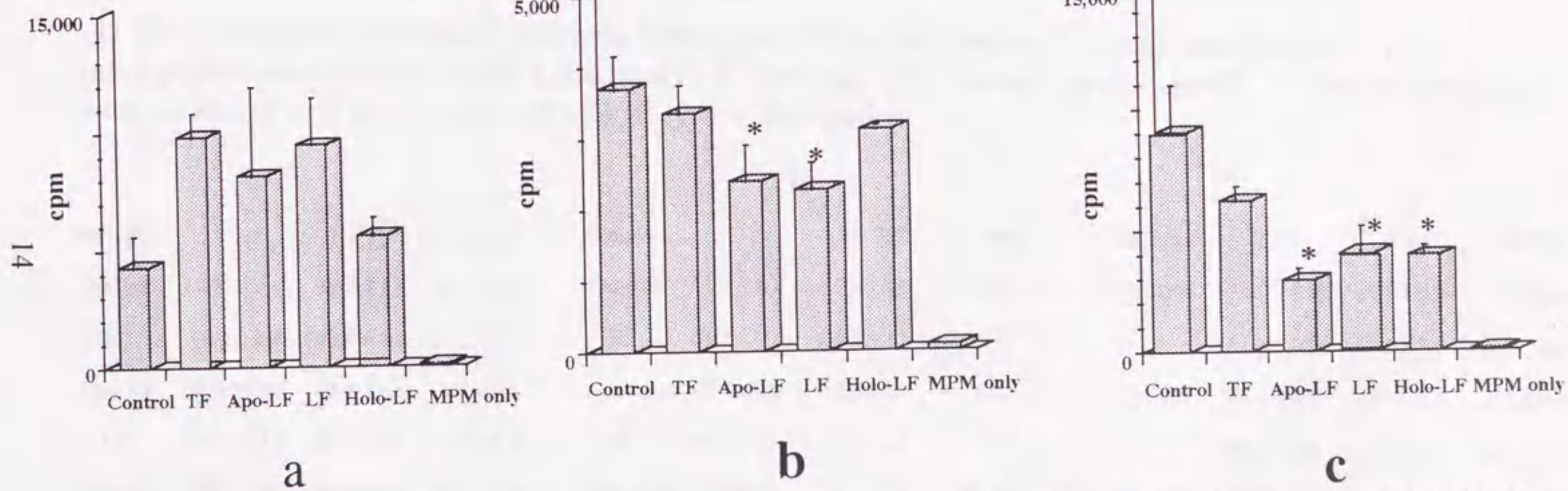


Fig. 3. Effect of LF to the growth of intracellular parasites in macrophages. a: The macrophages were incubated with 1,000 μg/ml LF for 24 h before *T. gondii* inoculation. b: The macrophages were incubated with 1,000 μg/ml LF simultaneously inoculation of *T. gondii*. c: The macrophages were incubated with 1,000 μg/ml LF for 24 h after *T. gondii* inoculation. Each value is the mean ± S.D. of triplicate measurement in a typical experiment (replicated twice). Control: Cultured D-MEM-BSA alone. *$p<0.05$, Control vs. Each sample (Student's *t*-test).

Table 1. Percent of infected macrophages post incubation for 24 h

A: The macrophages were incubated with 1,000 μg/ml LF for 24 h before *T. gondii* inoculation. B: The macrophages were incubated with 1,000 μg/ml LF simultaneously inoculation of *T. gondii*. C: The macrophages were incubated with 1,000 μg/ml LF after *T. gondii* inoculation.

A				B				C			
Samples	0 Tp	1-5 Tp[a]	≧6 Tp[b]	Samples	0 Tp	1-5 Tp[a]	≧6 Tp[b]	Samples	0 Tp	1-5 Tp[a]	≧6 Tp[b]
Control	53.2[c]±1.1[d]	32.7±1.3	14.1±2.4	Control	71.9[c]±9.3[d]	3.9±2.5	24.3±11.8	Control	70.4[c]±8.8[d]	26.9±6.9	2.7±1.8
TF	48.4±0.3	33.9±2.4	17.7±2.1	TF	70.7±15.3	0.9±0.1	28.5±15.3	TF	61.6±0.5	33.3±0.3	5.2±0.8
Apo-LF	59.0±11.0	32.9±6.6	8.1±4.4	Apo-LF	90.6±0.8	2.5±1.9	7.0±1.1	Apo-LF	84.2±2.5	15.0±2.8	0.8±0.3
LF	63.2±13.9	25.6±5.4	11.2±8.5	LF	95.3±1.1	1.1±0.1	3.7±1.2	LF	87.2±2.0	12.5±1.8	0.3±0.1
Holo-LF	62.5±5.8	23.4±9.6	14.1±3.8	Holo-LF	93.4±2.2	0.5±0.1	6.2±2.1	Holo-LF	84.4±0.3	14.8±1.1	0.8±0.8

a: Cells found 1-5 parasites in the cytoplasm.
b: Cells found more than 6 parasites in the cytoplasm.
c: Percentage of infected cells were calculated in 500 total cells.
d: Each value is the mean ± S.D. of triplicate measurement in a typical experiment that was replicated two times.

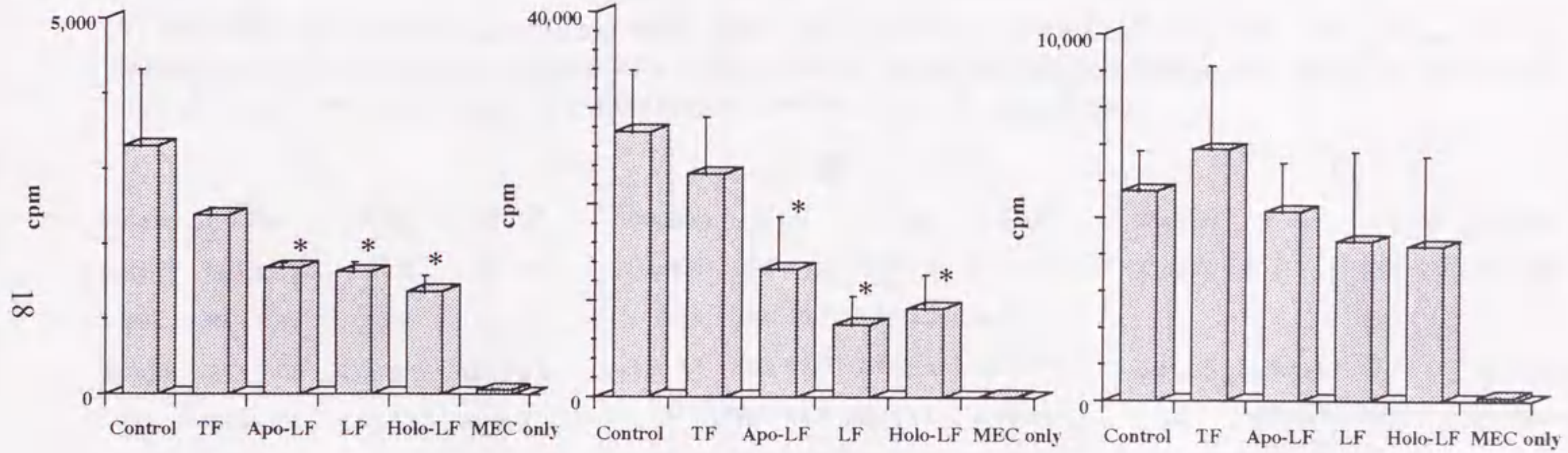
inoculation group (Fig. 4a and 4b, Table 2A and 2B). While, MECs incubated with LF after *T. gondii* inoculation did not induce any inhibitory effects. (Fig. 4c, Table 2C). Thus, one of non-phagocytic cells, MECs also showed the growth inhibitory effect to the intracellular parasites. This action seemed to be , however, different from that of MPM, because pretreatment of LF in MEC induced the growth inhibitory effect to the intracellular parasites.

4. DISCUSSION

In the present study, the author has no data or other observations to explain such different actions of the inhibitory effect of activated host cells. In regard with killing mechanisms against *T. gondii*, as host defense, human mononuclear macrophages possess oxygen-dependent and oxygen-independent anti-protozoan mechanism (14, 35, 45, 68). Primary response to intracellular *T. gondii* is mainly oxygen-dependent (60) and activation by lymphokines (interferron-γ) enhances oxygen-independent effect due to synthesis inorganic nitrogen oxide derived from L-arginine (1). While, suppression of the growth of *T. gondii* in human fibroblast activated by IFN-γ act through starvation for tryptophan (64). Thus, the inhibition of the growth of intracellular parasite is due to host cell defense system, and it may be also influenced by host cell condition. LF has binding capacity of divalent cations (Ca^{2+} and Mg^{2+}) to neutralized stabilize the negative charge of lipopolysaccharide (23). It is plausible to explain that the LF acts on parasites and/or host cells' metabolic function, resulting in the suppression of intracellular parasite growth. Hence induction of such anti-*Toxoplasma*

activity is Fe^{2+} ion independent, interaction of the Fe^{2+} ion would be negligible.

The region of LF molecule responsible for activating properties to MPM and MEC infected with *T. gondii* is not so obvious. To clarify these function and metabolisms, further experiments are under way to examine the interaction of LF and cell metabolic functions.



a **b** **c**

Fig. 4. Effect of LF to the growth of intracellular parasites in mouse embryonal cells. a: The mouse embryonal cells were incubated with 1,000 μg/ml LF for 24 h before *T. gondii* inoculation. b: The mouse embryonal cells were incubated with 1,000 μg/ml LF simultaneously inoculation of *T. gondii*. c: The mouse embryonal cells were incubated with 1,000 μg/ml LF for 24 h after *T. gondii* inoculation. Each value is the mean ± S.D. of triplicate measurement in a typical experiment (replicated twice). Control: Cultured D-MEM-BSA alone. *$p<0.05$, Control vs. Each sample (Student's *t*-test).

Table 2. Percent of infected mouse embryonal cells post incubation for 24 h

A: The mouse embryonal cells were incubated with 1,000 μg/ml LF for 24 h before *T. gondii* inoculation. B: The mouse embryonal cells were incubated with 1,000 μg/ml LF simultaneously inoculation of *T. gondii*. C: The mouse embryonal cells were incubated with 1,000 μg/ml LF after *T. gondii* inoculation.

A

Samples	0 Tp	1-5 Tp[a]	≧6 Tp[b]
Control	74.6[c]±8.8[d]	2.3±0	23.1±0
TF	81.4±3.6	1.5±0	17.1±3.5
Apo-LF	87.8±4.2	2.2±0.7	10.1±3.5
LF	83.0±5.4	2.6±1.3	14.5±4.2
Holo-LF	83.8±5.9	2.2±1.1	14.1±4.7

B

Samples	0 Tp	1-5 Tp[a]	≧6 Tp[b]
Control	73.6[c]±4.2[d]	9.3±1.6	17.6±3.4
TF	74.0±0.7	5.3±3.3	20.7±2.5
Apo-LF	92.5±1.6	2.2±0.1	5.3±1.7
LF	90.7±1.8	4.9±1.2	4.4±0.6
Holo-LF	87.4±1.8	3.3±0.2	9.4±1.6

C

Samples	0 Tp	1-5 Tp[a]	≧6 Tp[b]
Control	32.4[c]±2.4[d]	23.6±7.8	44.2±5.4
TF	53.2±4.2	22.1±5.5	24.8±1.3
Apo-LF	54.0±11.1	18.1±6.6	28.0±4.5
LF	50.1±8.4	13.0±1.5	37.0±6.9
Holo-LF	55.6±13.2	20.3±1.3	24.0±11.8

a: Cells found 1-5 parasites in the cytoplasm.
b: Cells found more than 6 parasites in the cytoplasm.
c: Percentage of infected cells were calculated in 500 total cells.
d: Each value is the mean ± S.D. of triplicate measurement in a typical experiment that was replicated two times.

Chapter 2

The Mechanism of Growth Inhibitory Effects of Bovine Lactoferrin to *Toxoplasma gondii* Parasites in Mouse Macrophages: Role of Radical Oxygen and Inorganic Nitrogen Oxide in *Toxoplasma* Growth Inhibitory Activity

1. INTRODUCTION

Lima and Kierszenbaum (46) reported that lactoferrin (LF) stimulates the phagocytic activity of mouse peritoneal macrophages and increases the intracellular killing of amastigotes of *Trypanosoma cruzi*, an intracellular parasitic protozoan in macrophages. Using scavengers of intermediates of oxygen reduction, they demonstrated that H_2O_2, and O_2^- were involved in the killing capacity of LF-treated macrophages.

As mentioned in chapter 1, somatic cells incubated with media containing LF display inhibitory activity against intracellular parasites of *Toxoplasma gondii*. The mechanism of this inhibitory activity induced by LF is, however, still unclear. Macrophages possess oxygen-dependent and oxygen-independent anti-protozoan mechanisms (45, 59, 60, 69). Murine macrophages activated with IFN-γ and/or lipopolysaccharide display killing activity against *T. gondii*, and this activity is associated with increased production of inorganic nitrogen oxides derived from L-arginine. A competitive inhibitor of the L-arginine-dependent effector pathway, N^G-monomethyl-L-arginine acetate (N^GMMA) abolishes such killing activity of activated macrophages (1).

To clarify the effector pathway of *Toxoplasma* growth-inhibitory activity induced by LF in macrophages, the author examined the production of free radical oxygen products, O_2^- and nitrogen oxide (NO) derived from L-arginine in murine macrophages stimulated with LF. To evaluate the role of NO derived from L-arginine in the mechanism of this activity, the culture medium was supplemented with N^GMMA. In these bioassays, the viability of the intracellular parasites in mouse macrophages was monitored.

2. MATERIALS AND METHODS

Preparation of lactoferrin and reagents

Bovine lactoferrin was prepared from cow's milk by the method of Law and Reiter (43). The amount of Fe^{2+} ion in the LF was 14.5 mg/100 g protein. Apo- and holo-lactoferrin were prepared by the method of Shimazaki and Hosokawa (73). Recombinant murine interferon-γ (Genzyme Co., Cambridge, MA, USA) was diluted in culture medium to obtain a final concentration of 500 unit/ml. N^G-monomethyl L-arginine acetate (N^GMMA: RBI Res. Biochemicals Inc., Natick, MA, USA) was dissolved in phosphate buffered saline (PBS) at 100 mM and stored at -80 ℃ until use. Nitrate and nitrite concentrations were measured using a Nitrite/Nitrate assay kit (Cayman Chemical Co., Ann Arbor, MI, USA) according to the manufacturer's recommendations.

Experimental animals

Seven weeks old male and female ICR mice were used throughout the experiments.

Monolayer cultures of peritoneal macrophages

Mouse peritoneal macrophages (MPMs) were harvested from the peritoneal cavity of mice inoculated with phosphate buffered saline (PBS) containing 0.2 % glycogen on the 5th day post-inoculation (p.i.). They were centrifuged at 800 ×g for 10 min and were suspended in Dulbecco's modified essential medium (D-MEM) containing 10 % fetal bovine serum (D-MEM-10FBS). The macrophage suspension was applied to round coverslips (15×15 mm diameter: Matsunami, Osaka, Japan) and 24-well tissue culture microplates (Corning IWAKI Inc., Osaka, Japan), at 5×10^5 cells/well. The macrophage suspensions were incubated at 37 ℃ for 2 h, then washed thoroughly to remove non-adherent cells, and further incubated at 37 ℃ overnight in D-MEM-10FBS. To avoid the influence of LF present in FBS, each MPM culture medium was substituted with D-MEM containing 1 % bovine serum albumin (D-MEM-BSA) for 24 h before the experiments.

Toxoplasma parasites

The RH strain of *T. gondii* tachyzoites was harvested from the peritoneal cavity of mice under anesthesia on the 3rd day p.i. The parasites were washed by centrifugation at 1,200 ×g for 10 min in PBS three times, and suspended in D-MEM-BSA at a concentration of 5×10^5 parasites/ml.

Measurement of O_2^- and NO

To assess the effects of O_2^- to MPM, MPMs on monolayered coverslips were incubated with LF at 10, 100 and 1,000 μg/ml or without LF for 24 h.

After incubation, the coverslips were washed in Krebs-Ringer phosphate buffer solution free of glucose (KRS) as described by Murray and Cartelli (58). Each coverslip was incubated in 1 ml of KRS containing 100 μM cytochrome c and 0.1 μg/ml phorbol myristate acetate (PMA: Sigma, Louis, MO, USA) at 37 ℃ for 1 h. Before triggering of O_2^- production with PMA, and after incubation, 400 μl of the culture supernatant was taken out and mixed with 1,600 μl of chilled H_2O. Measurement of O_2^- production in the cell culture was carried out by the method of McCord and Fridovich (54) immediately after collecting the samples. The values are expressed as the ratio of the amount per 100 μg of total protein of the MPMs tested.

Measurement of nitrite and nitrate production in the culture media was performed using a Nitrite/Nitrate assay kit as indicated above. The concentrations of nitrite and nitrate were calculated using a standard absorbance curve. The values are expressed as the ratio of the amount per 100 μg of total protein of the MPMs tested.

To measure the total amounts of protein contained in the MPMs tested, the MPMs on coverslips were washed in PBS thoroughly, and dissolved in 6 M urea at room temperature for 3 h. The total amount of protein in the solvent solution was measured using Coomassie^R protein assay reagent (Pierce Inc., Rockford, IL, USA).

Assays of the growth activity of the parasites in the host cells were performed by microscopic observation as described by Tanaka et al. (chapter 1). Prior to observation in this assay, MPM monolayers were incubated for 24 h with either D-MEM-BSA alone, D-MEM-BSA supplemented with LF at 10 to 1,000 μg/ml, or D-MEM-BSA supplemented with 100 μM N^G-

at 10 to 1,000 μg/ml, or D-MEM-BSA supplemented with 100 μM N^G-MMA.

