トキソプラズマ感染マウスにおけるガンマ･インターフェロンの産生増大

誌名	Japanese journal of veterinary science
ISSN	00215295
著者	白幡, 敏一 小島, 英理 石川, 潤
巻/号	51巻2号
掲載ページ	p. 380-388
発行年月	1989年4月

農林水産省 農林水産技術会議事務局筑波産学連携支援センター
Tsukuba Business-Academia Cooperation Support Center, Agriculture, Forestry and Fisheries Research Council Secretariat

AgriKnowledge

Augmented Production of Gamma Interferon in Mice Experimentally Infected with *Toxoplasma gondii*

Toshikazu SHIRAHATA, Eri KOJIMA, Hiroshi ISHIKAWA,[1)] and Hitoshi GOTO

Department of Veterinary Microbiology and [1)]Veterinary Hospital, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080, Japan

(Received 9 August 1988/Accepted 3 December 1988)

ABSTRACT. The gamma interferon (IFN-γ) production and the cell populations participating to this production were examined in Toxoplasma-infected mice. When spleen cells from Toxoplasma-infected mice were cultured with Concanavalin A (Con A) or OK-432, a Streptococcal preparation, they produced significantly high levels of IFN-γ as compared with that of noninfected mice. Such enhanced IFN titers were observed as early as at 5 days postinfection, reached at the maximum levels on 20 days around and declined gradually thereafter. Treatment of spleen cells from the infected mice with either monoclonal anti-Thy-1.2 antibody plus complement or macrophage-blocking agents virtually abolished the IFN production. The spleen cells producing IFN-γ were more susceptible to the treatment with monoclonal anti-Lyt-1.2 than anti-Lyt-2.2 antibodies, suggesting that $CD4^+$ T cells are main producers of this lymphokine. When mice infected with Toxoplasma 10 days previously were injected with lipopolysaccharide (LPS), a well-known inducer of IFN-α/β, the sequential production of IFN-α/β and IFN-γ was induced in their circulation.—KEY WORDS: IFN-α/β, IFN-γ, mitogen, toxoplasma.

Jpn. J. Vet. Sci. 51(2): 380–388, 1989

A number of studies have shown that gamma interferon (IFN-γ) plays an important role in resistance to *Toxoplasma (T.) gondii* infection by activating macrophages [1, 7, 8, 11–15]. McCabe *et al.* [7] demonstrated the enhanced protection of mice administered with recombinant IFN-γ from the challenging infection with a lethal dose of Toxoplasma. More recently, Suzuki *et al.* [15] reported that mice injected with a monoclonal antibody to murine IFN-γ died after inoculation with the low virulent strain of Toxoplasma, whereas mice infected with the same strain and received no antibody survived to develope the chronic infection. However, the significance of IFN-γ especially in relation to its role in the altered immune responses in Toxoplasma-infected host has not yet been fully understood.

Recently, it has been reported that certain bacteria alter the responsiveness of mice to mitogen stimulation with the enhanced production of circulating IFN-γ [9, 17]. Havell *et al.* [4] demonstrated that spleen cells from Listeria-infected mice produced the enhanced levels of IFN-γ after stimulation with T cell mitogens. On the basis of these observations, studies were carried out to evaluate the ability of Toxoplasma-infected mice to produce IFN in response to polyclonal mitogens. Here, we show the augmented production of IFN-γ induced nonspecifically by T- and B cell mitogens in mice during *T. gondii* infection.

MATERIALS AND METHODS

Mice: Female, 6-week-old ICR and C57BL/6 mice were purchased from a commercial breeder (Clea Japan Inc., Tokyo), and maintained in our laboratory under conventional condition throughout the experiment.

Infection with Toxoplasma: Mice (8–10 week old) were inoculated intraperitoneally (ip) with 2,000 tachyzoites of the S-273

strain of *T. gondii* which had been isolated from a clinically healthy pig (3). Mice were treated with sulphamonomethoxin (Daimeton B, Daiichi Pharmaceutical Co., Ltd., Tokyo) administrating ip at a dose level of 4 mg/mouse/day for 5 days from the 6th day after the infection to slow down parasite multiplication.

