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Effects of cytokines in the activation of peritoneal macrophages from mice infected with *Toxoplasma gondii*

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Abstract: The present study was undertaken to assess the role of cytokines in the activation of peritoneal macrophages from *Toxoplasma*-infected mice. Peritoneal macrophages from *Toxoplasma*-infected mice (10 cysts of Beverley strain/mouse) were harvested 8 weeks after infection, and incubated with the mitogen-induced lymphokine, recombinant mouse interferon-γ (IFN-γ), recombinant mouse tumor necrosis factor-α (TNF-α) alone or in combination with IFN-γ (IFN-γ/TNF-α) for 24hr at 37°C, 5% CO_2. Macrophage activation was measured by the amount of H_2O_2 and NO_2^- production, and anti-*Toxoplasma* activities of macrophages. IFN-γ or IFN-γ/TNF-α-treated macrophages from *Toxoplasma*-infected mice revealed significantly higher H_2O_2 production than resident macrophages from *Toxoplasma*-infected mice. The production of NO_2^- by TNF-α-, IFN-γ- or IFN-γ/TNF-α-treated macrophages from *Toxoplasma*-infected mice were significantly higher than that by resident macrophages, whereas lymphokine-treated group produced similar amount as that produced by resident macrophages. Anti-*Toxoplasma* activities of cytokine-treated macrophages from *Toxoplasma*-infected mice were significantly higher than those of resident macrophages. IFN-γ-treated macrophages were significantly increased production of H_2O_2 and NO_2^-, and anti-*Toxoplasma* activities of macrophages between normal and *Toxoplasma*-infected mice, whereas the other cytokine-treated groups were not significant differences between them. These data suggested that IFN-γ was the only one of cytokines capable of significantly activating the peritoneal macrophages from *Toxoplasma*-infected mice.

Key words: Cytokine, macrophage activation, H_2O_2, NO_2^-, mouse, *Toxoplasma gondii* (Beverley strain).

INTRODUCTION

T. gondii is an obligate intracellular protozoa that causes significant morbidity and mortality in both humans and animals. In immunocompromised hosts, severe infection is more commonly recognized, most often manifesting itself as a necrotizing encephalitis (McCabe and Remington, 1988). The most striking predilection for the development of significant *Toxoplasma* infection has been in patients with acquired immune deficiency syndrome (Centers for Disease Control, 1986).

Macrophages play a key role in the control of protozoan parasites as they are involved in innate resistance phenomena and effector mechanisms of specific host response but also they favor parasite multiplication (Nathan, 1983). Activated macrophages differ from resident macrophages in many respects, including their morphology, lysozomal enzyme

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content, expression of cell-surface receptors, enhanced production of reactive oxygen intermediate (ROI), reactive nitrogen intermediate (RNI), release of cytokines, tumoricidal activity, and microbicidal activity against intracellular pathogens (Adams and Hamilton, 1984; Nibbering *et al.*, 1991). The features most widely used as criteria for macrophage activation are the ability to inhibit intracellular proliferation of protozoa, the increased production of reactive oxygen intermediate, and the enhanced expression of Ia antigen (Saito *et al.*, 1987; Ding *et al.*, 1988; Nibbering *et al.*, 1991). Recently, it has been demonstrated that activated murine macrophages show enhanced production of reactive nitrogen intermediate, as measured by the proudcton of NO_2^- (Hibbs *et al.*, 1987; Green *et al.*, 1990; Sibley *et al.*, 1991; Langermans *et al.*, 1992).

However, it is not known whether the effects of cytokines in the activation of peritoneal macrophages from *Toxoplasma*-infected mice. The aim of the present study was undertaken to assess the role of cytokines in the activation of peritoneal macrophages from *Toxoplasma*-infected mice, and to assess the relation among production of H_2O_2 and NO_2^-, and anti-*Toxoplasma* activities of macrophages.

MATERIALS AND METHODS

1. Animals

Male BALB/c mice (8 to 10 weeks of age) obtained from Korea Research Institute of Chemical Technology (Taejon, Korea) were used. Each experimental group had three mice.

2. Parasites

The virulent RH strain and the avirulent Beverley strain of *T. gondii* were used. Tachyzoites of RH strain were used to infect the cultivated macrophages, whereas cysts of Beverley strain (10 cysts per mouse) were used to produce *Toxoplasma*-infected mice.