Statistical analysis

All experiments were done in triplicate and repeated at least twice. Data from each experiment was evaluated using Student's *t*-test. A value of $p<0.05$ was considered statistically significant.

3. RESULTS

Effect of O_2^- in macrophages treated with lactoferrin

Based on the author's previous study of the *T. gondii* growth inhibitory effect of MPM treated with LF (chapter 1), we anticipated that O_2^- and/or NO would play a role in this activity. To examine the O_2^- production capacity of macrophages incubated with LF, the concentration of O_2^- in the culture of MPMs incubated with 1,000 μg LF was compared with that of MPMs incubated without LF. As shown in Fig. 5, the amount of O_2^- produced in the culture of MPMs without LF increased gradually, and at 24 h post incubation, the rate of production was 39.2±11.0 nmol/100 μg protein/60 min. In contrast, the MPMs incubated with LF showed a lower O_2^- production capacity, and at 24 h post incubation the rate of production was 18.2±9.72 nmol/100 μg protein/60 min ($p<0.05$). The author next examined the effects of different doses of LF at 24 h post incubation. As shown in Fig. 6, MPMs incubated with LF showed a tendency to have a reduced rate of O_2^- production and the effect of LF was dose dependent. Since LF has iron binding capacity, one possibility to be considered was that

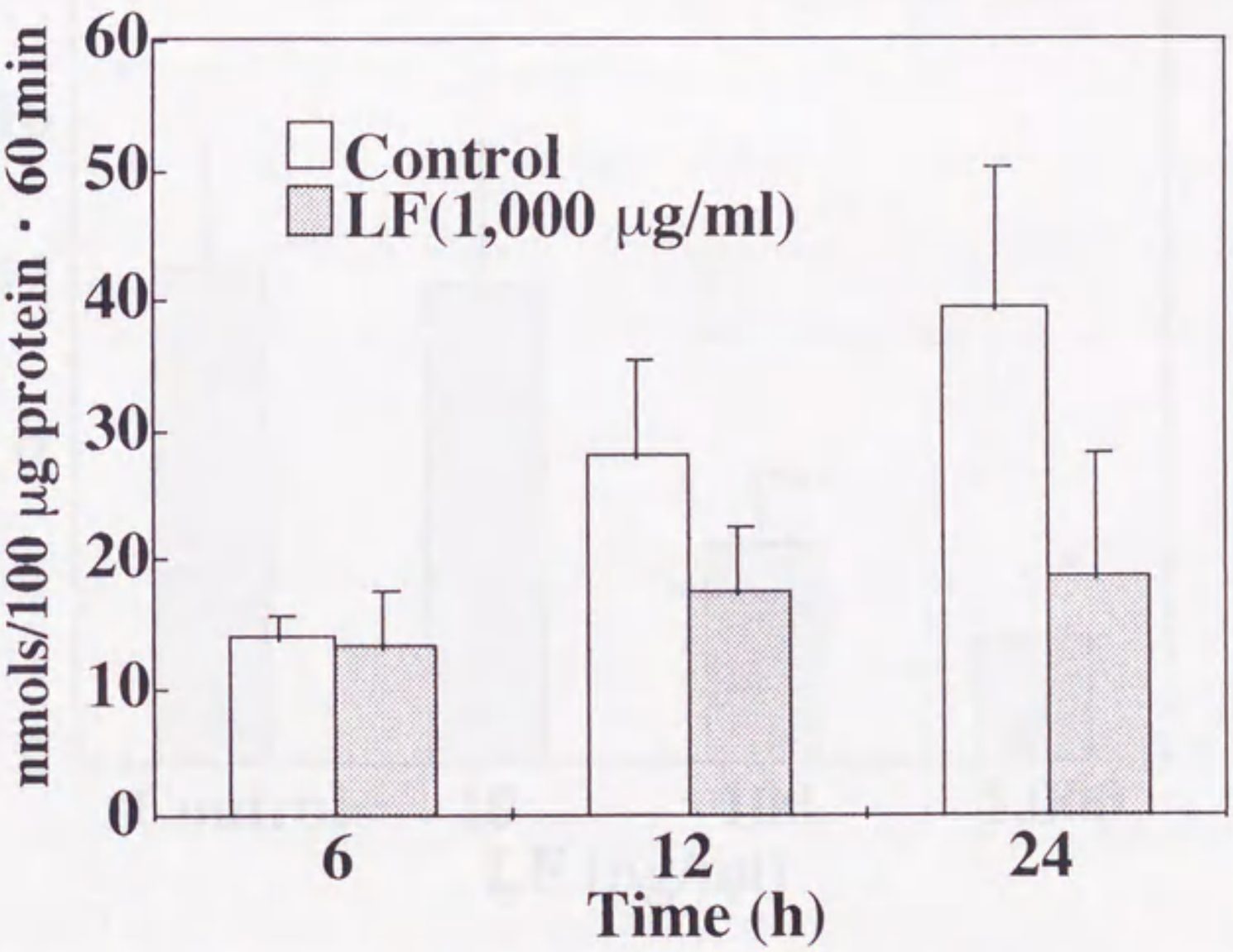


Fig. 5. Production of reactive oxygen intermediates from macrophages incubated with LF at the different time. Each values is the mean ±S.D. of triplicate samples.

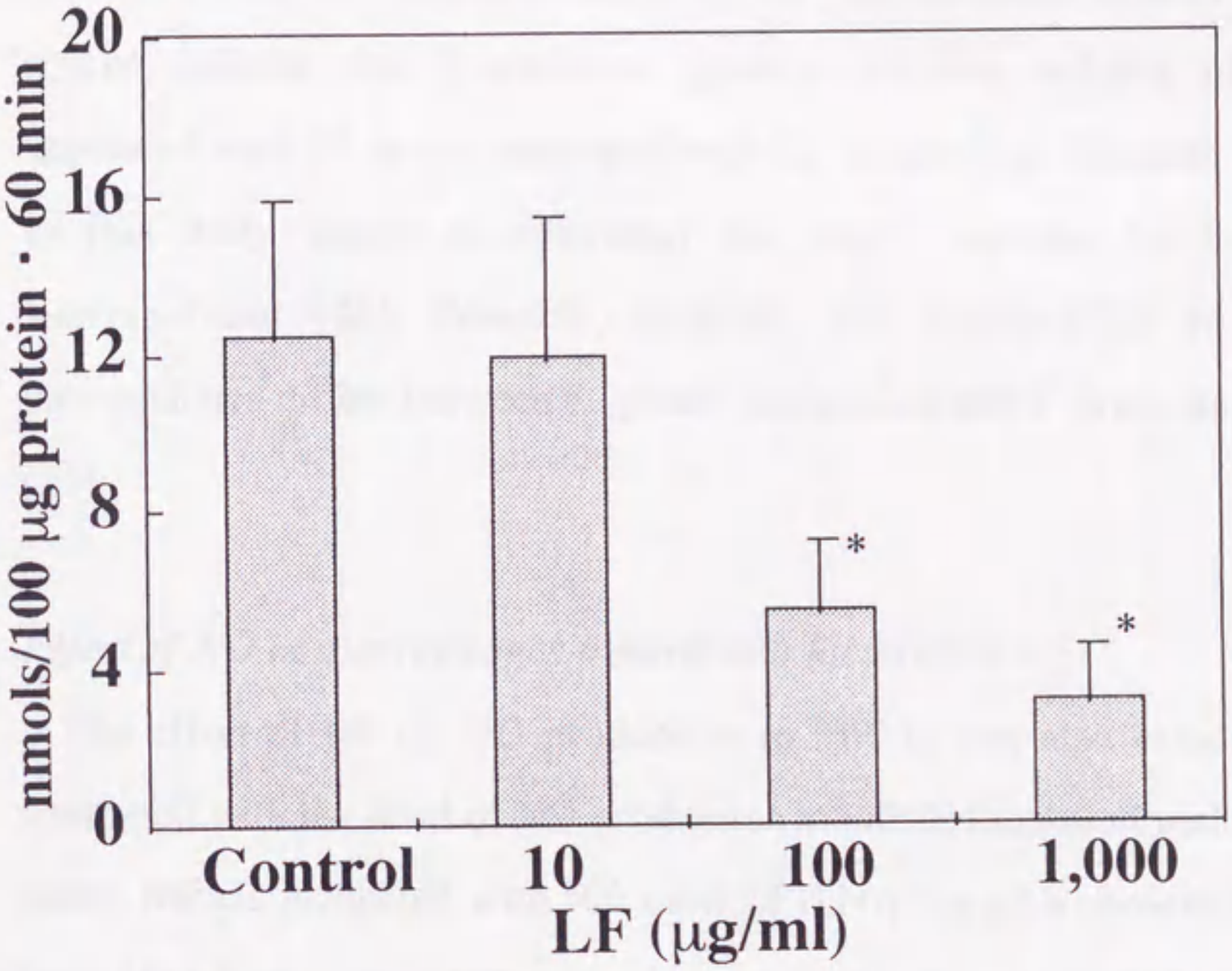


Fig. 6. Production of reactive oxygen intermediates from macrophages incubated with different concentration of LF after 24 h. Each value is the mean ±S.D. of triplicate samples. *$p < 0.05$, Control group vs. LF group (Student's *t*-test).

the O_2^- may have reacted with Fe^{2+} ions, resulting in a reduction in the concentration of O_2^- in the culture medium. The suppressive effect of LF was similar, however, in the case of MPMs incubated with the same dose of apo- or holo-LF (Fig. 7). Thus, the reduction of O_2^- production in MPMs incubated with LF was not mediated by Fe^{2+} ion contained in the LF. These results indicate that *Toxoplasma* growth-inhibitory activity of MPMs incubated with LF is not associated with O_2^- production. The data obtained in this study appear to contradict the results reported by Lima and Kierszenbaum (46). Probably, however, the susceptibility to oxygen intermediates differs between *T. gondii* tachyzoites and *T. cruzi* amastigotes (35).

Effect of NO in macrophages treated with lactoferrin

The effect of LF on NO production in MPMs was also examined. As compared with the level of NO production in MPMs incubated with medium alone, MPMs incubated with 500 units of IFN-γ for 48 h showed a higher level (432 ± 51.6 nmol/100 μg/protein). A slight increase in the level of NO production in MPMs incubated with LF at 1,000 μg/ml for 24 h was detected (32.9 ± 11.2 nmol/100 μg/protein), but the amount of NO produced was significantly lower than that in the presence of IFN-γ ($p<0.05$). Supplementation of the culture medium with N^GMMA strongly inhibited the production of NO in MPMs incubated with IFN-γ. A decrease in NO production following treatment of MPMs with N^GMMA was observed both in the presence and absence of LF (Fig. 8a).

To further evaluate the effect of N^GMMA on the *Toxoplasma* growth-

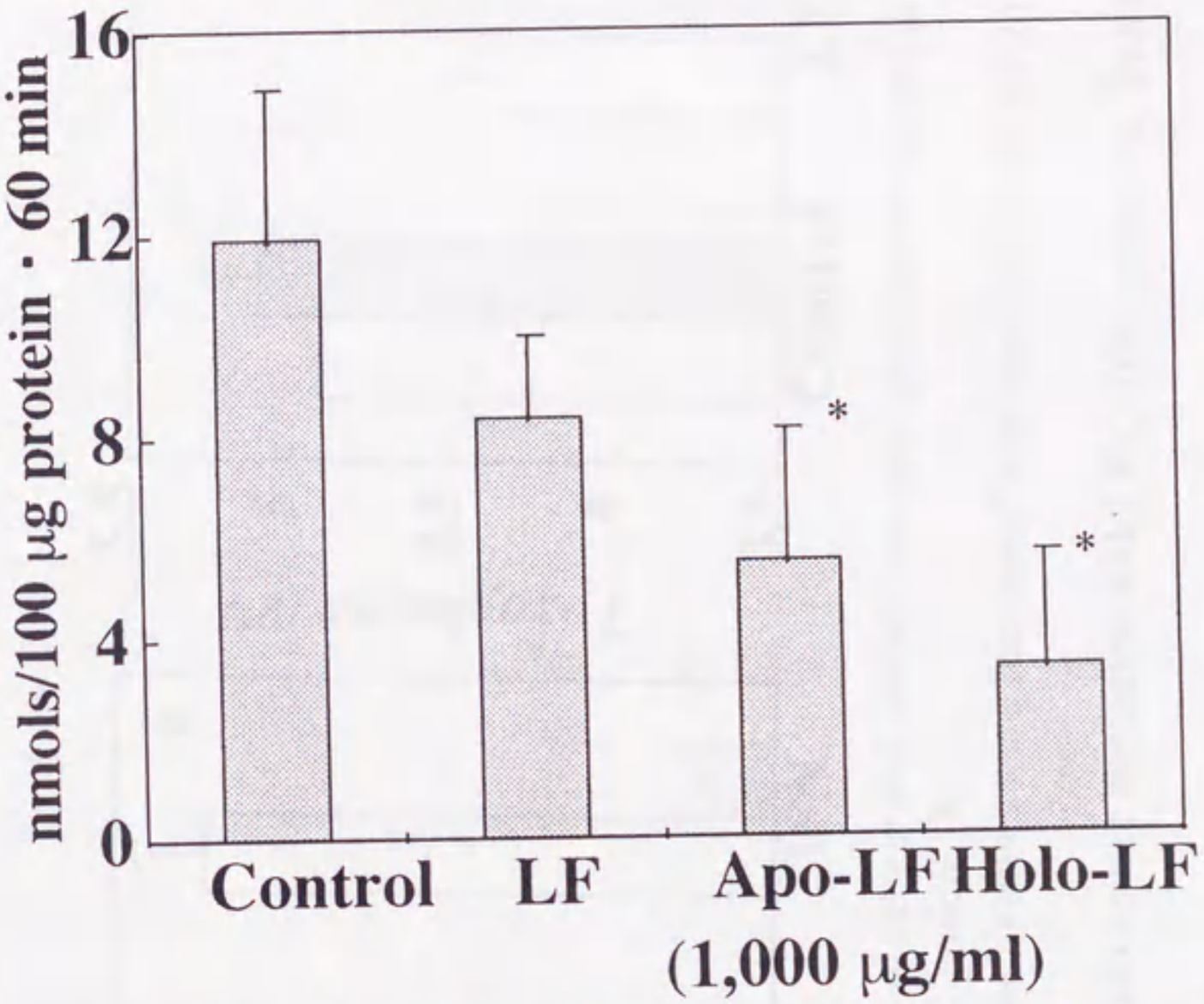


Fig. 7. Production of reactive oxygen intermediates from macrophages challenged tachyzoites and incubated with LF, Apo-LF, Holo-LF after 24 h. Each values is the mean ±S.D. of triplicate samples. *$p < 0.05$, Control group vs. LF group (Student's *t*-test).

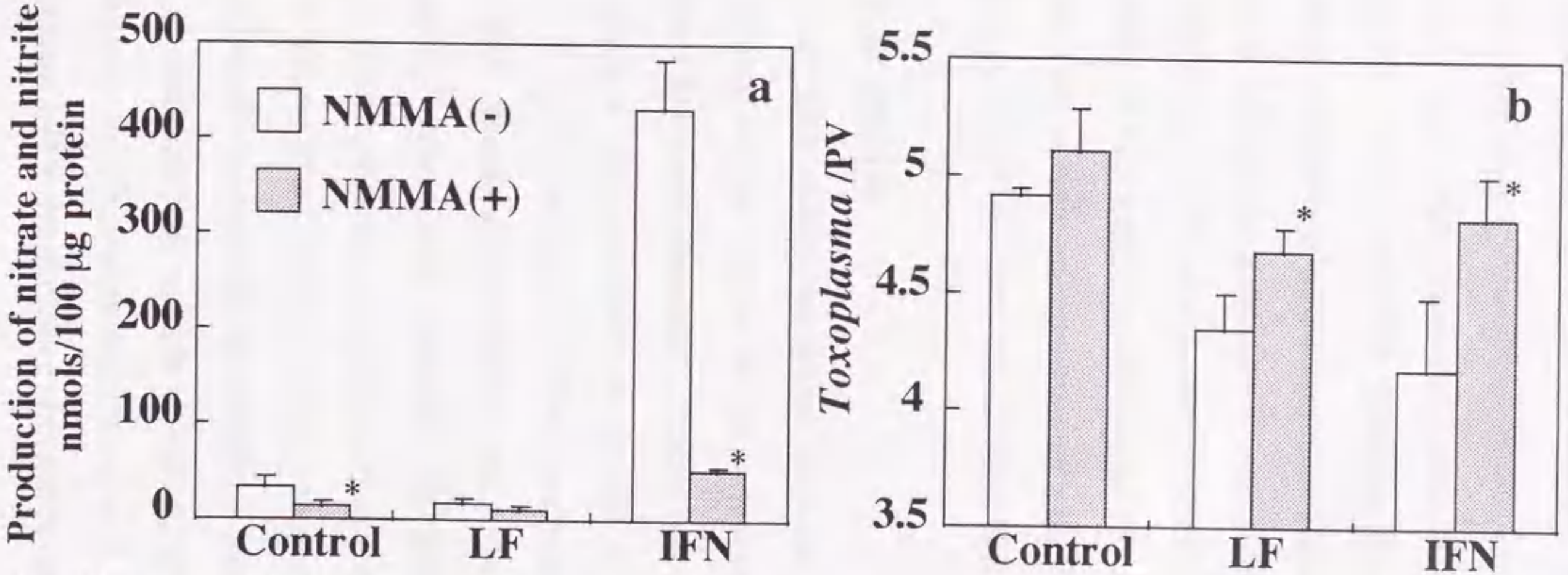


Fig. 8. Effects of NMMA on production of nitrate and nitrite from activated macrophages (a) and microbiostatic capacity of activated macrophages for *T. gondii* (b).
Control: Macrophages challenged with tachyzoites were incubated with medium alone for 24 h in absence (-) or presence (+) of NMMA (100 μM).
LF: Macrophages callenged with tachyzoites were incubated with LF (1,000 μg/ml) for 24 h in absence or presence of NMMA.
IFN: Macrophages incubated with IFN (500 units/ml) for 24 h were challenged with tachyzoites. After that these macrophages were incubated with IFN for 24 h in absence or presence of NMMA.
Toxoplasmastatic capacity is expressed as the number of tachyzoites/parasitophorous vacuole (PV) in 100 vacuoles. Each value is mean ±S.D. of triplicate samples. *$p < 0.05$, NMMA (-) group vs. NMMA (+) group (Student's *t* - test).

inhibitory activity of MPM, the viability of parasites in MPMs incubated with IFN-γ was compared to that in LF-treated MPMs. The inhibitory activity of MPMs incubated with IFN-γ was reduced in cultures supplemented with N^GMMA. Likewise, the *Toxoplasma* growth-inhibitory activity of MPMs incubated with LF decreased in cultures supplemented with N^GMMA. The possibility is negligible that the concentration of NO produced by MPMs treated with LF is sufficient to induce this *Toxoplasma* growth-inhibitory activity, because the NO concentration of MPMs treated with IFN-γ in medium supplemented with N^GMMA was higher than that in the case of MPMs treated with LF (Fig. 8b).