IFN inducers: Concanavalin A (Con A, Type IV, Sigma Chemicals Co., U.S.A) and lipopolysaccharide (LPS, *Escherichia coli* 0111: B4, Difco Laboratories, U.S.A.) were purchased from Wako Pure Chemical Industries, Osaka. OK-432 was kindly supplied by Dr. Niki of Chugai Pharmaceutical Co., Ltd., Tokyo.

Preparation of spleen cell cultures: Spleen cell suspensions were prepared as described previously [14]. Erythrocytes were removed by treatment with 0.83% NH_4Cl in 0.01 M Tris-HCl (pH 7.2). After washing twice with serum-free RPMI-1640 medium (RPMI-1640), the cells were resuspended in RPMI-1640 supplemented with 5% fetal calf serum (FCS), penicillin (100 U/m*l*) and streptomycin (100 μg/m*l*) and cultured at a concentration of 5×10^6 cells per m*l* in 12-well tissue culture plates (Linbro, Hamden, Conn., U.S.A). Various doses of Con A or OK-432 were put into each well and incubated at 37°C for designated periods in a CO_2 incubator. After incubation, the culture supernatants were collected and centrifuged. The fluids were stored at −80°C until assayed for IFN activity.

Assay for IFN activity: IFN activity was assayed by the plaque reduction method in L-929 cells with vesicular stomatitis virus (VSV) as described previously [13]. One unit in this assay was approximately equivalent to 1.2 U of the reference mouse IFN-α/β (Lot No., G–002–904–511, National Institute of Allergy and Infectious Disease, NIH, Bethesda, Md., U.S.A.).

Neutralization test of IFN: Sheep anti-mouse IFN-α/β serum (Lot No., G–204–501–568) was kindly provided by the Animal Substance Program, National Institute of Allergy and Infectious Disease, NIH, U.S.A. The anti-IFN-α/β serum was diluted in 1: 100 with RPMI-1640 to estimate 3,000 U/m*l* of IFN-neutralizing titer. One neutralizing unit of the antiserum can neutralize 8 to 10 international unit (IU) of mouse IFN-α/β. The IFN samples were mixed with either an equal volume of 1: 100 diluted anti-mouse IFN-α/β serum or RPMI-1640. These mixtures were then incubated at 37°C for 1 hr before being assayed for residual IFN activity.

Stability of IFN at pH 2: The IFN samples were dialyzed against 0.05 M glycine-HCl buffer containing 0.1 M NaCl (pH 2.0) at 4°C for 24 hr and then redialyzed against RPMI-1640 at 4°C for 24 hr. Control samples were obtained by dialysis against 0.01 M PBS (pH 7.4) instead of pH 2 buffer. After the dialysis, the samples were assayed for residual IFN activity.

Cytotoxicity technique: The two-step cytotoxicity assay [6] was used for the evaluation of lymphocyte subpopulation in the whole spleen cell suspensions. The monoclonal anti-Thy-1.2, anti-Lyt-1.2, anti-Lyt-2.2 antibodies and low-toxicity rabbit complement were obtained from Cedarlane Laboratories Ltd., Ontario, Canada. Ten million spleen cells were suspended in 2 m*l* of each monoclonal antibody diluted in 1: 20 with RPMI-1640 and incubated at 4°C for 1 hr. After removal of antibody by centrifugation, the cells were treated with complement diluted in 1: 10 at 37°C for 45 min and washed twice with RPMI-1640. The percentage of cellular cytotoxicity was calculated by trypan blue dye exclusion test described by Macario *et al* [6]. Macrophages were depleted by treatment with either silica (2 mg/m*l*) at 37°C for 15 hr or 1mM of 2-choroadenosine at 37°C for 2 hr as reported previously [18].