3. Cytokines

Recombinant mouse interferon-γ (IFN-γ) and recombinant mouse tumor necrosis factor-α (TNF-α) were supplied by Genzyme (U.S.A.), and lymphokine was prepared by following methods. Lymphocytes were isolated from BALB/c mice and suspended at a concentration of 1×10^6 cells/ml in RPMI medium containing 10% fetal bovine serum with antibiotics (100 units/ml of penicillin G, 100 μg/ml of streptomycin) and concanavalin A (5μg/ml). They were incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 48hr, the supernatant was centrifuged, and was sterilized by filtration (0.2 μm; Gelman Science). Lymphokine was used at final concentration of 1/100×, 1/10×, and 1 ×.

4. Macrophage culture

Mouse peritoneal macrophages were harvested by method of Makioka and Kobayashi (1986). Peritoneal macrophages from normal and *T. gondii*-infected mice (mice infected with Beverley strain 8 weeks before) were harvested by repeated lavages of the peritoneal cavity *in situ* until 8 to 10 ml of lavage fluid was collected from each mouse. Lavage fluid was used calcium- and magnesium-free Hanks' balanced salt solutions (HBSS). After centrifugation at 350 ×g for 10 min at 4°C, the cell pellet was resuspended in complete media; RPMI 1640 containing 10% heat-inactvated fetal bovine serum and antibiotics. When erythrocytes were visible, the cell pellet was treated with distilled water for 30 second. Peritoneal cells were incubated in 16-well chamber slide (Nunc, Denmark) for 2 hr at 37°C in 5% CO_2. Nonadherent cells were removed by warm HBSS and freshly prepared complete media were added, and then cultured for 3 days. After culture for 3 days, resident macrophages were obtained by plating the cell in 16-well chamber slide at 5×10^5 cells per well and cultured in complete medium with or without cytokines for 24 hr.

5. Administration of cytokines

Resident macrophages from normal and *Toxoplasma*-infected mice were incubated in medium alone. For *in vitro* studies of the dose response to cytokines, peritoneal macrophages obtained from normal and *Toxoplasma*-infected mice were incubated with various concentrations of lymphokine (1/100, 1/10 and 1×), TNF-α (20, 200 and 2,000 units/ml),

IFN-γ (10, 100 and 1,000 units/ml) and IFN-γ in combination with TNF-α (IFN-γ/TNF-α; 10/20, 100/200 and 1,000/2,000) for 24 hr at 37°C, respectively.

6. Measurement of hydrogen peroxide (H_2O_2)

H_2O_2 in the culture supernatant was assayed by a colorimetric method (Nibbering *et al.*, 1991). At the end of the culture each well was added 1 ml of phenol red solution [0.05M potassium phosphate buffer (pH 7.0), 0.56mM phenol red, 20U/ml of horseradish peroxidase] and 10 ng/ml of phorbol myristate acetate. The plate was returned to the incubator for an additional hour. The reaction was stopped by addition of 10 μl 1N NaOH. The cell free supernants were collected and read in a photometer at 600 nm. The results were referred to a standard curve and expressed as μmoles of H_2O_2 per 5×10^5 cells per 1 hr.

7. Measurement of nitrite (NO2-) production

Nitrite concentration in cell culture supernatant was assayed by a standard Griess reaction (Vincendeau and Daulouede, 1991). The Griess reagent was prepared by mixing equal volumes of sulfanilamide (0.15% in 1N HCl) and N-(1-naphtyl)ethylenediamine dihydrochloride (0.15% in H_2O_2). A volume of 600 μl of reagent was mixed with 100 μl of sample and incubated 30 min in the dark. Absorbance of the chromophore formed was measured at 540 nm and expressed as μmoles of NO_2^- per 5×10^5 cells per 1 hr. $NaNO_2$ was used as a standard.