4. DISCUSSION

From these results, the author speculate that the *Toxoplasma* growth-inhibitory activity induced by LF in macrophages might be mediated by another L-arginine-dependent effector pathway that does not lead to NO production. As mentioned in chapter 1, MPMs showed *Toxoplasma* growth-inhibitory when the MPMs were treated with LF after challenge with *T. gondii*. In contrast, the MPMs pretreated with LF showed no such activity against *T. gondii* after challenge. This means that LF acts against the intracellular parasites, but not directly on extracellular parasites, LF has no parasiticidal effect, as shown in the author's previous report. Regarding the host-parasite relationship, intracellular *T. gondii* tachyzoites are enveloped by a parasitophorous vacuole membrane (PVM) which accounts for their resistance to phagolysosomal fusion and this membrane may play an important role in the exchange of nutrients and metabolic products. A recent

study has demonstrated that host cell vimentin binds to the parasitophorous vacuole and may serve to dock the parasite compartment to the host cell nuclear surface (28). It has been observed that LF interacts avidly with nucleic acids. A recent study has documented that LF taken up by leukemia cells becomes bound to DNA and this binding event leads to transcriptional activation (33). One possibility that should be considered is that LF might be taken up by MPMs and then act on PVM to block its biological functions. Further studies of the effects of LF should address the relevance of host cell conditions.

Chapter 3

The Mechanism of Growth Inhibitory Effects of Bovine Lactoferrin to *Toxoplasma gondii* Parasites in Mouse Macrophages: Phosphorylation of Tyrosine in Mouse Macrophages Induced by Bovine Lactoferrin

1. INTRODUCTION

Macrophages possess oxygen-dependent and oxygen-independent anti-protozoan mechanisms and activation by interferon-γ (IFN-γ) enhance oxygen-dependent killing effect to the intracellular *Toxoplasma gondii* (*T. gondii*) due to synthesis of inorganic nitrogen oxide from L-arginine. While, human fibroblast activated by IFN-γ suppress the growth of the intracellular parasites due to starvation for tryptophan (64). The mechanism of the growth inhibitory effects induced by lactoferrin (LF) in macrophages is not mediated by oxygen-dependent anti-protozoan activity (chapter 2). The mechanism of the growth inhibitory effects is however, unclear.

Recently, the intracellular events triggered by LPS, which ultimately lead to anti-bacterial responses in macrophages have been identified. Also, murine macrophages stimulated with LPS and soluble antigens of *T. gondii* showed rapid induction of the tyrosine-phosphorylation of several proteins in murine macrophages (88).

To clarify the mechanisms of anti-*T. gondii* activity induced by LF, the present study was examined whether LF promoted the phosphorylation of tyrosine in mouse peritoneal macrophages.

2. MATERIALS AND METHODS

Preparation of lactoferrin and reagends

Bovine lactoferrin was prepared from cow's milk by the method of Law and Reiter (43). The amount of Fe^{2+} ion in the LF was 14.5 mg/100 g protein. Genistein (Wako Co., Osaka, Japan) was dissolved in Dulbecco's modified essential medium (D-MEM) containing 10 % fetal bovine serum (D-MEM-10FBS). Genistein is a inhibitor of protein tyrosine kinase (PTK).

Experimental animals

Seven weeks old male and female ICR mice were used throughout the experiments.

Monolayer cultures of peritoneal macrophages

Mouse peritoneal macrophages (MPMs) were harvested from the peritoneal cavity of mice inoculated with phosphate buffered saline (PBS) containing 0.2 % glycogen on the 5th day post-inoculation (p.i.). They were centrifuged at 800 ×g for 10 min and were suspended in D-MEM-10FBS. The macrophage suspension was applied onto round coverslips (15 × 15 mm diameter: Matsunami, Osaka, Japan) and 24-well tissue culture microplates (Corning IWAKI Inc., Osaka, Japan), at 1×10^6 cells/well. The macrophage suspensions were incubated at 37 ℃ for 2 h, then washed thoroughly to remove non-adherent cells, and further incubated at 37 ℃ overnight in D-MEM-10FBS. To avoid the influence of LF presented in FBS, each MPM culture medium was substituted with D-MEM containing 1 % bovine serum albumin (D-MEM-BSA) for 24 h before the experiments.

Toxoplasma parasites

The RH strain of *T. gondii* tachyzoites was harvested from the peritoneal cavity of mice under anesthesia on the 3rd day post inoculation (p.i.). The parasites were washed by centrifugation at 1,200 ×g for 10 min in PBS three times, and suspended in D-MEM-BSA at a concentration of 1×10^6 parasites/ml.

Macrophages stimulated by lactoferrin

The MPMs (1×10^6 cells/ml) were incubated with the concentration of 1, 10, 100 and 1,000 μg/ml LF for 30 min. In the same way, they were incubated with 1,000 μg/ml LF for 5, 15, 30 and 60 min.

Assays of the growth activity of the parasites in the host cells were performed by microscopic observation as described by Tanaka et al. (chapter 1). Prior to observation in this assay, MPM monolayers were incubated with either D-MEM-BSA alone, or D-MEM-BSA supplemented with LF at 1,000 μg/ml for 24 h. To examine the effect of tyrosine-phophorylase inhibitor, some of MPM cultures were supplemented with genistein at 0.1, and/or 1.0 μg/ml .

Immune peroxidase staining

MPMs stimulated with LF were fixed with 1 % paraformaldehyde for 15 min at 4℃. After washing with PBS, the cells were treated with 0.3 % H_2O_2-methanol for 15 min at 4 ℃ to inactivate endogenous peroxidase. After washing PBS, they were blocked with 3 % BSA-PBS overnight. After washing with PBS, they were incubated with anti-phosphotyrosine antibody

various fold diluted in PBS for 30 min at 37 ℃. After washing with PBS, they were incubated with goat anti-mouse IgG conjugated HRPO (1/500 dilution: BIO RAD, Hercules, CA, USA) for 30 min at 37 ℃. After washing with PBS, the peroxidase reaction was visualized using diaminobenzidine 4 HCl in 0.1 M Tris-HCl (pH 7.4) containing 0.03 %.

SDS-PAGE

After washing with PBS, the MPMs stimulated with LF were solubilized in 50 μl SDS-PAGE sample buffer. After shaking overnight, the suspension were heated to 95 ℃ for 5 min and sonicated to lysis DNA. Each sample was applied to 10 % polyacrylamide gel for SDS-PAGE.

Immunoblotting

The samples used by SDS-PAGE were transferred from the gels onto PVDF membranes for 50 min at 12 V. After washing with PBS-Tween 20, the membranes were blocked with 3 % BSA-PBS at 4 ℃ overnight. After washing with PBS-Tween 20, they were incubated with anti-phosphotyrosine mouse monoclonal antibody (1/500 dilution: Clone PY20, ICN Biomedicals, Inc., Costa Mesa, CA, USA) at 4 ℃ overnight. After washing with PBS-Tween 20, they were incubated with peroxidase conjugated anti-mouse IgG conjugted HRPO. After washing with PBS-Tween 20, binding of secondary antibody was detected with the enhanced chemiluminescence detection methods (DuPont NEM Res., Boston, MA, USA).

Statistical analysis

All experiments were done in triplicate and repeated at least twice. Data from each experiment was evaluated using Student's *t*-test. A value of $p<0.05$ was considered statistically significant.

3. RESULTS

Induction of protein tyrosin-phosphorylation in macrophages

First, phosphorylation of tyrosine was examined by immune peroxidase test using anti-phosphorylated tyrosine antibody. In some of cells in MPMs incubated with LF, peroxidase reaction was detected on cytoplasm of them (Fig. 9). In the culture incubated without LF, specific reaction was non-detectable.

Further, phosphorylation of tyrosine was examined by immunoblotting analysis. As shown in Fig. 10, some bands were detected on the lane of the phosphorylated protein. Specific band, of which molecular was approximately 30 kDa was detected on the lane of MPMs incubated with LF (1, 10, 100, 1,000 μg/ml) for 30 min. Particularly, when the MPMs incubated with the concentration of 1,000 μg/ml LF, the band was appeared at the highest density.

The change of incubation time, 30 kDa was detected on the lane of MPMs incubated with LF for either 30 or 60 min (Fig. 11).

Effect of tyrosin-phosphorylation in macrophages supplemented with genistein

The number of tachyzoites in parasitophorous vacuole (PV) of MPM

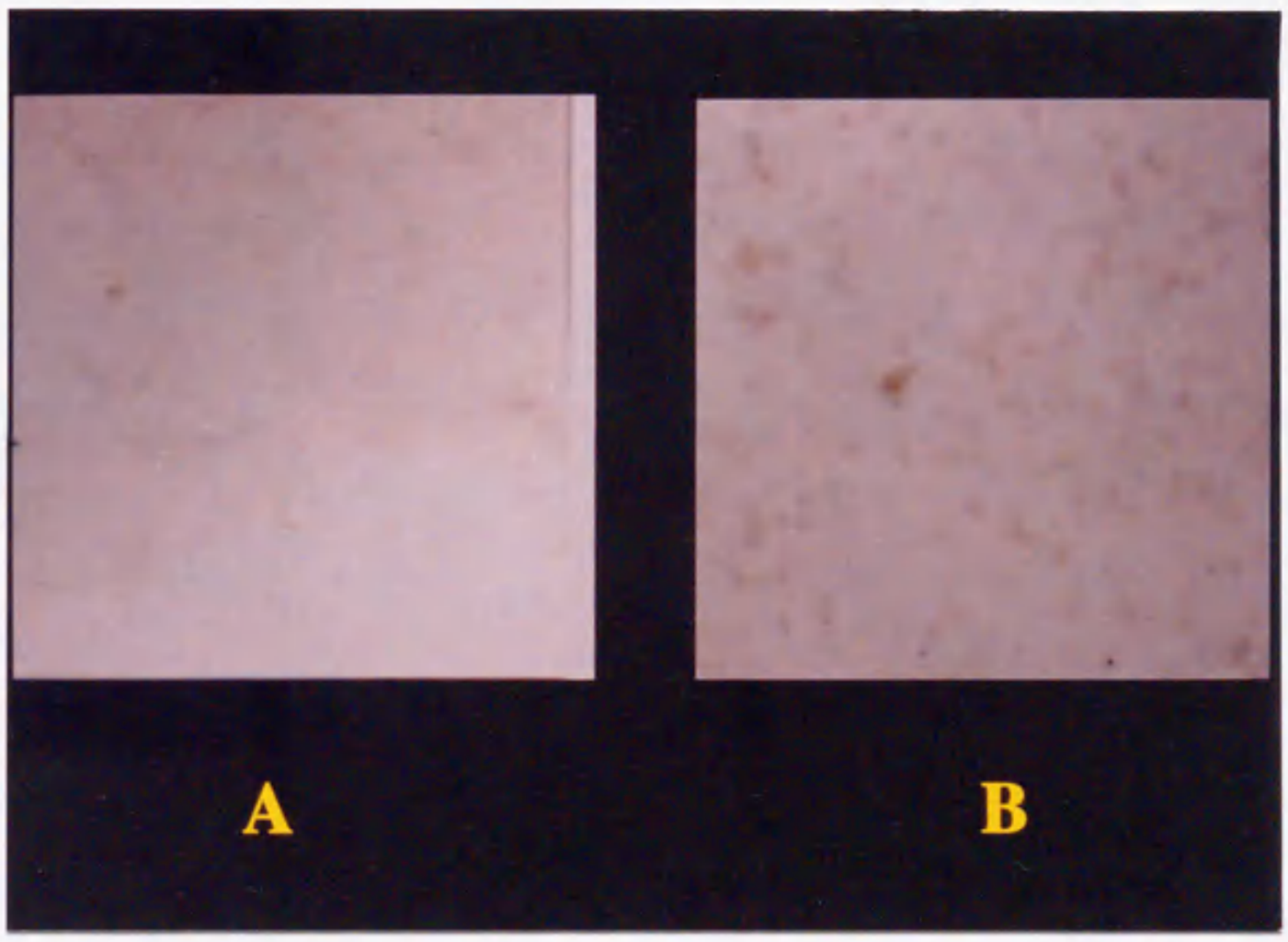


Fig. 9. Phosporylation of tyrosine in macrophages by immune peroxidase staining. The macrophages were incubated with either medium alone (A) or LF (B) for 30 min.

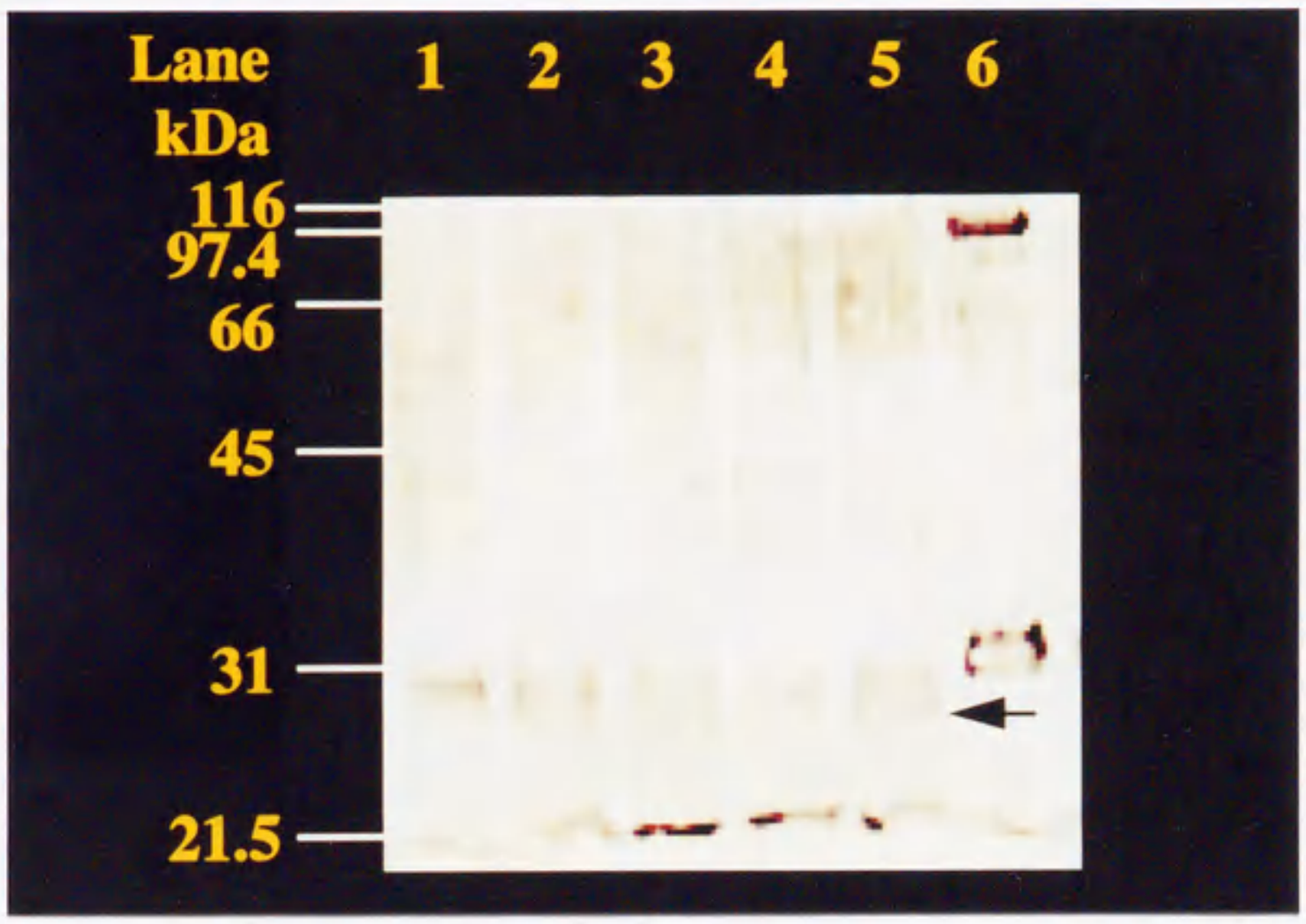


Fig. 10. Kinetics of tyrosin phosphorylation in macrophages stimulated with LF. The macrophages were incubated either with or without LF for 30 min. Lane 1: Medium alone. Lane 2: LF (1 μg/ml) . Lane 3: LF (10 μg/ml) . Lane 4: LF (100 μg/ml) . Lane 5: LF (1,000 μg/ml) . Lane 6: Molecular standards.