Statistical analysis: Student's *t* test was

used to calculate the statistical significance. A statistical significant difference was considered to be present at $P<0.05$.

RESULTS

IFN production: The IFN production in the spleen cell cultures stimulated with Con A or OK-432 were observed similarly on both of Toxoplasma-infected and noninfected mice (Fig. 1). The IFN production reached to the maximum level at 48 hr. The IFN titers in the immune cells stimulated with Con A or OK-432 were significantly higher than those in the nonimmune controls at any hrs of incubation periods ($P<0.05$). The effect of concentration of the inducers on IFN production is shown in Fig. 2. Among the cultures, the maximum IFN titers were produced in the groups of cells stimulated with concentration of 10 μg of Con A or OK-432. However, no statistical difference in the IFN titers were noted between immune and nonimmune cell cultures stimulated with 1.25 μg or 10 μg of Con A. From the results, spleen cells were cultured with 5 μg of Con A or 10 μg of OK-432 for 48 hr, in a concentration of 5×10^6 cells per m*l*, in the subsequent experiments.

Enhanced production of IFN in spleen cells of Toxoplasma-infected mice: The IFN production in cultures of spleen cells taken from mice at varying days after infection with *T. gondii* is shown in Fig. 3. A titer of IFN in the culture of spleen cells was developed as early as at 5 days after the infection and peaked at 20 days in Con A and around 15 days in OK-432, and declined gradually thereafter. The peak titer of IFN in the immune spleen cell culture with Con A was approximately 10 times higher than those in the noninfected mice. OK-432 also induced significantly higher levels of IFN in the cultures of immune cells from 10 to 20 days postinfection as compared with those in the control mice ($P<0.05$).

Characteristics of IFN: The characteristics of IFN produced by spleen cells from Toxoplasma-infected mice are shown in Table 1. The IFN activities induced by Con A or OK-432 were not neutralized by anti-mouse IFN-α/β serum. In addition, treatment of these IFN preparations with

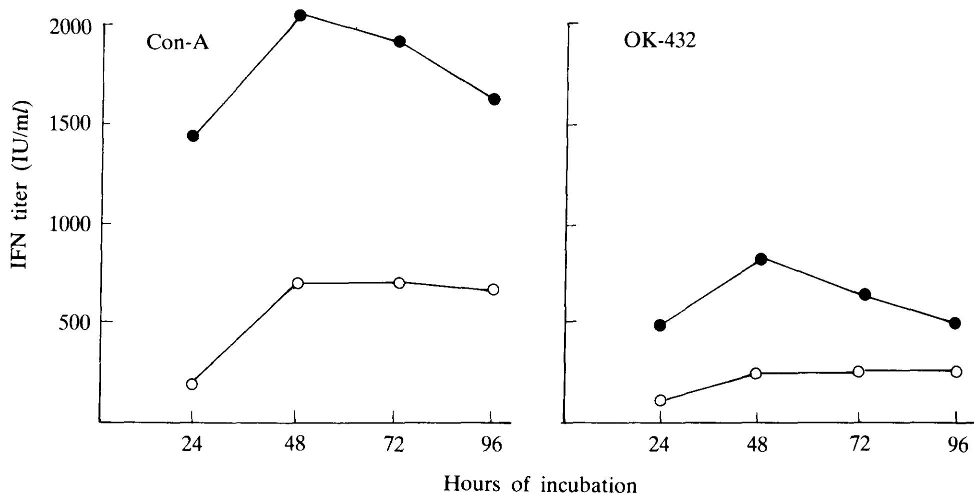

Fig. 1. IFN titers in the spleen cell cultures induced by Con A or OK-432. Spleen cells (5×10^6 cells/m*l*) from ICR mice infected with Toxoplasma 15 days previously (●) and from noninfected mice (○) were incubated for various times after the addition of Con A (5 μg/m*l*) or OK-432 (10 μg/m*l*) and the resultant supernatants were assayed for IFN activity. Each point represents the mean titer from 5 mice.

pH 2 buffer resulted in the loss of more than 90% of the activity.