8. Measurement of anti-*Toxoplasma* activities of macrophages

Anti-*Toxoplasma* activities of peritoneal macrophages was assayed by method as described Makioka and Kobayashi (1986). The virulent RH strain of *T. gondii* was maintained in mice. After 3~4 days, *T. gondii* were collected and a suspension of 5×10^6 tachyzoites/ml in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicllin G and 100 μg/ml streptomycin was prepared. Each 100 μl of a *Toxoplasma* suspension was plated on 16-well chamber slide. After culture for 2 hr at 37°C in 5% CO_2, free *T. gondii* were removed by three washes with warm HBSS. The cells on chamber slide were fixed in methanol and stained with Giemsa solution for microscopical determination of the percentage of macrophages infected with *T. gondii* per 100 macrophages. Anti-*Toxoplasma* activities of peritoneal macrophages are expressed as follows,

anti-*Toxoplasma* activities (%)=

$$= \left(1 - \frac{\text{No. of macrophages infected with } T.\ gondii}{100 \text{ macrophages.}}\right) \times 100$$

9. Statistical analysis

The results were expressed as the mean±standard deviations of 3 experiments. The significance of differences between the values for various types of cytokines was assessed by the ANOVA (analysis of variance) test. The results of macrophage activation were compared by multiple regression analysis. The level of significance was set at $p<0.05$.

RESULTS

1. Hydrogen peroxide (H_2O_2) production by macrophages

In normal resident macrophages, amount of H_2O_2 production was 41.5±6.7 $\mu M/5\times10^5$ cells/hr. The production of H_2O_2 by IFN-γ/TNF-α-treated macrophages from normal mice were significantly enhanced amount as compared with the normal resident macrophages. However TNF-α- and IFN-γ-treated macrophages from normal mice released similar amount of H_2O_2 relative to normal resident macrophages, except 1,000 U/ml IFN-γ-treated group. In resident (medium-treated) macrophages from *Toxoplasma*-infected mice, amount of H_2O_2 production was 50.5±7.2 $\mu M/5\times10^5$ cells/hr. H_2O_2 productions of IFN-γ- or IFN-γ/TNF-α-treated macrophages from *Toxoplasma*-infected mice were significantly higher than that by resident macrophages from *Toxoplasma*-infected mice, whereas lymphokine- and TNF-α-treated macrophages produced similar amount to medium-treated macrophages. H_2O_2

productions were not revealed in a dose-dependent fashion in *Toxoplasma*-infected mice. H_2O_2 productions obtained with medium- and IFN-γ-treated macrophages (except 1,000 U/ml IFN-γ-treated macrophage) from *Toxoplasma*-infected mice were significantly different from those obtained from normal mice, however lymphokine- and IFN-γ/TNF-α-treated groups were not significant differences between normal and *Toxoplasma*-infected mice (Fig. 1).

2. Nitrite (NO_2^-) production by macrophages

In normal resident macrophages, amount of NO_2^- production was 17.2±2.1 $\mu M/5\times10^5$ cells/hr. The production of NO_2^- by TNF-α-, IFN-γ- or IFN-γ/TNF-α-treated macrophages from normal mice were significantly enhanced, however lymphokine-treated group was similar amount of NO_2^- relative to normal resident macrophages. In resident macrophages from *Toxoplasma*-infected mice, amount of NO_2^- production was 16.0±3.1 $\mu M/5\times10^5$ cells/hr. NO_2^- productions of cytokine-treated macrophages from *Toxoplasma*-infected mice were significantly higher than that by resident macrophages from *Toxoplasma*-infected mice, except lymphokine-treated group. IFN-γ-treated macrophages from *Toxoplasma*-infected mice revealed significantly higher NO_2^- production than IFN-γ-treated macrophages from normal mice, however the other cytokine-treated groups were not significant differences between normal and *Toxoplasma*-infected mice (Fig. 2).

3. Anti-*Toxoplasma* activities of macrophages

Anti-*Toxoplasma* activities of normal resident macrophages was 43±4%/5×10^5 cells/hr. Anti-*Toxoplasma* activities of cytokine-treated macrophages from normal mice were significantly enhanced as compared with the normal resident macrophages, except TNF-α-treated group. In *Toxoplasma*-infected mice, anti-*Toxoplasma* activity of resident macrophages was 48±5%/5×10^5 cells/hr, and cytokine-treated macrophages from