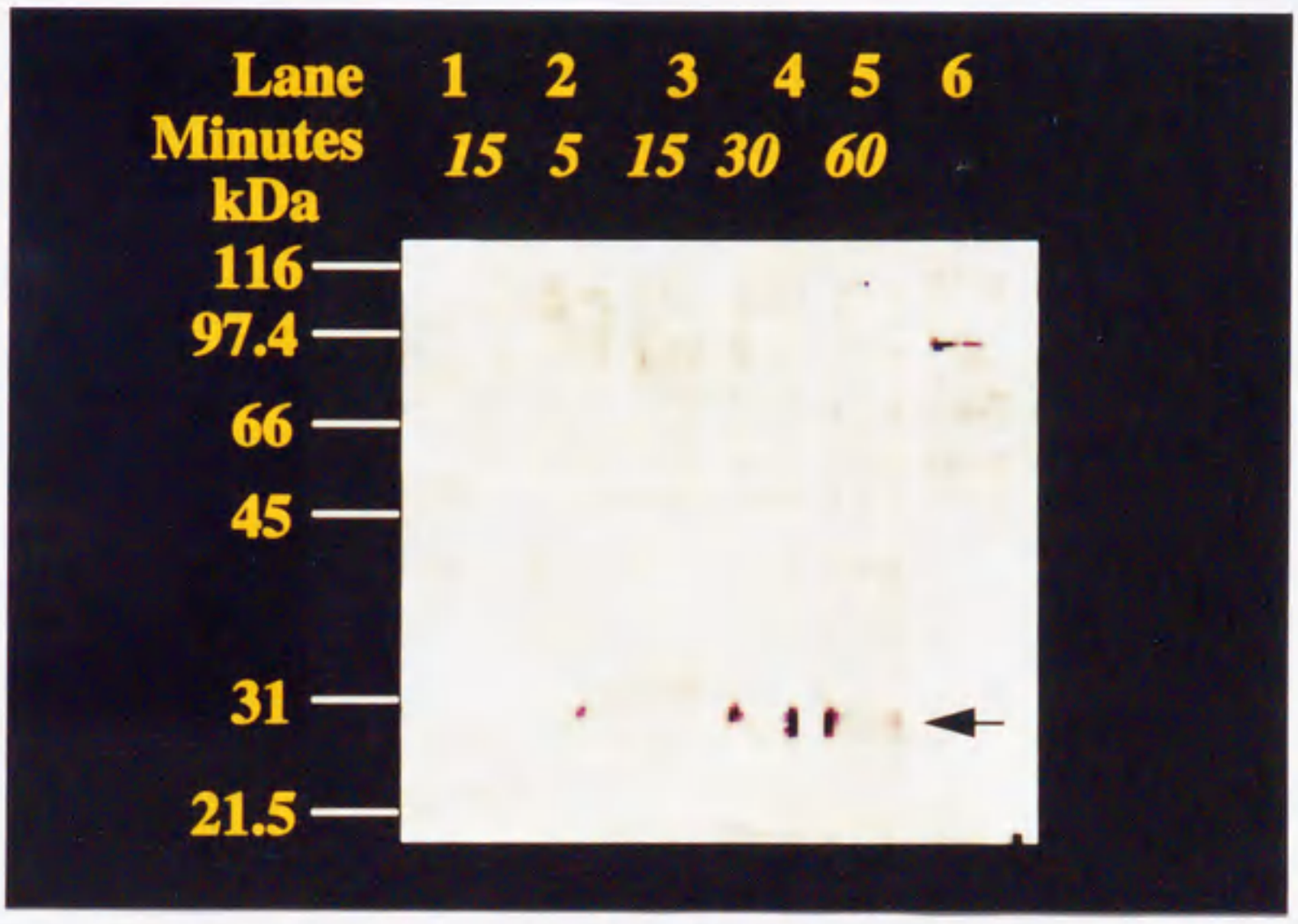


Fig. 11. Kinetics of tyrosin phosphorylation in macrophages stimulated with LF. Lane 1: Medium alone. Lanes 2~6: The macrophages were incubated with LF (1,000 mg/ml) for the time indicated above each lane. Lane 6: Molecular standards.

supplemented with LF-genistein was shown higher than that of treated LF. From this results, it is plausible that the tyrosine-phosphorylation in MPMs treated with LF would be associated with the growth inhibitory activity to *T. gondii* (Fig. 12). It is determined that phospholation of 30 kDa protein was related with growth inhibitory activity. As shown Fig. 13, 30 kDa bands of the macrophages stimulated with LF for 30 min were disappeared, when the cell culture contained 1.0 μg/ml genistein.

4. DISCUSSION

Raju and Hutchens (65) reported that Glutathione-S-transferase (GSTpi) synthesis was increased by both human LF and bovine LF in leukemia cell line. GST plays an important role in cellular detoxification processes. A 28-kDa protein in leukemia cell line was decreased in response to human LF. This protein was identified as GDP dissociation inhibition, a protein involved in regulatory functions of GTP binding proteins. Genistein is a tyrosine-phospholylation inhibitor which blocks ATP-dependent phospholylation pathway. It is demonstrated that the 30 kDa phosphorylation protein must be similar to these proteins. Moreover, it is plausible that the tyrosine-phosphorylation in MPM treated with LF would be associated with the growth inhibitory activity to *T. gondii*.

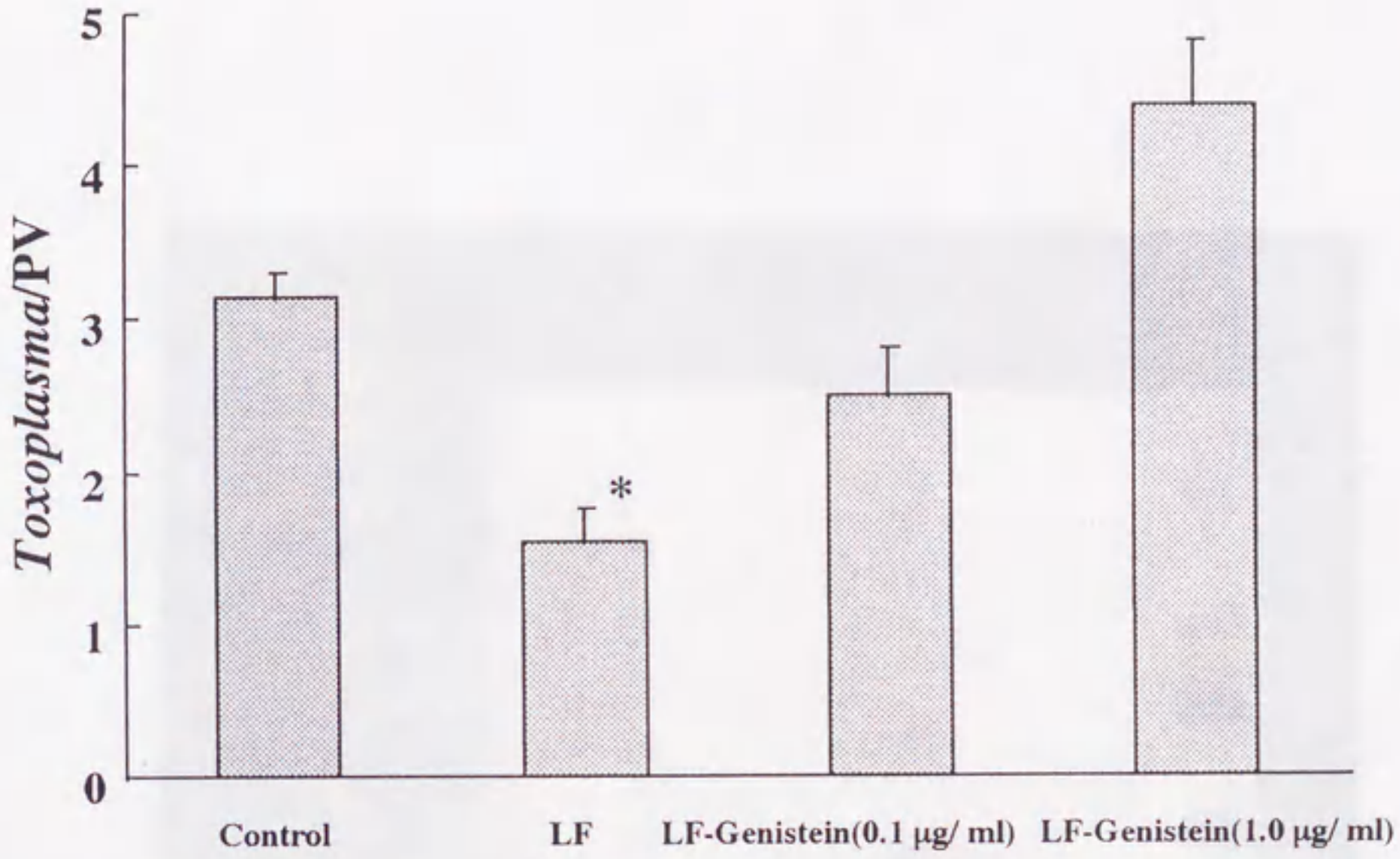


Fig. 12. Effect of genistein to the growth of *T. gondii* in macrophage. Each value is mean ±S.D. of triplicate masurement in a typical experiment. Control: Cultured D-MEM-BSA alone. *$p<0.05$, Control vs. Each sample (Student's *t* - test).

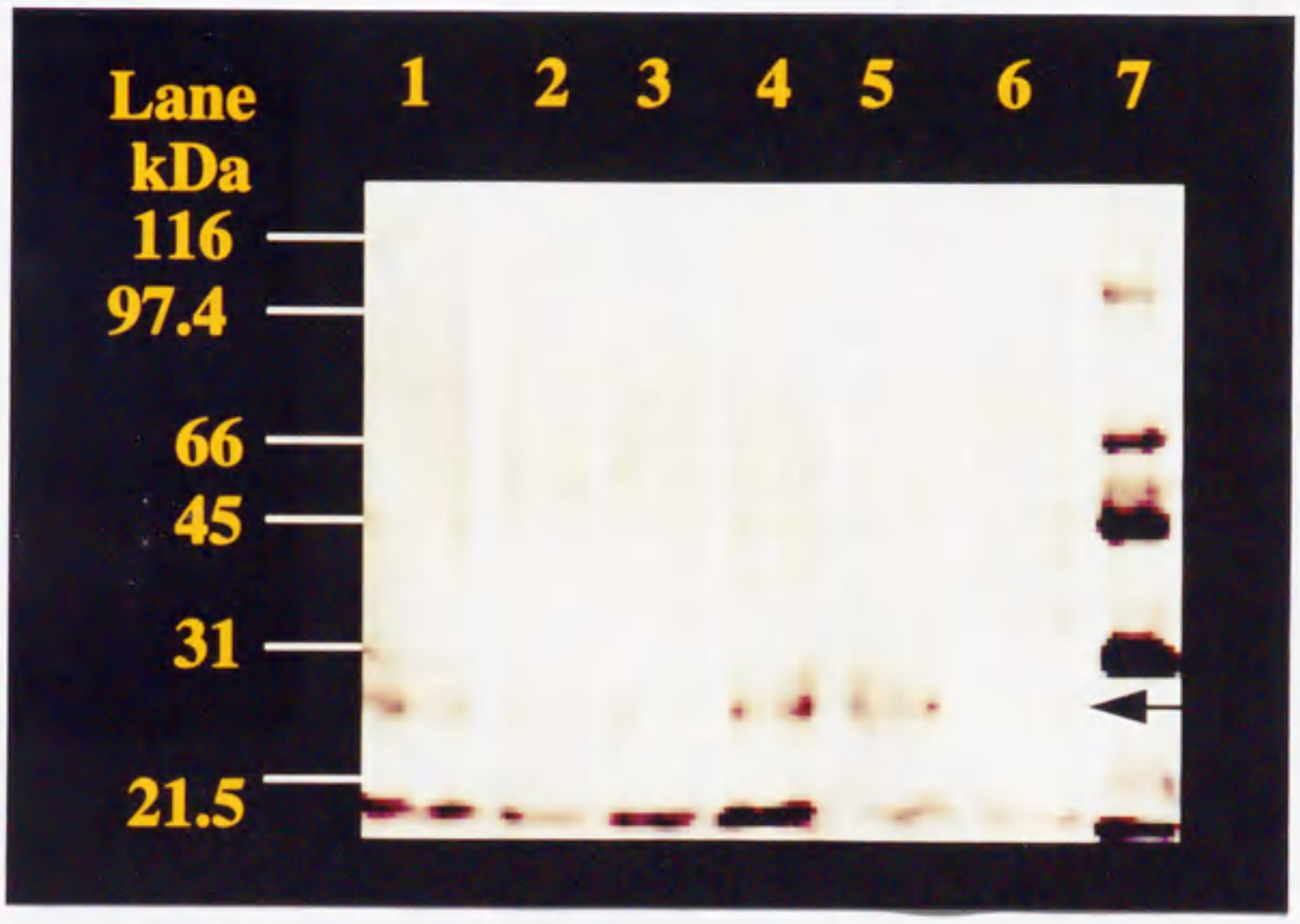


Fig. 13. Kinetics of tyrosine phosphorylation in macrophages supplemented with genistein. Lane 1: Medium alone. Lane 2: Medium alone supplemented with 0.1 µg/ml genistein. Lane 3: Medium alone supplemented with 1.0 µg/ml genistein. Lane 4: LF. Lane 5: LF supplemented with 0.1 µg/ml genistein. Lane 6: LF supplemented with 1.0 µg/ml genistein. Lane 7: Molecular standards.

Chapter 4

Protective Effect of Bovine Lactoferrin in Mice Infected with *Toxoplasma gondii* Parasites

1. INTRODUCTION

Lactoferrin (LF), an iron binding protein found in exocrine secretions, has wide spectrum bacteriostatic effects due to destruction of membrane of microorganisms (21), and is capable of destabilizing the outer membrane of gram-negative bacteria and binding capacity to lipid A (2, 21). The effects of LF *in vivo* has also been reported (80). Furthermore, LF has been reported to play important roles in various immunological function, including production of IL-1, IL-2, IL-6, TNF-α, natural killer cell cytotoxicity and lymphocyte proliferation (70). Actually, Lima and Kierszenbaum (47) have demonstrated that LF stimulated macrophages to kill amastiogotes of *Trypanosoma cruzi*. It has been shown that LF had growth inhibitory effect *in vitro* against *Toxoplasma gondii* (*T. gondii*), an obligatory intracellular parasitic protozoa, even though LF had no parasiticidal effects directly on extracellular parasites (chapter 1). One possibility was guessed that LF may activate macrophages into a parasitistatic state. It is of interest whether the action of LF *in vitro* reflect to the relevance of host defense systems *in vivo*.

In the present study, the author report that administration of LF prevent death of acute toxoplasmosis. In the protective response against *T. gondii*, interferon-γ (IFN-γ) produced by both $CD4^+$ and $CD8^+$ cells plays an

essential role for activation murine macrophages to display killing activity against the parasites (24, 77). Natural killer cells also produce IFN-γ stimulated with TNF-α. Further, recent studies showed that interleukin-12, a heterodimeric cytokine is also essential for IFN-γ production. Thus, the author also studied the role of protective immune responses and examined whether LF influences serum levels of IFN-γ.

2. MATERIALS AND METHODS

Experimental animal

Female ICR mice at age 8 weeks, weighing 23-28 g were used throughout the experiments. In selected *in vivo* experiments, female ICR nu/nu mice on age 8 weeks (Charles River Inc., Yokohama, Japan) were also used.

Preparation of lactoferrin

Bovine lactoferrin was prepared from bovine milk whey as described by Law and Reiter (43), and dissolved in phosphate-buffered saline (PBS) at a concentration of 20 mg/ml just before use.

Preparation of cysts of T. gondii

Cysts of the Beverley strain of *T. gondii* were obtained by homogenization of cerebral tissue from infected mice on 2-3 months postinfection. The number of cysts in the homogenates of cerebral tissue were determined by counting microscopically. Briefly, 10 μl of a homogenate made from one brain was dropped onto a slide glass and observed under 18×18-mm coverslip. All samples were done in triplicate.

In vivo assay

The protective effect of LF was examined by experimental *T. gondii* inoculation. Twenty mice were orally challenged with LD_{90} of cysts and divided four groups. Thirty min before challenge, and once again on each of the first 7 days postinfection, two groups of each five mice were orally administered with either 1.0 or 10.0 mg/0.5 ml of LF in 0.5 ml of saline. Another two groups were administered intraperioneally either 0.1 or 1.0 mg of LF in 0.5 ml of saline. As a negative control, five mice were orally inoculated with saline as well as test groups.

After challenge, the mortality and survival period of the mice were monitored up to 35 days postchallenge. On day 35, the survivors were sacrificed and their brains were removed and homogenized gently in 3 ml of PBS with a glass homogenizer to count the number of cysts.

To address the question whether immune effector cells, especially T cells are involved in the resistance in mice administered with LF, the author examined the experimental *T. gondii* inoculation in nude mice administered intraperitoneally with LF.

Interferon-γ assay

To examine the serum levels of interferon-γ in the mice, each tested mice in the experiment as mentioned above was bled and approximately 100 μl /mouse was obtained from the supraorbital vein at various time. The sera were stored at -80 ℃ until use. Assay of interferon-γ activity in the serum was performed by ELISA using assay kit (mouse ELISA IFN-Endogen Inc., Cambridge, MA, USA). All experiments were done at least twice.

Statistical analysis

Significance of differences were evaluated using χ^2 test. A value of $p<0.05$ was considered statistically significant.