Cell populations participating in IFN-γ production: As indicated in Table 2, treatment of spleen cells from infected mice with monoclonal anti-Thy-1.2 antibody plus complement virtually abolished the production of IFN-γ by Con A stimulation. In Table 2, marked reduction of IFN was noted by the treatment of spleen cells with anti-Lyt-1.2 antibody plus complement. In addition, the IFN production was almost completely abrogated when the spleen cells were treated with either silica or 2-chloroadenosine.

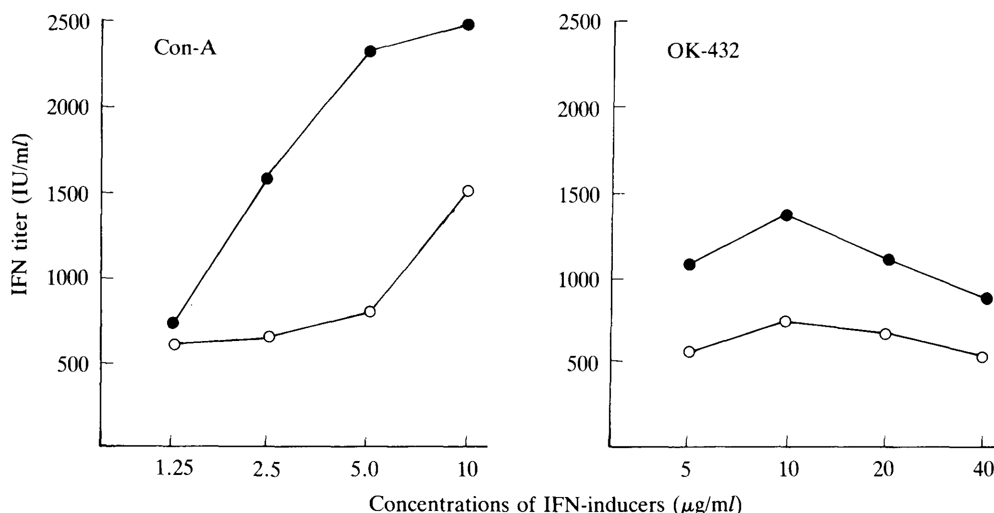

Fig. 2. Effect of concentrations of inducers on IFN production in the spleen cell cultures of Toxoplasma-infected and noninfected mice. Spleen cells (5×10^6 cells/ml) from ICR mice infected with Toxoplasma 21 days previously (●) and from noninfected mice (○) were incubated with different concentrations of Con A or OK-432 for 48 hr and the resultant supernatants were assayed for IFN activity. Each point represents the mean titer from 5 mice.

Table 1. Characterization of IFN produced by spleen cells from Toxoplasma-infected mice

Inducers	Days after infection	IFN titers (IU/ml) after treatment with			
		anti-IFN-α/β serum	RPMI-1640	pH 2	pH 7.4
Con A	5	450 (75)[a]	600	<10	132
	10	660 (92)	720	18 (2)	840
	15	700 (88)	800	32 (8)	408
	20	1,560 (76)	2,040	60 (9)	672
	25	1,320 (110)	1,200	72 (6)	1,200
OK-432	5	144 (80)	180	<10	180
	10	180 (83)	216	<10	540
	15	148 (100)	144	<10	384
	20	77 (80)	96	<10	156
	25	108 (114)	94	<10	180
NDV[b]		<10	4,560	3,600	3,600

a) Numbers in parentheses are percentage of residual IFN activity after treatment with anti-IFN-α/β serum or pH 2 buffer.

b) Newcastle disease virus (NDV)-induced L cell IFN was used as a reference of murine IFN-α/β.