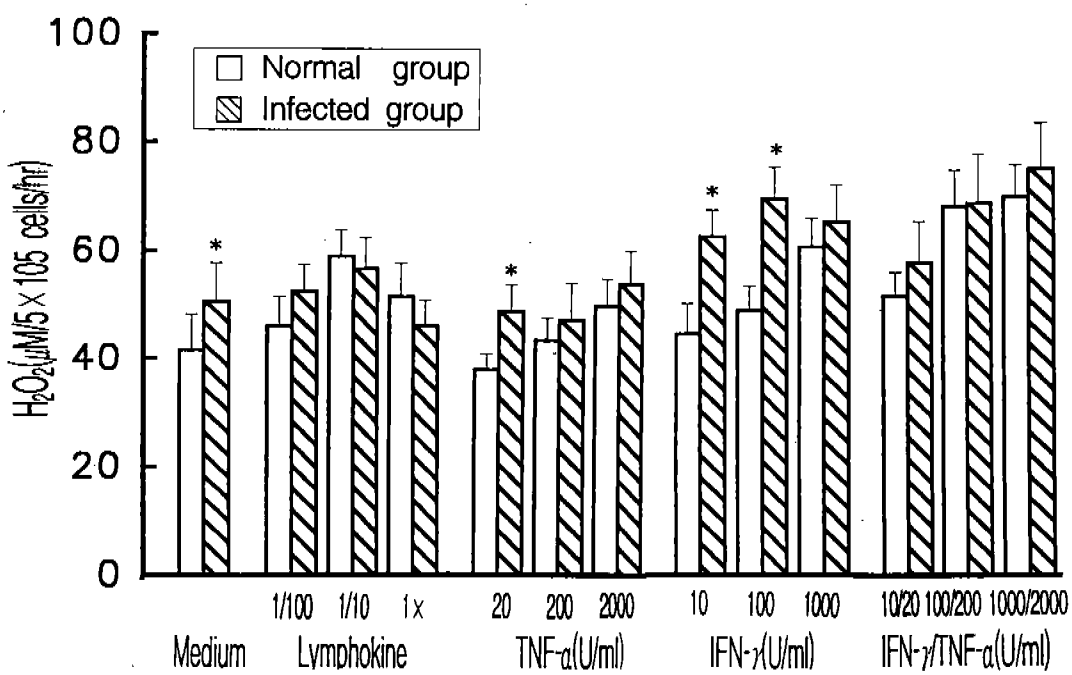

Fig. 1. Production of hydrogen peroxide by cytokine-treated macrophages from normal and *Toxoplasma*-infected mice. The peritoneal macrophages were incubated 24 hours after treatment of cytokine. All results are mean ± standard error of 3 experiments. *Indicates values significantly different ($p<0.05$) from those for resident macrophages.

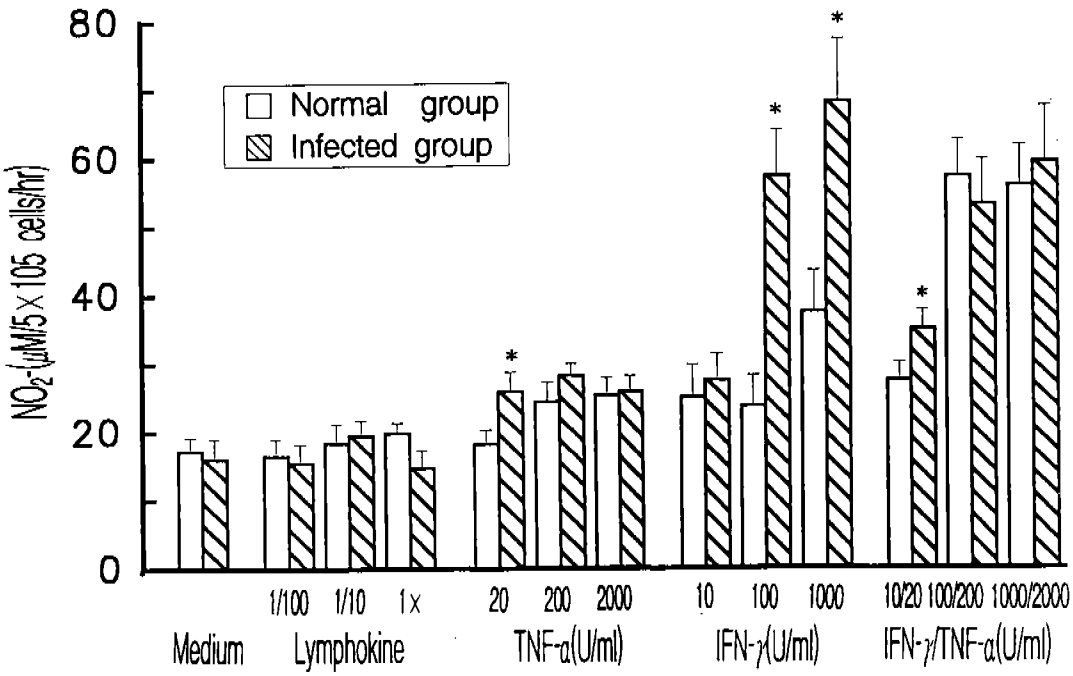

Fig. 2. Production of nitrite by cytokine-treated macrophages from normal and *Toxoplasma*-infected mice.