3. RESULTS

Effect of oral administration with lactoferrin

Oral administration of LF showed preventive effect against acute toxoplasmosis. All of mice orally administered with 1.0 mg of LF survived until the end of the experiment. Sixty % of mice administered orally with 10.0 mg LF also survived until the end of the experiment and the survival period of the dead mice showed prolongation, as compared with that of control (Fig. 14a).

Effect of intraperitoneal administration with lactoferrin

In control groups, 60 % of the mice died of acute toxoplasmosis within 14 days post challenge. In the peritoneal cavity of the mice, many parasites and mononuclear cells were found. Whereas, intraperitoneal administration of 1.0 mg LF prevent death of acute toxoplasmosis. Eighty % of the treated mice survived until the end of the experiment (Fig. 14b). Although 60 % of the mice administered intraperitoneally with 0.1 mg LF eventually died of toxoplasmosis between 13 to 18 days postchallenge, the survival time of those mice was longer than that of control.

The number of cysts in brain of survived mice administered with LF was significantly reduced, compared with the survivor of control (Table 3).

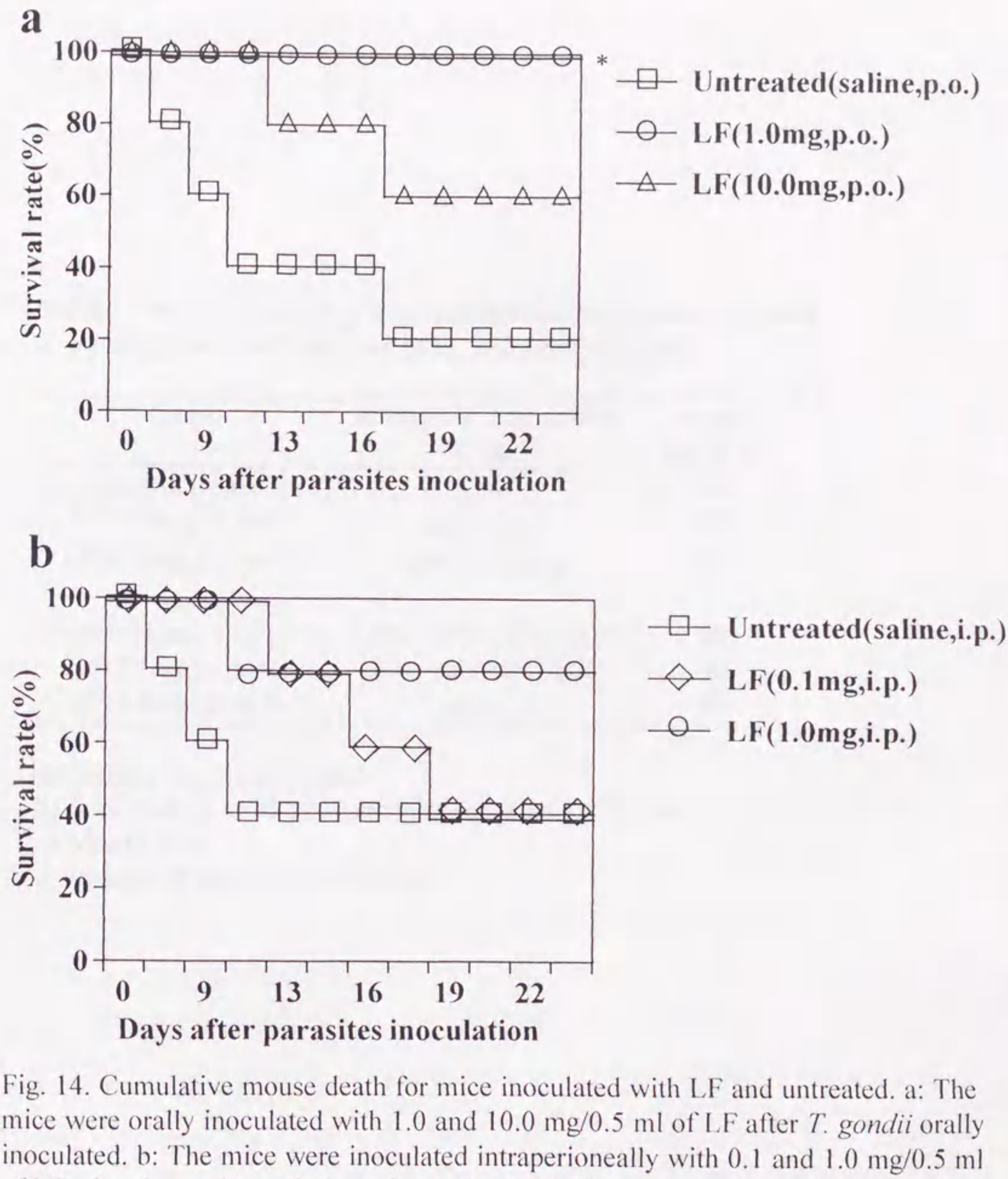


Fig. 14. Cumulative mouse death for mice inoculated with LF and untreated. a: The mice were orally inoculated with 1.0 and 10.0 mg/0.5 ml of LF after *T. gondii* orally inoculated. b: The mice were inoculated intraperioneally with 0.1 and 1.0 mg/0.5 ml of LF after *T. gondii* orally inoculated. *$p<0.05$, Untreted group vs. LF group (χ^2 test).

Table 3. The survival rate of mice and the number of cerebral tissue *T. gondii* cysts in administered group and untreated group

Group	Average of total number of cysts [a)]	Survival rate[b)](%)
Untreated(saline,p.o.,n=1)	3,900	20
LF(1.0mg,p.o.,n=5)	567±257[c)]	100
LF(10.0mg,p.o.,n=3)	1,550±1,014	60
Untreated(saline,i.p.,n=2)	3,300, 3,400(AVE=3,350)	40
LF(0.1mg,i.p.,n=2)	800, 800(AVE=800)	40
LF(1.0mg,i.p.,n=4)	950±34	80

a) Cerebral tissues per mouse.
b) Survivors up to 35 day post inoculation/total of tested.
c) Mean±S.D.
n: Number of counted mouse brain.

Effect of nu/nu mice by lactoferrin administration

In control group, all of nu/nu mice died of acute toxoplasmosis within 10 to 20 days post challenge. Similarly, the most of the nu/nu mice administered with intraperitoneal inoculation of 1.0 mg of LF also died of acute toxoplasmosis within 17 days post challenge (Fig. 15).

Effect of IFN-γ production by lactoferrin administration

As shown in Fig. 16, all of challenged mice produced IFN-γ. The survivors orally and intraperitoneally administered with LF produced IFN-γ between 5 and 7 days post challenge. IFN-γ production of control was as same level as that of LF administration. The mice either perorally or intraperitoneally administered with LF alone showed no production of IFN-γ during the sampling period (not detected).

4. DISCUSSION

In the present study, both of oral and intraperitoneal administration of LF showed preventive effects for toxoplasmosis in mice. One mg of LF is sufficient to induce the protective activity. These results suggest that administration of LF may be inducible of activation of host defense systems which have prevention activity to the parasite growth.

To examine more details of the host defense mechanism in mice administered with LF, the author measured IFN-γ activity in the serum of mice. The survivors orally and intraperitoneally administered with LF produced similar amount of IFN-γ to that of control and the production was relative later, compared to the control. These findings suggest that the

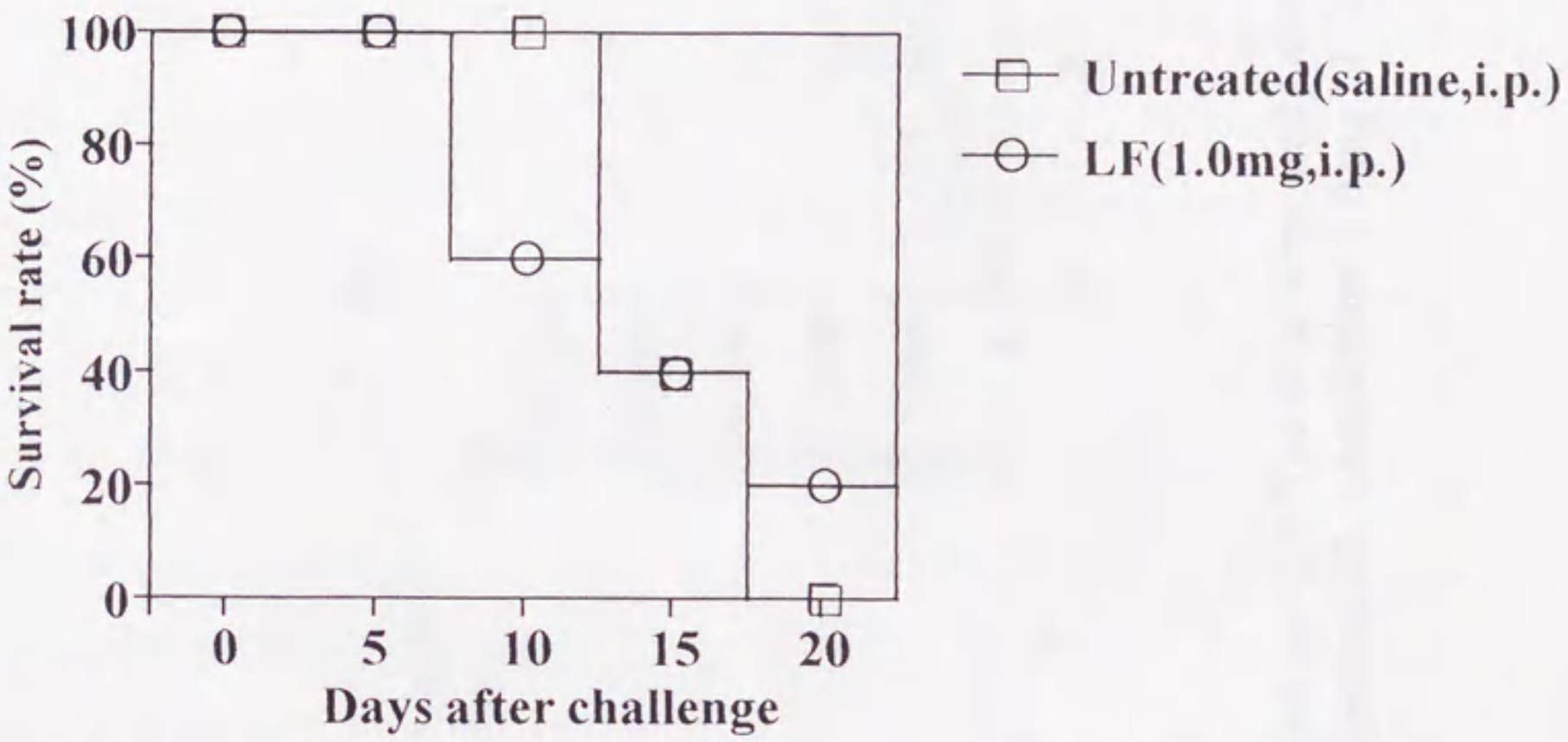


Fig. 15. Effect of administered LF on resistance of nu/nu mice to *T.gondii* infection. The mice were intraperitoneally administered 1.0 mg of LF after oral challenge with *T.gondii*.

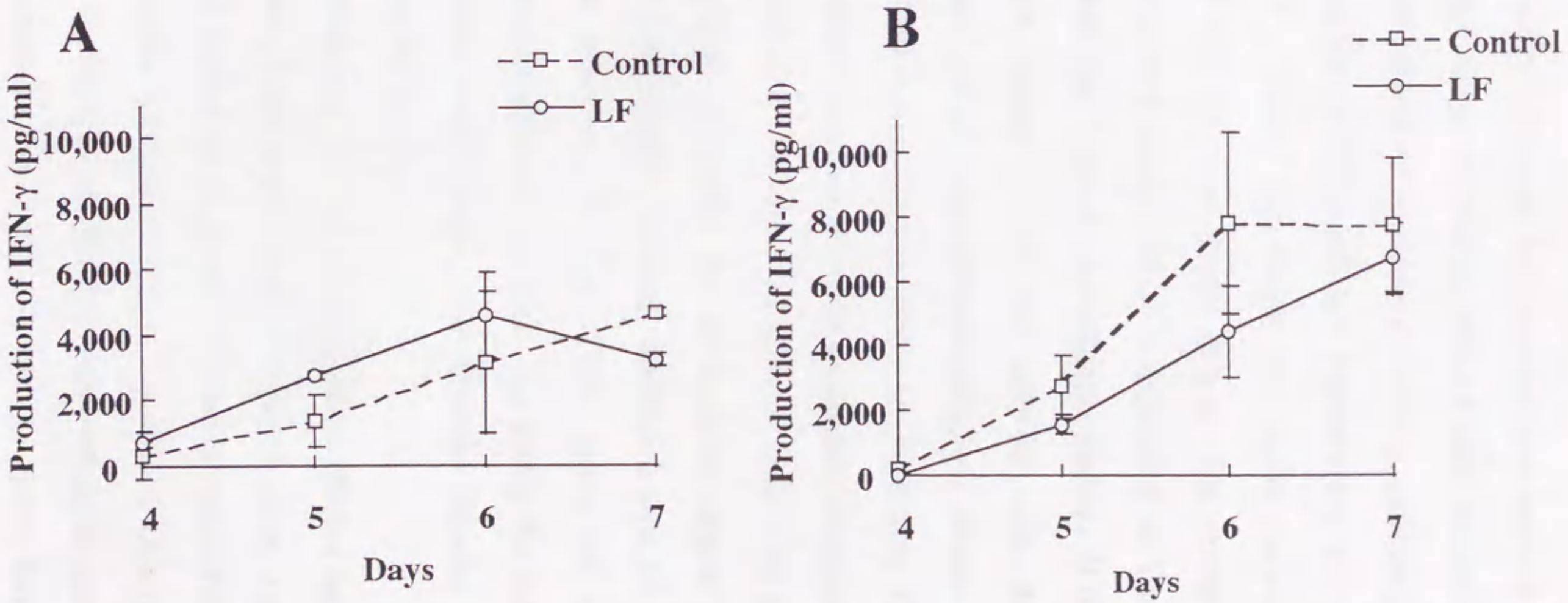


Fig. 16. Production of IFN-γ in serum of mice (n=3). A:The mice were orally administered 10.0 mg of LF for 7 days post infection. B: The mice were intraperitoneally administered 1.0 mg LF for 7 days post infection.

resistance induced by LF would be associated with not only the production of IFN-γ, but also action of immune effector cells. Interestingly, the mice administered with LF had no production of IFN-γ, indicatingly that LF itself may have no activity as IFN-γ inducer. Furthermore, we investigated the experimental *T. gondii* inoculation in nu/nu mice administered intraperitoneally with LF. The results indicate that resistance against *T. gondii* infection in mice induced by LF is dependent on T cells. Sher et al. (72) demonstrated that *T. gondii* tachyzoites stimulate IFN-γ synthesis in NK cells, which require TNF-α and adherent cells. Moreover, they documented that *T. gondii* triggered macrophages to produce IL-12, which synergize with TNF-α to stimulate IFN-γ production by NK cells. From these, the possibility be considered is that the defense mechanism induced by LF may be interacted by at least two effector cells. One is that LF may activate macrophages to inhibit the intracellular parasite's growth and enhance antigen presentation function. Another is that LF may stimulate immune effector cells, such as $CD4^+$, $CD8^+$, and/or NK cells which are cytotoxic for parasite-infected host cells. To clarify the role of LF in the interaction between macrophages and immune effector cells, further experiment should be necessary.

Oral administration of LF also had preventive effects against *T. gondii* infection. Recently, it has been shown that lactoferricin, a peptide derived from acid-pepsin hydrolysis of bovine LF had parasiticidal effect against extracellular parasites and those in cyst of *T. gondii in vitro* (chapter 5), and *in vivo* (chapter 6). From these, two possibility would be speculated: One is that LF may be absorbed in the bloodstream as native form and activate

macrophages and/or immune effector cells. Another is that LF may have cleaved by proteolytic enzymes in the stomach and became pepsin-generated hydrolysate which has parasiticidal activity. Kuwata et al. (42) observed that LFcin was liberated from LF by gastrointestinal proteolytic activity. To confirm these possibilities, immune responses in mice peroral administered with LF should be investigated.

Chapter 5

Parasiticidial Effects of Lactoferricin and C-lobe in Bovine Lactoferrin to *Toxoplasma gondii* Parasites

1. INTRODUCTION

Recently, an antimicrobial peptides was isolated and sequenced from pepsin digestion of lactoferrin (LF). The active peptides of bovine LF (named lactoferricin: LFcin) is derived from the N-terminal region of the molecule, and composed of 25 amino acid residues. It has inhibitory effects to the growth of Gram-negative and Gram-positive microorganisms due to direct damage to the outer membrane of them (6, 7, 38). Usefulness of the peptides to inactivation for food-borne pathogen is considerably potential exist.

Toxoplasma gondii (*T. gondii*), an obligatory intracellular parasitic protozoa transmit by uncooked infected meat as oral infection and cause congenital infection and opportunistic infection in immunodifiecient individuals. It is, however, unknown that LFcin has parasiticidal effect to such parasitic protozoa.

In the present study, the author attempted to examined the effect of LFcin to the infectivity and viability of *T. gondii* parasites *in vitro* and *in vivo* experiments.

2. MATERIALS AND METHODS

Preparation of lactoferricin

Lactoferricin was prepared from bovine LF as described by Bellamy et al.,(5). The purity of LFcin was confirmed by high performance liquid chromatography and amino acid sequencer.

Preparation of lactoferrin C-lobe

The C-terminal region of bovine lactoferrin was prepared by procedures descried by Shimazaki et al. (74).