Table 2. Effect of depletion of T cells and macrophages on IFN production in splenic cell cultures

Spleen source	Treatment[a)]	IFN titer (IU/m*l*)
Infected	None	830
	Complement alone	850
	Anti-Thy-1.2 antibody+complement	<10
	Anti-Lyt-1.2 antibody+complement	120
	Anti-Lyt-2.2 antibody+complement	352
	Recombination of anti-Lyt-1.2+C′-treated cells with anti-Lyt-2.2+C′-treated cells	630
	Silica	<30
	2-chloroadenosine	38
Noninfected[b)]	None	57±7.4

a) Spleen cells (1×10^7 cells/m*l*) of C57BL/6 mice infected with Toxoplasma 21 days earlier were treated with either monoclonal antibody plus complement (C′) or reagents as described in Materials and Methods. After treatment, 5×10^6 cells per m*l* were cultured with Con A (5 μg/m*l*) for 48 hr. Data are mean values from two experiments.

b) Spleen cells (5×10^6 cells/m*l*) from noninfected C57BL/6 mice were cultured individually with Con A (5 μg/m*l*) and IFN activities in culture supernatants were determined. Data are mean titer ±S.D. from 5 mice.

Changes in Thy-1-bearing cell population: The total number and the percentage of Thy-1$^+$ cells increased both significantly in the spleens of infected mice as compared with that of noninfected mice during 10 to 20 days postinfection (Fig. 4). During this period, a huge splenomegaly was noted in the infected mice (data not shown). However, at 45th day there was no significant difference in the number as well as the percentage of Thy-1$^+$ cells between infected and noninfected mice.

Induction of IFN-γ by LPS in the circulation of Toxoplasma-infected mice: When the mice infected with Toxoplasma 10 days before were injected intravenously with 1 μg of LPS, the enhanced and prolonged production of IFN was induced in their circulation as compared with that of noninfected mice (Table 3). In the infected mice, the IFNs produced at 2 and 3 hr were α/β type and IFN-γ was predominantly induced from 4 to 7 hr after injection of LPS. In contrast, no significant amount of IFN-γ was induced when the mice infected with Toxoplasma 21 days previously were injected with the same amount of LPS. Most of the IFN activities induced in noninfected mice and mice infected 21 days before were neutralized by anti-mouse IFN-α/β serum (Table 3).

DISCUSSION

The results presented in this paper show that infection of mice with *T. gondii* sensitize their spleen cells to augment *in vitro* production of IFN upon stimulation with Con A or OK-432. The enhancement of IFN production by immune spleen cells was pronounced during the early phase of Toxoplasma infection (Fig. 3). Although the anti-mouse IFN-γ serum was unavailable, the type of IFN induced by Con A or OK-432 in the spleen cell cultures of Toxoplasma-infected mice was identified as IFN-γ by the properties that the antiviral activity was acid labile and was not neutralized by anti-mouse IFN-α/β serum.

The cellular mechanism operative in such enhanced IFN-γ production in the early phase of Toxoplasma infection is still