Toxoplasma-infected mice were significantly higher anti-*Toxoplasma* activities than that by resident macrophages from *Toxoplasma*-infected mice. Anti-*Toxoplasma* activities obtained with IFN-γ- and TNF-α-treated macrophages from *Toxoplasma*-infected mice were significantly different from those obtained from normal mice, however the other cytokine-treated groups were not significant differences between normal and *Toxoplasma*-infected mice (Fig. 3).

4. Correlations among the production of H_2O_2 and NO_2^-, and anti-*Toxoplasma* activities of macrophages

Amounts of H_2O_2 and NO_2^- production, and anti-*Toxoplasma* activities of macrophages showed close correlations among them in normal mice. Good correlations were found between H_2O_2 and NO_2^- production by peritoneal macrophages from *Toxoplasma*-infected mice ($r=0.84162$, $p<0.0003$). Statistical analysis revealed no correlation between NO_2^- production and anti-*Toxoplasma* activities of macrophages ($r=0.53744$, $p<0.0582$), whereas the H_2O_2 production correlated significantly with anti-*Toxoplasma* activities ($r = 0.69417$, $p < 0.0085$) (Table 1).

DISCUSSION

Activation of macrophages can be induced by various cytokines, such as IFN-γ, TNF-α, interleukin-2 (IL-2), IL-4, granulocyte-colony stimulating factor, but also by various bacterial products, such as lipopolysaccharide (LPS), BCG, *Corynebacterium parvum* (Adams and Hamilton, 1984; Stuehr and Maretta, 1987). The aim of this study was to assess the effects of cytokines in the activation of peritoneal macrophages from *Toxoplasma*-infected mice according to concentrations and kinds of cytokines. H_2O_2 production by TNF-α-treated macrophages from normal mice and NO_2^- production by IFN-γ-treated macrophages from *Toxoplasma*-infected mice were dose-dependent in the enhancement of their synthesis, however the other cytokine-treated groups were not significant differences according to the concentration of cytokines.

Peritoneal macrophages from mice acutely and chronically infected with *T. gondii* have a