Preparation of parasites

Toxoplasma gondii parasites of RH strain were obtained from the peritoneal cavity of mice on the 3rd day post inoculation. The parasites enriched peritoneal exudate was washed by centrifugation at 1,200 ×g for 10 min in PBS 3 times. The parasites were isolated by Percoll-sucrose solution density gradient centrifugition (gravity: 1.070) at 40,000 ×g for 20 min, then were suspended in Dulbecco's modified minimum essential medium (D-MEM) containing 1 % bovine serum albumin (D-MEM1%BSA). Cysts of S-273 strain of *T. gondii* were obtained from brains of chronically infected mice by Percoll-PBS density gradient centrifugation as described by Corrnelisen et al (17) and were suspended in D-MEM containing 10 % fetal bovine serum (D-MEM10FBS).

Preparation of mouse embryonal cells

Mouse embryonal cells (MECs) were prepared as described elsewhere

(63) and cultured in D-MEM10FBS. For experiments, MECs were harvested from culture flask by incubation with PBS containing 0.025 % trypsin, centrifuged at 800 ×g for 10 min and were suspended in D-MEM 10FBS to adjust 5×10^4 cells/ml. On each oval cover slip (diameter, 15 mm, Matsunami glass Inc. Osaka, Japan), 0.2 ml of cell suspension was mounted and incubated at 37 ℃ overnight.

Effect of lactoferricin to the parasites

To examine the parasiticidal effect of LFcin, bovine LF and/or the C-terminal region of bovine LF, parasites suspension (1×10^6/ml) was mixed and incubated at 37 ℃ for 15 min to 4 h either with various concentration of LFcin (0, 100, and 1,000 μg/ml) or 1,000 μg/ml of C-terminal region of bovine LF and/or 1,000 μg/ml of bovine LF in a series of tubes containing D-MEM 1% BSA. Each series of tubes were washed thoroughly by centrifugation at 1,200 ×g for 10 min 3 times in PBS , then were resuspended in D-MEM 1% BSA to adjust 1×10^6/ml parasites.

To examine the exclusion of trypan blue dye as a criterion of parasite viability, a 5 μl aliquot of the parasite suspension was mixed with the same volume of PBS containing 0.5 % trypan blue and staining was immediately assessed under a light microscope. The number of stained parasites was counted among a total of 200 parasites in four samples of each series. The ratio of stained parasites was represented as the mean of percentages.

For assay of infectivity of the parasite to host cells, 0.1 ml of either treated or untreated parasites was mounted onto the MEC monolayered coverslips

and incubated at 37 ℃. At 18 h post inoculation (p.i.), the coverslips were washed in PBS thoroughly, fixed with methanol and stained with Giemsa. The number of infected cells was counted among a total of 500 cells and the percentage of infected cells was calculated.

The infectivity of treated parasites and/or cystozoites in cysts after incubation with LFcin for 4 h was also examined by an inoculation test in mice. Five mice in each group were inoculated intraperitoneally with 10^2 parasites either untreated or pretreated with various concentrations of LFcin and the survival time and mortality of the mice were monitored for up to 30 days p.i. Samples from the mice that died during the monitoring period were examined microscopically for the presence of parasites in the peritoneal cavity. To examine the infectivity of cysts, five mice per group were inoculated with 10^2 cysts either untreated or pretreated with LFcin as described above, and the survival time of the mice was monitored for up to 30 days p.i. The number of cysts in the brains of individual mice was counted using microscope. To confirm the infection, the level of anti-*T. gondii* IgG in the serum of the mice was measured by an indirect immunofluorescence assay as described elsewhere (63).

All experiments described above were done in triplicate.

3. RESULTS

Effect of lactoferricin on the viability of parasites

A preliminary experiment was carried out to determine the putative anti-toxoplasma activity of LFcin. Viability of *T. gondii* was examined after incubation of the parasite suspension with LFcin for various periods. As

shown in Fig. 17, after treatment of *T. gondii* with LFcin at 100 μg/ml for 1 h, 64 % of the parasites became oval in shape and had lost the ability to exclude trypan blue dye. Their nuclei showed little staining by Giemsa. At 4 h p.i, more than 96 % of the parasites showed no dye exclusion ability. Similarly, approximately 96 % of the parasites treated with LFcin at 1,000 μg/ml for 0.5 h lost the dye exclusion ability. After 2 h of incubat ion with LFcin at 1,000 μg/ml, no unstained parasites were detected. In contrast, more than 80 % of the parasites incubated with bovine LF 1,000 μg/ml for 4 h retained the dye exclusion ability. Likewise, the parasites incubated with D-MEM alone for 4 h maintained this ability.

Effect of lactoferricin on the infectivity of parasites in mouse embryonal cells

The activity of the parasites to penetrate MECs was represented by the percentage of infected cells. The parasites had significantly less penetration activity after preincubation with LFcin at 1,000 μg/ml for 0.5 h (Fig. 18). The parasites treated with LFcin at 100 μg/ml showed gradually decreasing penetration activity and the percentage of infected cells became less than 10 % after 4 h of treatment. However, the untreated parasites retained this activity after similar incubation.

Infectivity of parasites in mice after treatment with lactoferricin

The loss of infectivity of the parasites and/or cystozoites in cyst was confirmed by inoculation of mice. (Table 4) All five mice inoculated with 10^2 untreated parasites died within 9 days post-challenge. Similarly,

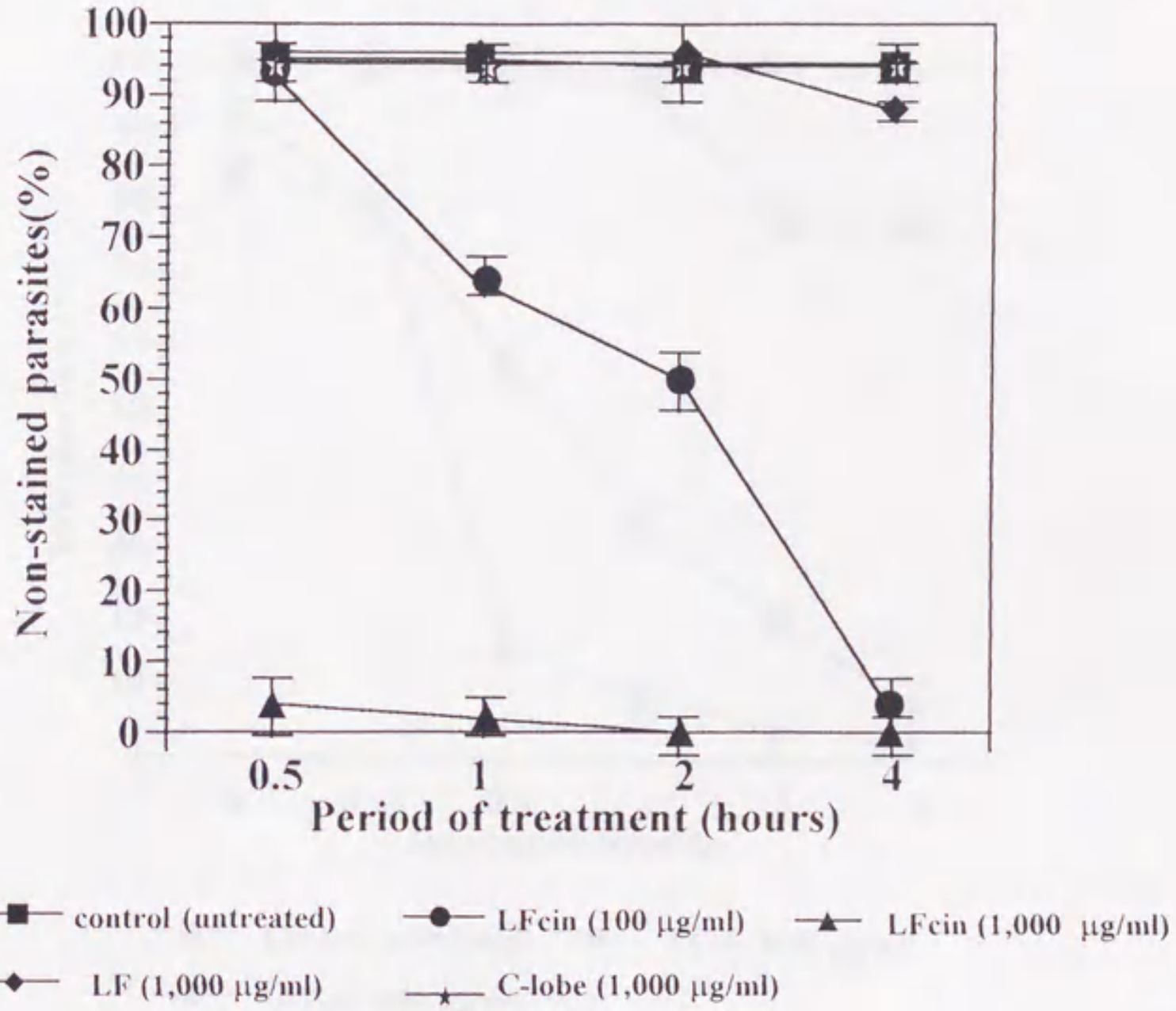


Fig. 17. Effect of LFcin on the viability of *T. gondii*. Parasites were incubated with LFcin at defined concentrations for the periods indicated and then viability was examined by the trypan blue dye-exclusion assay. The mean percentage of non-stained parasites is shown. Values shown are mean±S.D. of triplicate samples.

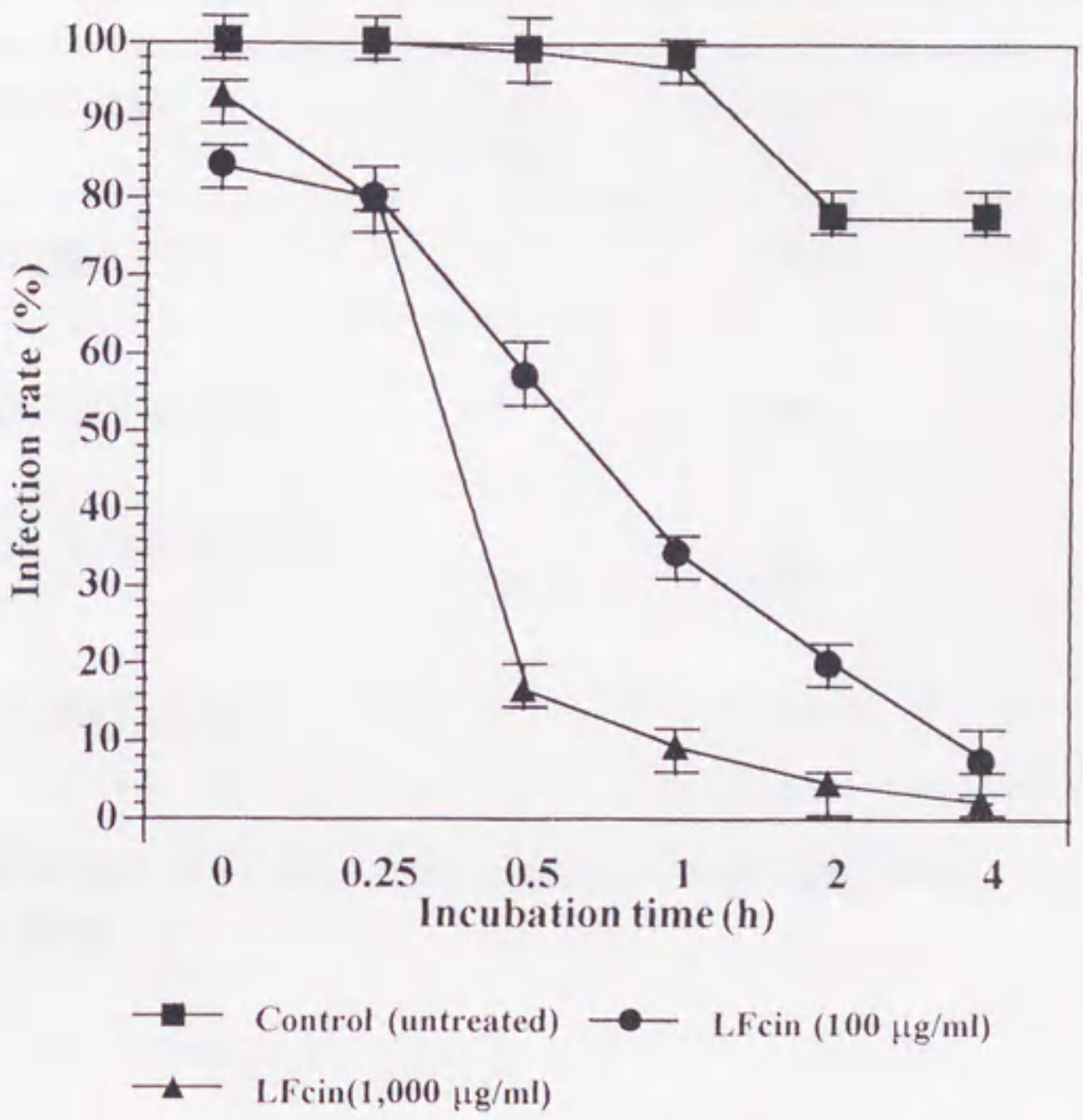


Fig. 18. Effect of LFcin on the infectivity of *T. gondii* in MEC . Parasites were incubated with LFcin at 37 ℃ for the time indicated and then were applied onto MEC monolayered cover slips for 18 h. The infection rate shown is the percentage of infected cells among a total of 500 cells. Mean±S.D. of triplicate samples.

Table 4. Infectivity of *T. gondii* in mice after treatment with LFcin

Treatment of parasites	No. of mice inoculated	Mortality(%)	Anti-*T. gondii* IgG in serum 1)
Untreated	5	100	ND
LFcin (100 μg/ml)	5	80	<4
LFcin (1,000 μg/ml)	5	20	<4
LF (1,000 μg/ml)	5	100	ND

1) Anti-*T.gondii* IgG titers in the serum of surviving mice.
ND: Not done.

parasites pretreated with bovine LF at 1,000 μg/ml 100 % mortality of inoculated mice within 9 days post-challenge. In contrast, four of five mice inoculated with the same dose of parasites pretreated with LFcin at 1,000 μg/ml survived for more than 30 days post-challenge. In the case of parasites pretreated with LFcin at 100 μg/ml, one of five mice survived up to 30 days post challenge. All mice inoculated with cysts either pretreated with LFcin or untreated, survived for more than 30 days. The mean number of cysts in the brains of the mice inoculated with untreated cysts was approximately 1,500, while that of the group inoculated with LFcin-treated cysts was only 300 per mouse.

4. DISCUSSION

In the present study, treatment of *T. gondii* with LFcin caused an irreversible loss of trypan blue dye exclusion activity and inactivation of the host cell-penetration capacity of the parasites. These observations are positive evidence of the parasiticidal effects of LFcin. Concentration of LFcin of 100 μg/ml or higher achieved marked efficacy against *T. gondii* and this is consistent with the effective dose against other microorganisms (6). LFcin contains asymmetric clusters of basic amino acid residues such as lysine and arginine. Similar cationic peptides, such as defensins, magainins and the antibiotic polymyxin, are known to show affinity for biological membranes and act to disrupt the cytoplasmic membrane of various microorganisms (21, 22). The biochemical similarity and/or dissimilarity of the membranes of *T. gondii* and microbial membranesis obscure. Nonetheless, it is conceivable that LFcin may have affinity for membranes

of *T. gondii* and act to disrupt their normal functional properties. Further studies aimed to evaluate the susceptibility of other parasitic protozoa to LFcin are under way.

Lactoferrin and C-lobe of LF had no effects inhibitory effect on the parasites, whereas LFcin displayed this activity. These results suggest that an anti-*T. gondii* sequence within LF, located near N-terminus is released from the folded protein molecule by certain proteolytic enzymes. It is plausible that proteolytic enzymes in the phagosomes of host cells cleave the LF molecules and generate potent parasiticidal peptides. In order to clarify the mechanisms of the parasiticidal effect of LFcin, further studies are necessary to investigate the kinetics of interaction between LFcin and the membrane of the parasite.

Chapter 6

Protective Effect of Bovine Lactoferricin in Mice Infected with *Toxoplasma gondii* Parasites

1. INTRODUCTION

Toxoplasma gondii (*T. gondii*), an obligatory intracellular parasitic protozoan of the apicomplexa, penetrates tissues of vertebrates as tachyzoites and forms cysts containing a number of bradyzoites to escape host defense systems.

Felidae, a definitive host of *T. gondii* shed environmentally resistant oocysts in their feces after primary infection. Ingestion of uncocked meat contaminated with cysts or sporulated oocysts is a common source of *T. gondii* infection. To prevent such transmission of toxoplasmosis, food additives which have anti-*Toxoplasma* activity should be developed.

It has recently been shown that lactoferricin (LFcin) had parasiticidal activity *in vitro* against *T. gondii* (chapter 5). The present study aimed to examine the effect of administered LFcin in mice acutely infected with this parasite. Since *T. gondii* is one of interferon-γ (IFN-γ) inducer and IFN-γ produced by both $CD4^+$ and $CD8^+$ cells plays an essential role in the protective response against *T. gondii* (24, 77), the author also investigated whether administered LFcin influences serum levels of IFN-γ.

2. MATERIALS AND METHODS

Animals

Female ICR mice at 8 weeks of age, weighing 23-28 g were used throughout the experiments. In selected *in vivo* experiments, female ICR nu/nu mice on age 8 weeks (Charles River Inc., Yokohama, Japan) were also used.

Preparation of lactoferricin

Lactoferricin was prepared from lactoferrin as described by Bellamy et al. (7), and dissolved in phosphate-buffered saline (PBS) a concentration of 10.0 mg/ml or 1.0 mg/ml just before use.

Preparation of cysts of T. gondii

Cysts of the Beverley strain of *T. gondii* were obtained by homogenization of cerebral tissue from infected mice 2-3 months post infection. The number of cysts in the homogenates of cerebral tissue was determined by counting microscopically. Briefly, in each instance, 10 μl of a homogenate made from one brain was dropped onto a slide glass and observed under an 18×18-mm coverslip. All samples were prepared in triplicate.

In vivo assay

In vivo experiments examining the effects of administered LFcin were performed by experimental *T. gondii* inoculation. Thirty mice were orally challenged with cysts at a dose of either LD_{50} or LD_{90} and divided into three groups. Thirty min before challenge, and once again on each of the first 7

days post infection, two groups of five mice were orally administered either 0.5 mg or 5.0 mg of LFcin in 0.5 ml of saline. Another two groups of five mice were administered intraperitoneally either 0.1 or 1.0 mg of LFcin in 0.5 ml of saline on each of the first 7 days post infection. As a negative control, five mice were orally administered saline. To examine a different route of the infection, five mice orally administered 5.0 mg of LFcin were challenged intraperitoneally with cysts at a dose of LD_{50}.

After challenge, the mortality and survival period of the mice were monitored up to 35 days postchallenge. On day 35, the survivors were sacrificed and the brain of each mouse was removed and homogenized gently in 3 ml of phosphate buffered saline (PBS) with a glass homogenizer in order to count the number of cysts.

To address the question whether immune effector cells, especially T cells are involved in the resistance in mice administered with LFcin, we examined the experimental *T. gondii* inoculation in nude mice administered orally with LFcin.

Interferon-γ assay

To examine the serum levels of interferon-γ in the mice, each mouse in the experiment described above was bled and approximately 100 μl/mouse was obtained from the supraorbital vein at various times post inoculation. The sera were stored at -80 ℃ until use. Assay of interferon-γ in the serum was performed by ELISA using an assay kit (mouse ELISA IFN-Endogen Inc., Cambridge, MA, USA).

Statistical analysis

Significance of differences were evaluated using χ^2 test. A value of $p<0.05$ was considered statistically significant.

3. RESULTS

Effect of oral administration with lactoferricin

As shown in Fig. 19, in the case of mice orally inoculated with *T. gondii* cysts, all of the five mice orally administered 5 mg of LFcin survived for at least 35 days post challenge, whereas, 60 % of the mice in the group orally administered 0.5 mg LFcin died of acute toxoplasmosis within 14 days post challenge (Fig. 19a). In the case of mice orally administered 5.0 mg of LFcin and challenged intraperitoneally with one LD_{50} dose of cysts, 20 % of the mice died of acute toxoplasmosis within 7 days post challenge. In the control group, 40 % of the mice also died within 7 to 18 days post challenge (Fig. 19c).

Effect of intraperitoneal administration with lactoferricin

Substantial numbers of tachyzoites were found in the peritoneal cavity of mice administered 1.0 mg of LFcin intraperitoneally, and 40 % of the mice in this group died of toxoplasmosis within 22 to 31 days post challenge. In contrast, all of the mice administered 0.1 mg of LFcin intraperitoneally survived until the end of the experiment (Fig. 19b). In the control group, 60 % of the mice administered saline died of acute toxoplasmosis within 14 days post challenge.

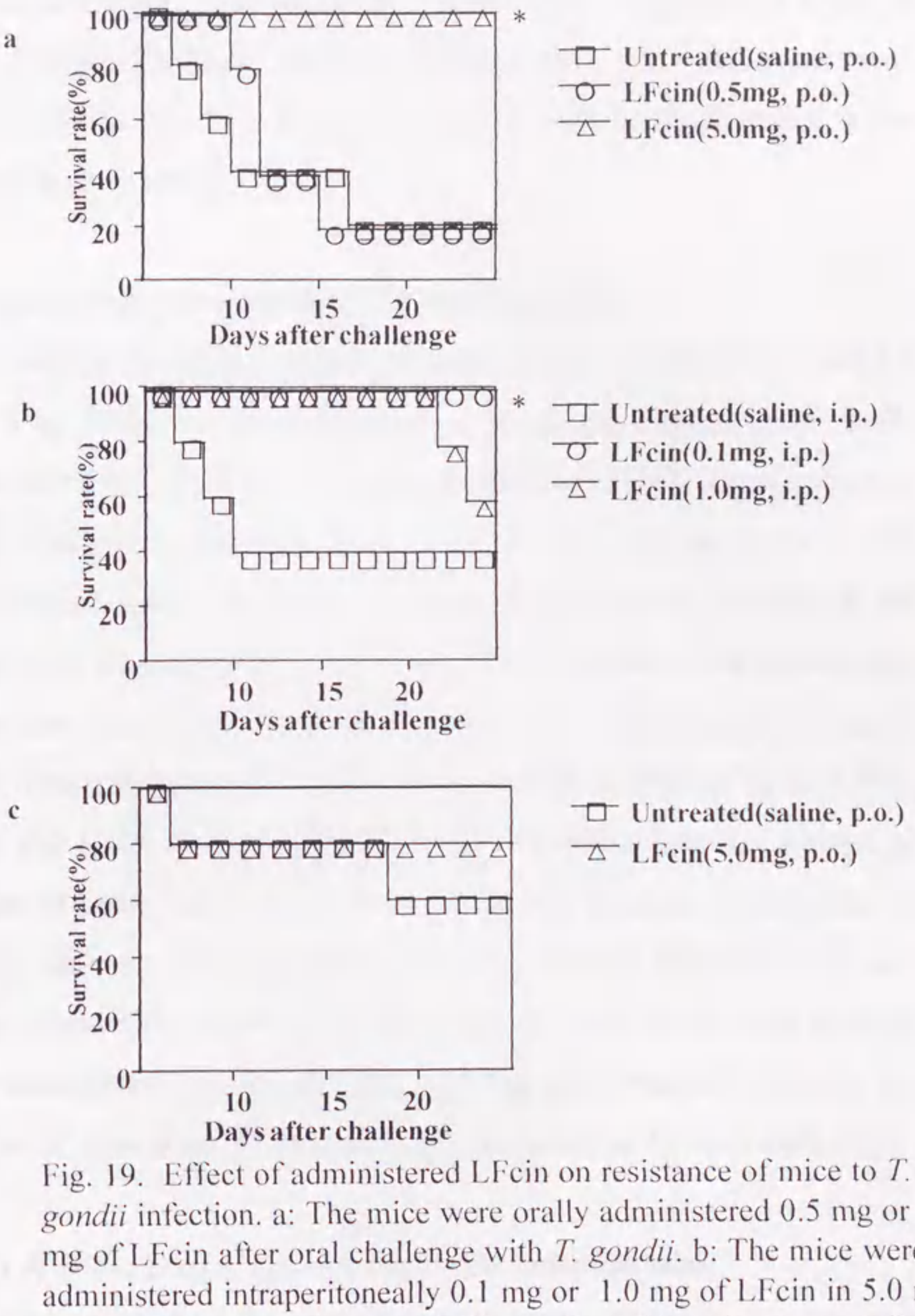


Fig. 19. Effect of administered LFcin on resistance of mice to *T. gondii* infection. a: The mice were orally administered 0.5 mg or 5.0 mg of LFcin after oral challenge with *T. gondii*. b: The mice were administered intraperitoneally 0.1 mg or 1.0 mg of LFcin in 5.0 ml of saline after oral challenge with *T. gondii*. c: The mice were orally administered 5.0 mg of LFcin in 5.0 ml of saline after intraperitoneal challenge *T. gondii*. *$p<0.05$, Untreted group vs. LF group (χ^2 test).

Effect of nu/nu mice by lactoferricin administration

In control group, all of nu/nu mice died of acute toxoplasmosis within 10 to 14 days post challenge. Similarly, all of the nu/nu mice administered with oral inoculation of 5.0 mg of LFcin also died of acute toxoplasmosis within 14 days post challenge (Fig. 20).

The number of cysts by lactoferricin administration

The number of cysts found in the brain of mice in each group tested is shown in Table 5. The number of cysts in the case of mice orally administered 5.0 mg of LFcin was significantly lower (233-100 cysts/mouse brain), compared with that of survivors in the control group (3,900 cysts/mouse brain). The number of cysts in the case of survivors in the group orally administered 0.5 mg of LFcin (600 cysts/mouse brain) was also lower than that of the control. In the case of mice administered 1.0 mg of LFcin intraperitoneally, individual differences in the number of cysts (4,000, 3,600 and 1,200 cysts/mouse brain) were significant and the average of number of cysts was similar to that of the control mice. The number of cysts in the case of mice administered 0.1 mg of LFcin intraperitoneally also varied substantially (1,000-6,300 cysts/mouse brain). In the case of mice orally administered 5.0 mg of LFcin following intraperitoneal challenge, the number of cysts found in the brain was similar to that of the control mice.

Effect of IFN-γ production by lactoferricin administration

IFN-γ was detected in the serum of all of the infected mice, whether untreated or treated with LFcin. The levels of IFN-γ in the mice treated with

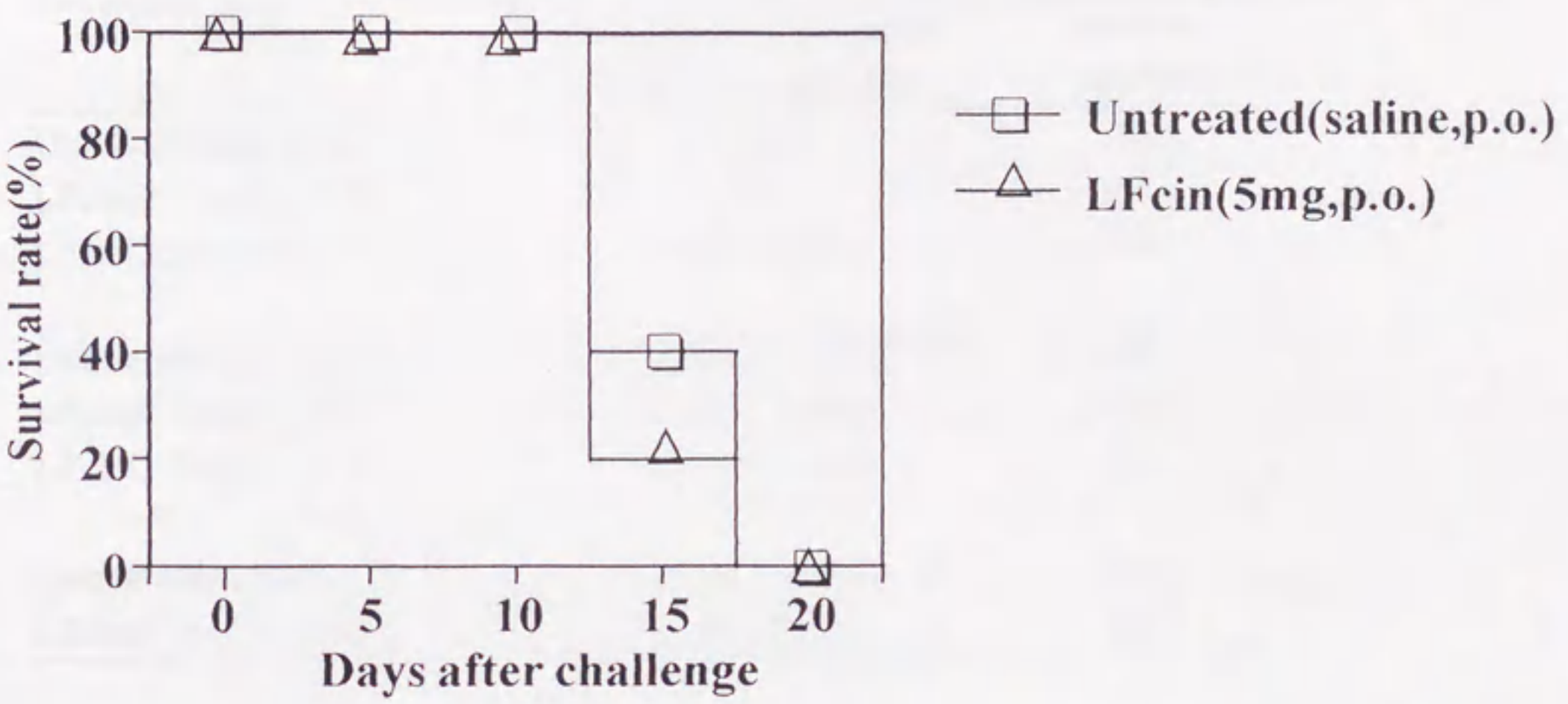


Fig. 20. Effect of administered LFcin on resistance of nu/nu mice to *T. gondii* infection. a: The mice were orally administered 5.0 mg of LFcin after oral challenge with *T. gondii*.

Table 5. The survival rate of mice and the numbers of *T. gondii* cysts in cerebral tissue of mice in LFcin-treated and untreated groups

Group	Average of total number of cysts [a)] in the brain	Survival rate[b)](%)
Untreated(saline,p.o.,n=1)	3,900	20
LFcin(0.5mg,p.o.,n=1)	600	20
LFcin(5.0mg,p.o.,n=5)	147±56[c)]	100
Untreated(saline,i.p.,n=2)	3,300, 3,400,(AVG=3350)	40
LFcin(0.1mg,i.p.,n=5)	2,767±2,498	100
LFcin(1.0mg,i.p.,n=3)	2,933±1,514	60
Untreated(saline,p.o.,n=3)	2,100±1,552	60
LFcin(5.0mg,p.o.,n=4)	1,825±618	80

a) Cerebral tissue per mouse.
b) Survival up to day 35 post inoculation/total mice tested.
c) Mean±S.D.
p.o.: Peroral.
i.p.: Intraperitoneal.
n: Number of mouse brains examined.

LFcin were lower than control groups (Fig. 21). No IFN-γ was detected in the serum of non-infected mice treated with LFcin (not detected).

4. DISCUSSION

As mentioned in chapter 5, LFcin has parasiticidal activity against *T. gondii* tachyzoites and bradyzoites in cysts *in vitro*. In the present study, orally administered LFcin was shown to exert a protective effect against *T. gondii* infection *in vivo*, and its protective effect was dose dependent. Based on these results, two possibilities should be considered. One possibility is that LFcin may interact directly with *T. gondii* cysts and bradyzoites in the intestinal tract, resulting in a decrease in infectivity of the parasites. Another possibility is that LFcin itself and/or structural components released from the parasite as a result of its interaction with LFcin may activate the host's defense systems. Regarding the former possibility, it has been reported that LFcin is a cationic peptide having a strong capacity to bind to anionic surface components on the surface of biological membranes (7, 15, 48). The cell surface of *T. gondii* tachyzoites is known to have a strong negative charge and binds cationic substances (15, 16). It has been reported that liposomes consisting of stearylamine, a positively charged lipid have parasiticidal activity against *T. gondii* (78). Thus, it is plausible that LFcin has the capacity to bind to the surface of the parasite in the intestinal tract, and this interaction may lead to a disruption of biological function of the parasites' membrane, resulting in loss of infectivity.

In addition to the oral route of challenge, the author examined the resistance of mice to intraperitoneal infection with *T. gondii* cysts at dose of

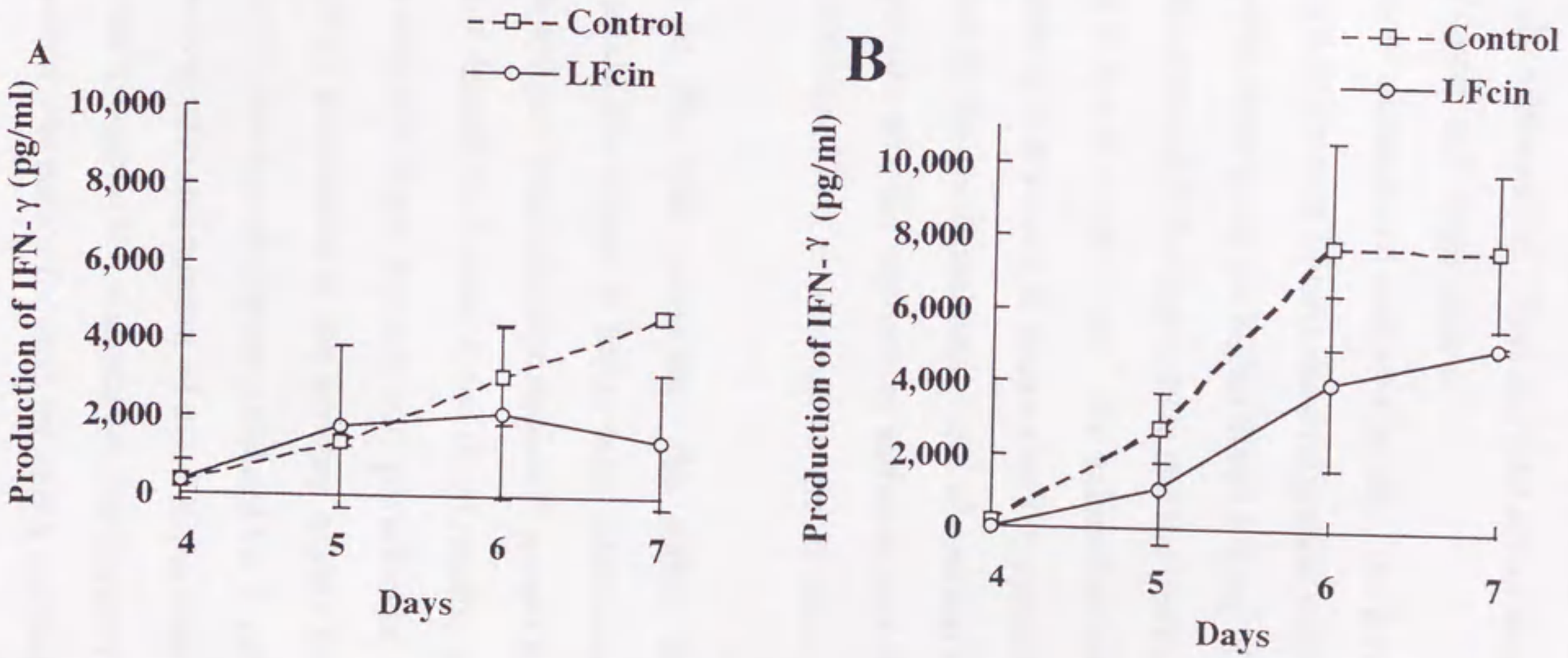


Fig. 21. Production of IFN-γ in serum of mice (n=3). A:The mice were orally administered 5.0 mg of LFcin for 7 days post infection. B: The mice were intraperitoneally administered 1.0 mg LFcin for 7 days post infection.