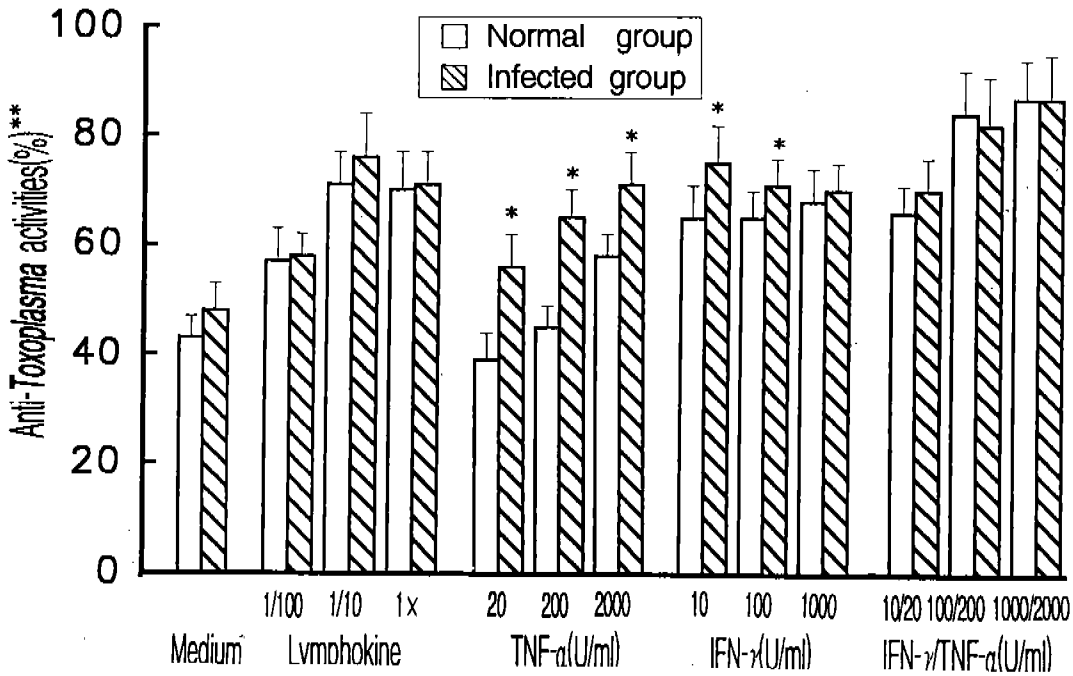

Fig. 3. Anti-*Toxoplasma* activities of cytokine-treated macrophages from normal and *Toxoplasma*-infected mice.

**Anti-*Toxoplasma* activities of macrophages (%)

$$=(1-\frac{\text{No. of macrophages infected with } T.\ gondii}{100 \text{ macrophages}}) \times 100$$

Table. 1. Correlation coefficient for the production of hydrogen peroxide, nitrite, and anti-*Toxoplasma* activities of macrophages from normal and *Toxoplasma*-infected mice

	Normal mice			*Toxoplasma*-infected mice		
	H_2O_2	NO_2^-	ATA**	H_2O_2	NO_2^-	ATA
H_2O_2	1.0000 (0.0)	-	-	1.0000 (0.0)	-	-
NO_2^-	0.83902 (P=0.0003)	1.000 (0.0)	-	0.84162 (P=0.0003)	1.000 (0.0)	-
ATA	0.91569 (P=0.0001)	0.73378 (P=0.0043)	1.0000 (0.0)	0.69417 (P=0.0085)	0.53744 (P=0.0582)	1.0000 (0.0)

** ATA: Anti-*Toxoplasma* activities (%)

$$= (1-\frac{\text{No. of macrophages infected with } T.\ gondii}{100 \text{ macrophages.}}) \times 100$$

remarkable ability to kill not only intracellular *Toxoplasma* (Murray and Cohn, 1980) but also other unrelated phylogenetically distinct facultative and obligate intracellular pathogens (Vincendeau and Daulouede, 1991) as well as tumor target cells (Hibbs *et al.*, 1987). In our model, cytokine-treated macrophages from *Toxoplasma*-infected mice were revealed similar amount of H_2O_2 production and anti-*Toxoplasma* activities of macrophages. However NO_2^- productions by IFN-γ-and IFN-γ/TNF-α-treated macrophages from *Toxoplasma*-infected mice were increased more than two fold as compared with lymphokine- and TNF-α-treated groups. These results suggested that the pathways leading to secretion of H_2O_2 and NO_2^- are independent (Ding *et al.*, 1988), and the assessment of NO_2^- production by macrophages is preferred as a measure of macrophage activation because it is rapid and easy to perform and peritoneal cells other than macrophages do not generate large amounts of reactive nitrogen intermediates (Adams *et al.*, 1990). Nibbering *et al.* (1991) described that measurement of the amount of NO_2^- produced by murine macrophages stimulated with a calcium-ionophore offers the most practical criterion for distinction between activated macrophages and resident macrophages.

IFN-γ/TNF-α-treated group was revealed similar production of H_2O_2 and NO_2^-, and anti-*Toxoplasma* activities of macrophages between normal and *Toxoplasma*-infected mice. These results suggested that IFN-γ in combination with TNF-α was a potent stimulant of macrophages both normal and *Toxoplasma*-infected mice, so there were no apparent differencs between them. Also in these studies, the synergy between IFN-γ and TNF-α was revealed in normal mice, but the synergy was not revealed in *Toxoplasma*-infected mice, especially IFN-γ-treated macrophages from *Toxoplasma*-infected mice were similar results as compared with IFN-γ/TNF-α-treated group. These data suggested that macrophages from *Toxoplasma*-infected mice are activated by infection, so some cytokines are produced during *T. gondii* infection (Murray and Cohn, 1980). Similar results were revealed at the H_2O_2 production of medium-treated macrophages from *Toxoplasma*-infected mice.