LD_{50}. The results showed that the resistance of mice orally administered 5.0 mg of LFcin was incomplete, compared with the case of oral challenge. This suggests that orally administered LFcin has little effect on parasites present in the peritoneal cavity and tissue organs.

When LFcin was administered intraperitoneally , the group of mice given the lower dose (i.e. 0.1 mg of LFcin) showed greater resistance against *T. gondii* infection than those given the higher dose (1.0 mg of LFcin). A lower survival ratio was observed at the higher dose and the number of cysts in the brain was similar to that of control mice. The author has insufficient data to explain these results in this report. It seems that the optimal dose of LFcin against the parasite in the peritoneal cavity and abdominal organs is below 1.0 mg/mouse (perhaps near 0.1 mg/mouse) and an excess of LFcin appears to result in suppressive effects or damage to the defense systems of the mouse.

With Regard to the latter possibility, the author investigated the experimental *T. gondii* inoculation in nu/nu mice administered orally with LFcin. The results indicate that resistance against *T. gondii* infection in mice induced by LFcin is depend on T cells. From these results, it is conceivable that LFcin in the intestinal ducts has not only parasiticidal activity which inhibits the parasites's penetration of the intestine organs of the mice, but also induces protective immune responses mediated via T cells. The results concerning the survival ratio and number of cysts in the brain in the case of the treated group tend to support this speculation. Furthermore, to clarify the role of T cells' function, the author focused the IFN-γ production activity in mice. Because the protective immune response against *T. gondii* infection

which mediated by T cells is generally associated with IFN-γ, it was anticipated that the group of mice given LFcin and showed resistance against the infection would produce higher level of IFN-γ. To the author's surprise, lower levels of IFN-γ were detected in the serum of mice orally treated with LFcin as compared with the levels in untreated mice. It is conceivable that the parasiticidal activity of LFcin in the intestinal tract might be highly effective and, consequently, the number of parasites which penetrate the intestinal organs of the mice may be significant fewer than that in the case of control mice, and those few parasites would stimulate only a slight protective immune response resulting in lower serum levels of IFN-γ.

Oral administration of LFcin had no effect on serum levels of IFN-γ in non-infected mice.The author has no data in this report to suggest that LFcin has the capacity to regulate IFN-γ production. It is also obscure whether LFcin can pass through the intestinal mucosa into tissues and serum. Further study is required to elucidate the biological activity of LFcin, especially its regulatory role in the immune system.

GENERAL DISCUSSION

Lactoferrin has many kind of biological functions, which are associated with host defense mechanisms (bacteriostatic effect, direct killing of microorganism, regulation of immune system etc.).

First, the author focused his interest to determine the possibility of anti-*Toxoplasma* of activity of lactoferrin in murine somatic cells. Experiments described in chapter 1 showed that lactoferrin inhibited the intra-cellular *Toxoplasma gondii* (*T. gondii*) multiplication in murine somatic cells. Interestingly, lactoferrin itself had no parasiticidal activity and the action of lactoferrin to macrophage seemed to be different from that of murine embryonal cells. The induction of such anti-*Toxoplasma* activity induced by lactoferrin is Fe independent.

In regard with killing mechanisms against *T. gondii*, macrophages posses oxygen-dependent and oxygen-independent anti-protozoan mechanism (14, 35, 45, 68, 69). To clarify the effector pathway of *Toxoplasma* growth inhibitory activity induced by lactoferrin in macrophages, the author examined the production of free radical oxygen products (O_2^-), and nitrogen oxide (NO) in murine macrophages stimulated by lactoferrin (chapter 2). Production of O_2^- was not detected in cultures of macrophages supplemented with lactoferrin. Likewise, production of NO was not enhanced in cultures of macrophages supplemented with lactoferrin. From these, it is suggested that *Toxoplasma* growth inhibitory activity induced by lactoferrin in macrophages is not mediated by oxygen-dependent cascade systems. A recent study has documented that lactoferrin taken up by

leukemia cells was bound to DNA and the binding event lead to transcriptional activation (33). The possibility that should be considered is that lactoferrin may be taken up by macrophages and it acts DNA, result in to blocking *T. gondii* multiplication. To confirm mechanism of the growth inhibitory the parasites activity induced by lactoferrin, the author examined whether lactoferrin promoted phosphorylation of tyrosine residues of proteins in macrophages (chapter 3). Macrophages treated with lactoferrin have induced phosphorylation of tyrosine. Supplement of genisteine, one of tyrosine phosphorylation inhibitor in the macrophages, reduced them to no-inhibitory activity. From these results, it is plausible that tyrosine phosphorylation in macrophages induced by lactoferrin would be associated with the growth inhibitory activity to *T. gondii*.

The purpose of the experiments in chapter 4 is to examine whether the action of lactoferrin *in vitro* reflect to relevance of host defense systems *in vivo*. The results indicate that both oral and intraperitoneal administration of lactoferrin in mice prevents hosts from *T. gondii* infection. Whereas, IFN-γ level in the serum of mice administered with lactoferrin was similar to that of control. These findings suggest that the resistance induced by lactoferrin would be associated with not only the production of IFN-γ, but also action of immune effector cells.

Recently, an antimicrobial peptides was isolated from pepsin digestion of N-lobe of lactoferrin. This peptide was named lactoferricin (6). As shown in chapter 5, lactoferricin showed parasiticidal effects to *T. gondii*. However, native lactoferrin and C-lobe of lactoferrin had no effect on the parasites. These results suggest that the anti-*T. gondii* activity, located in N-terminals

(N-lobe) and is released from the native protein.

Moreover, the author examined the effect of administration of lactoferricin in mice acutely infected with *T. gondii*. The data demonstrated that oral administration of lactoferricin induced resistance to *T. gondii* infection in mice (chapter 6).

From the data in chapters 4 and 6, two possibilities would be speculated: One is that lactoferrin may activate immune effetor cells. Another is that lactoferrin might be cleaved by proteolytic enzymes in the stomach, result in lactoferricin which kill *T. gondii*. It is demonstrated that *T. gondii* has super-antigenic properties (19). As a result, the reactive cells proliferate, secrete high levels of inflammatory cytokines (IFN-γ, TNF-α etc.), and ultimately die or become anergic to further stimulation. Because lactoferrin is an inflammatory protein which inhibits TNF-α and IFN-γ (18). Therefore, lactoferrin might block cytokine overproduction in mice infected with *T. gondii*, and then the survival rate of mice treated with lactoferrin is higher than that of mice untreated. On the other hand, the survival rate of nu/nu mice administered with lactoferrin or lactoferricin were similar to that of control group. From these results, the important protective factor of lactoferrin or lactoferricin to *T. gondii* infection might be T cells.

In the future, lactoferrin is expected to be biological response modifiers (BRM) with no side effect substance that is different from antibiotic to toxoplasmosis. However, the author has to investigate about function of lactoferrin to explain his findings.

CONCLUSION

1. The aims of the experiments reported in this thesis is summarized to effects of bovine lactoferrin to growth of *Toxoplasma gondii* inside cell and mice infected with parasites.

2. The effect of bovine lactoferrin on the intracellular growth *Toxoplasma gondii* parasites were examined in murine macrophage and embryonal cells. The host cells infected with the parasites were incubated with the supplement of either lactoferrin, apo-lactoferrin, holo-lactoferrin or transferrin in the culture media for varying periods. The growth activity of intracellular parasites was determined by the measurement of selective incorporation of ^{3}H-uracil to parasites. Supplement of lactoferrin had no effect on the penetration activity of the parasites, while development of intracellular parasites was inhibited linearly in concentration of lactoferrin. Supplement of apo-lactoferrin and holo-lactoferrin, but not transferrin showed similar effects. These suggest that lactoferrin induces the inhibitory effects on the development of intracellular parasites. Pretreatment of lactoferrin to the macrophages, however, did not show any inhibitory effects. Whereas, mouse embryonal cells preincubated with lactoferrin suppressed the intracellular growth. Thus, the action of lactoferrin to macrophages would be different from that of mouse embryonal cells (chapter 1).

3. To study the effector pathway of *Toxoplasma* growth inhibitory activity induced by lactoferrin in murine macrophage, the role of reactive oxygen intermediates (O_2^-) and inorganic nitric oxide (NO) was examined. Production of O_2^- was diminished in cultures of macrophages supplemented with lactoferrin and the effect of lactoferrin was dose and time dependent. Production of NO was enhanced in cultures of macrophages supplemented with interferon-γ, but not with lactoferrin. These findings suggest that the *Toxoplasma* growth inhibitory activity induced by lactoferrin in macrophages is not mediated by O_2^- or NO molecules. A competitive inhibitor of the L-arginine-dependent effector pathway, N^G-monomethyl-L-arginine (N^GMMA), virtually abolished the inhibitory effects induced by interferon-γ. Similarly, the inhibitory activity induced by lactoferrin was also diminished in cultures supplemented with N^GMMA. From these findings, it appears that *Toxoplasma* growth-inhibitory activity induced by lactoferrin in macrophages may be mediated by an L-arginine-dependent effector pathway that does not involve NO production (chapter 2).

4. The mechanism of the growth inhibitory effects induced by lactoferrin is not mediated oxygen-dependent anti-protozoa. To clarify the mechanism of anti-*Toxoplasma gondii* activity induced by lactoferrin, this study was examined whether lactoferrin promoted the phosphorylation of tyrosine in macrophages. By immnoblotting, phosphorylation of tyrosine induced by lactoferrin was detected in approximately 30 kDa protein(s). The

number of tachyzoites in macrophages supplemented with lactoferrin and genistein was shown higher than that treated with lactoferrin. Moreover, phosphorylation of 30 kDa protein(s) in macrophages stimulated with lactoferrin were disappeared in the addition of 1.0 μg/ml genistein. Genistein is a tyrosine-phosphorylation inhibitor which blocks ATP-dependent phosphorylation pathway. It is plausible that the tyrosine-phosphorylation in macrophages induced by lactoferrin would be associated with the growth inhibitory activity to *Toxoplasma gondii* (chapter 3).

5. Resistance activity of lactoferrin to *Toxoplasma gondii* infection was examined by *in vivo* treatment in experimental murine toxoplasmosis. All of mice orally administered with 1.0 mg of lactoferrin survived at least 35 days post challenge of *Toxoplasma gondii* cysts (Beverley strain). Eighty % of mice intraperitoneally administered with 1.0 mg of lactoferrin also survived for long periods post challenge. In contrast, 80 % of untreated mice died of acute toxoplasmosis within 14 days post challenge. The number of cysts in the brain of mice administered with lactoferrin was significantly lower than that found in untreated mice. Athymic nude mice, intraperitoneally administered with lactoferrin had no resistance to the infection. All of mice produced interferon-γ in the serum of survivors administered with lactoferrin was similar to that of untreated mice. Whereas, the mice administered with lactoferrin alone had no production of interferon-γ. These results indicate that administration of lactoferrin induces resistance against the infection in mice and lactoferrin

itself had no activity as interferon-γ inducer, and T cell may have important role to protect *Toxoplasma gondii* infection (chapter 4).

6. Lactoferricin is an active peptide produced by pepsin digestion of lactoferrin which displays bactericidal activity against both of Gram-negative and Gram-positive bacteria and other microorganisms. In the present study, the effect of lactoferricin on the infectivity of *Toxoplasma gondii* parasites was examined both *in vitro* and *in vivo*. Parasites incubated with lactoferricin had lost trypanblue dye exclusion activity and the ability to invade mouse embryonal cells. When mice were inoculated with a highly virulent strain of the parasite pretreated with lactoferricin, they survived for more than 30 days without anti-*Toxoplasma gondii* antibody production. The number of cysts in the brains of mice inoculated with cysts pretreated with lactoferricin was significantly lower than that of mice inoculated with untreated cysts. These data indicate that lactoferricin exerts a parasiticidal effect against *Toxoplasma gondii* (chapter 5).

7. The protective effect of lactoferricin against *Toxoplasma gondii* infection was examined in this study of experimental murine toxoplasmosis. When mice were orally administered 5.0 mg of lactoferricin, and challenged with cysts of *Toxoplasma gondii* (Beverley strain) at a dose of LD_{90}, 100 % of the treated mice survived at least 35 days post challenge. Intraperitoneal administration of 0.1 mg of lactoferricin also prevented death in 100 % of treated mice orally challenged with *Toxoplasma gondii*

cysts . In contrast, 80 % of untreated mice died of acute toxoplasmosis within 14 days post challenge. In the mice treated perorally with lactoferricin, the number of cysts in the brain was significantly lower than that found in untreated mice. Athymic nude mice, administered with lactoferricin had no resistance to the infection. Levels of interferon-γ in the serum of infected mice treated perorally with lactoferricin were lower than those in the infected mice without treatment. These results demonstrate that oral administration of lactoferricin induces resistance to *Toxoplasma gondii* infection in mice, and T cells may have important role for the induction of resistance (chapter 6).

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Our ref. Vetpar 1154 (revised) Your ref. 7th June 1996

Dear Dr. Tanaka

I am pleased to inform you that the revised version of your paper entitled ' Growth inhibitory effect of bovine lactoferrin on *Toxoplasma* gondii tachyzoites in murine macrophages: role of radical oxygen and inorganic nitrogen oxide in *Toxoplasma* growth-inhibitory activity ' is acceptable for publication in Veterinary Parasitology.

Thank you for your prompt revision.

Yours sincerely,

S. M. Taylor

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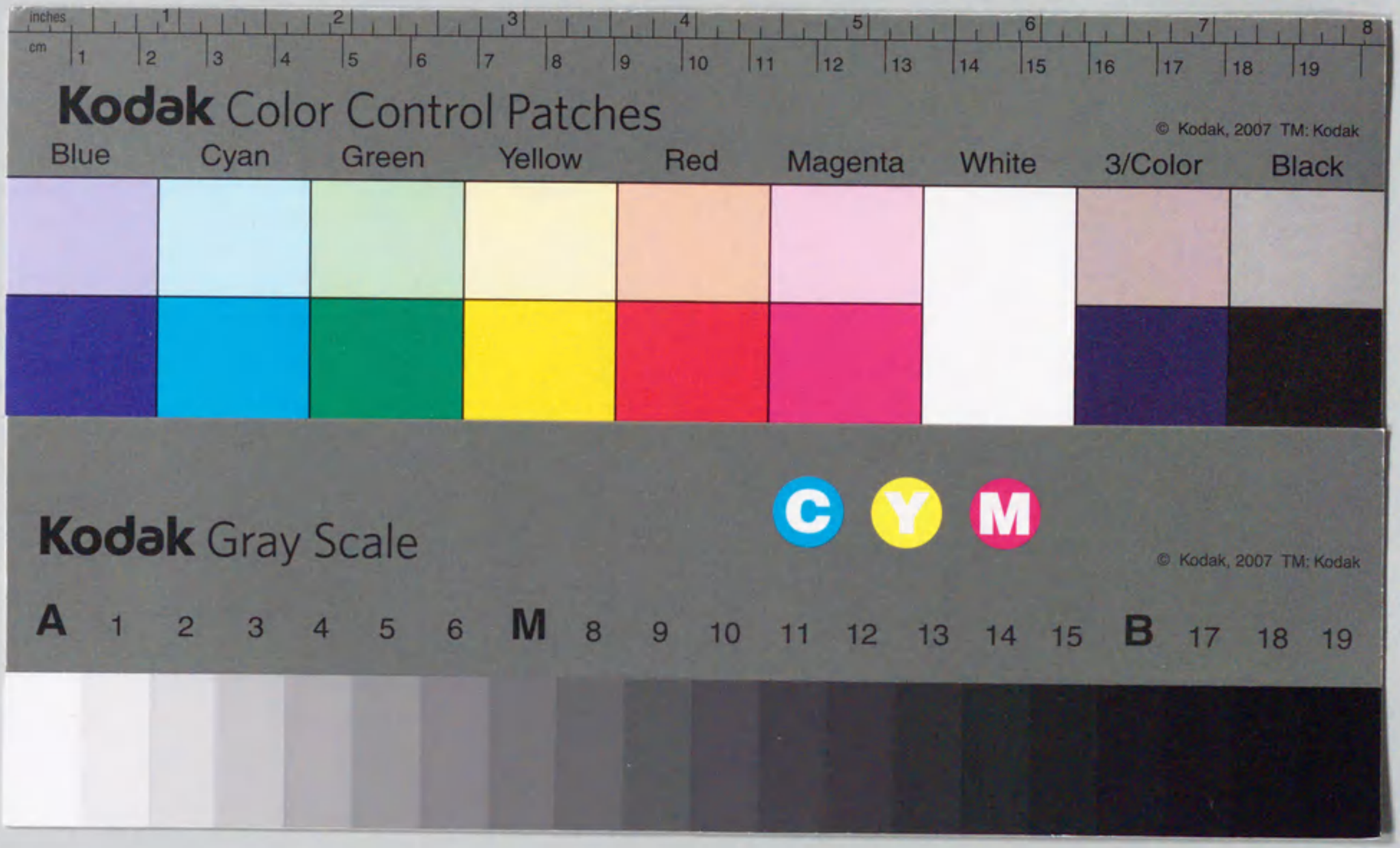
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