Measurement of anti-*Toxoplasma* activities of macrophages has a few drawbacks such as this test can only be determined *in vitro* in adherent macrophages. Furthermore, strict criteria must be applied for standardized evaluation of the results, precautions must be taken to protect the investigator against infections, and maintenance of tachyzoites requires biweekly passage through mice which is a time consuming procedure. In these studies, cytokine-treated macrophages from normal and *Toxoplasma*-infected mice were significantly increased anti-*Toxoplasma* activities as compared with medium-treated macrophages from normal and *Toxoplasma*-infected mice, respectively. These results showed that cytokine-treated macrophages are reduced the entry of *T. gondii* as compared with resident macrophages. Chao *et al.* (1994) reported that IFN-γ/LPS-treated microglial cells limited *T. gondii* growth by reducing entry of this parasite rather than intracellular multiplication.

NO_2^- productions of cytokine-treated macrophages from *Toxoplasma*-infected mice were not correlated with the values of anti-*Toxoplasma* activities of macrophages, but H_2O_2 productions were closely correlated with anti-*Toxoplasma* activities of macrophages. These results were closely resembled with Peetermans *et al.* (1993), but there were not closed with Nibbering *et al.* (1992). Murray and Cohn (1980) and Peetermans *et al.* (1993) described that the ability of the murine macrophage to kill or inhibit the intracellular multiplication of *Toxoplasma* has been directly correlated to the amount of oxygen metabolites released after stimulation of phorbol myristate acetate. But Nibbering *et al.* (1991) reported that nitrite production by activated murine macrophages correlates with their toxoplasmaststic activity, Ia antigen expression, and production of H_2O_2.

At present study, IFN-γ-treated macrophages from *Toxoplasma*-infected mice were significantly increased production of H_2O_2 and NO_2^-, and anti-*Toxoplasma* activities of macrophages as compared with macrophages from normal mice, whereas the other cytokine-treated groups were not significant differences between normal and *Toxoplasma*-infected

mice. Ding *et al.* (1988) described that only recombinant mouse IFN-γ could activate macrophages for increased secretion of both NO_2^- and H_2O_2 in mouse model, also Suzuki *et al.* (1988) and Liew (1991) reported that IFN-γ is capable of activating macrophages for enhanced antitoxoplasmal activation *in vitro* and *in vivo* and forms the primary regulator of cellular immunity in mice, largely through activation of macrophages. These results revealed that IFN-γ was a sufficient stimulant for the activation of macrophages and did not require second signals in the macrophages from *Toxoplasma*-infected mice.

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= 국문초록 =

Cytokine이 *Toxoplasma*감염 마우스 복강대식세포의 활성화에 미치는 영향

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*T. gondii*의 Beverley주를 감염시킨 마우스(감염군)로부터 분리한 복강대식세포에 cytokine의 종류 및 농도에 따른 대식세포의 활성화 정도를 평가하기 위하여 복강대식세포 단세포층에 medium, 조제 lymphokine, 재조합 tumor necrosis factor-α (TNF-α), 재조합 interferon-γ (IFN-γ), 및 재조합 IFN-γ와 TNF-α를 함께(IFN-γ/TNF-α) 처치한 후, 각 처치군별 H_2O_2 생산량, NO_2^- 생산량 및 *T. gondii*의 대식세포내 침투억제능을 측정하였다. 감염군의 복강대식세포에 IFN-γ 처치시 NO_2^- 생산량은 농도에 따라 유의하게 증가하였으나 그외의 처치군에서는 농도에 따른 유의한 차이가 없었다. 감염군 대식세포에 IFN-γ나 IFN-γ/TNF-α 처치시 H_2O_2 생산량이 medium처치군보다 유의하게 증가하였으며, NO_2^- 생산량은 TNF-α, IFN-γ 나 IFN-γ/TNF-α 처치시 유의하게 증가하였다. 감염군에 cytokine 처치시 *T. gondii*의 대식세포내 침투억제능은 medium 처치시보다 모두 증가되었다. 또한 정상군과 감염군의 H_2O_2 생산량, NO_2^- 생산량 및 *T. gondii*의 대식세포내 침투억제능을 상호 비교시 IFN-γ 처치군은 유의한 차이를 나타냈으나 그 외의 cytokine 처치군에서는 유의한 차이를 나타내지 않았다. 이상의 성적으로 보아 IFN-γ가 *Toxoplasma* 감염 마우스 복강대식세포의 활성화에도 중요한 역할을 함을 알 수 있었다.

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