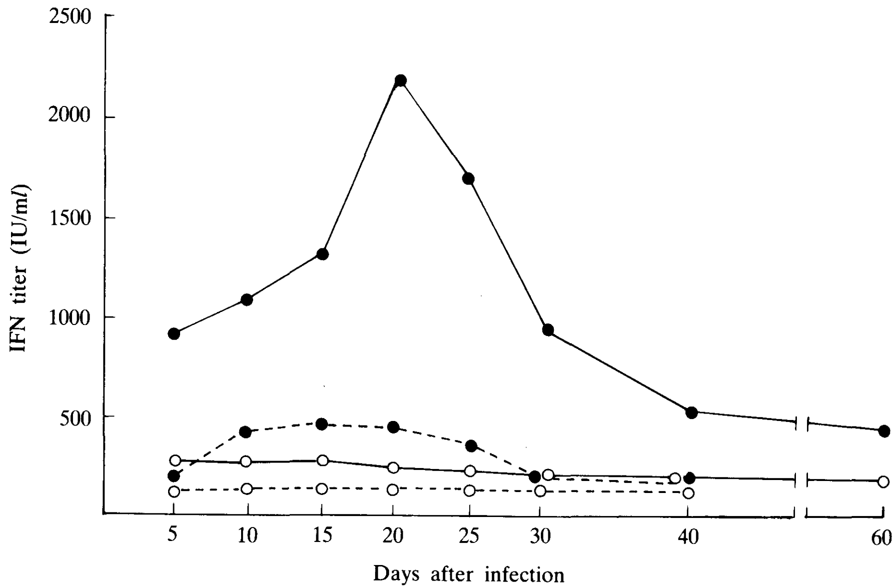

Fig. 3. Development of the capacity for enhanced IFN production by spleen cells from Toxoplasma-infected mice. At indicated day after infection of Toxoplasma, spleen cells (5×10^6 cells/ml) from groups of either infected (●) or noninfected (○) ICR mice matched in age were cultured with Con A (—) or OK-432 (---) for 48 hr and IFN activities in the culture supernatants were determined. Each point represents mean titer from 4 mice.

obscure. However, it may be suspected that Toxoplasma infection might have resulted in the activation of reticuloendothelial system. Actually, the enlargement of Thy-1-bearing cells in the spleen, probably of producing IFN-γ, and a huge splenomegaly (data not shown) were observed during the acute phase of infection. Therefore, it seems likely that macrophages are also increased in number and activated individually to act more functionally in IFN-producing system. The splenic cells responsible for the enhanced production of IFN-γ was apparently T cell population, since the IFN production was completely abolished by the treatment of spleen cells with monoclonal anti-Thy-1.2 antibody plus complement. In addition, treatment of spleen cells with lethal agents for macrophages reduced greatly their ability to produce this lymphokine. Taken together, these data suggest that the production of IFN-γ by Toxoplasma-immune spleen cells is carried out by T cells in cooperation with macrophages and are compatible with the findings observed in other murine systems [5, 10, 14, 18]. At present, however, little is known how macrophages participate in the enhanced production of IFN-γ. The analysis of cellular source of IFN-γ indicates that $CD4^+$ T cells are major producer of this lymphokine. A minor but significant role of $CD8^+$ lymphocytes for the IFN-γ production was also recognized in this system (Table 2). These results are consistent with the recent reports on the CD phenotypes of lymphocytes responsible for murine IFN-γ production [2, 4, 16].

Although LPS has been generally accepted as an inducer of IFN-α/β, there have been recent studies on the induction of IFN-γ by this mitogen [9, 10, 17]. In this study, we showed that the sequential production of IFN-α/β and IFN-γ could be induced in the circulation of mice infected with Toxoplasma 10 days previously upon stimulation with LPS (Table 3). However,

Table 3. Production and characterization of IFNs induced by LPS in the circulation of Toxoplasma-infected and noninfected mice

Source of IFN	Hours after injection of LPS	IFN titer (IU/m*l*) after neutralization with[a]	
		RPMI-1640	anti-mouse IFN-α/β serum
Noninfected	2	190	<20 (<10.5)[b]
	3	265	<20 (<7.6)
	4	300	<20 (<6.6)
	5	68	NT[d]
10-day infected	2	370	40 (11)
	3	470	175 (37)
	4	515	427 (83)
	5	890	740 (83)
	7	630	590 (94)
21-day infected	2	240	<20 (<8.3)
	3	315	<20 (<6.3)
	4	285	<20 (<7.0)
	5	80	NT
	7	<20	NT
L cells[c]		2,300	<20 (<1.0)

a) The neutralization test was carried out using pooled sera from four mice.
b) Numbers in parentheses are percentages of residual IFN activities after treatment with anti-mouse IFN-α/β serum.
c) NDV-induced IFN was used as a reference of mouse IFN-α/β.
d) Not tested.

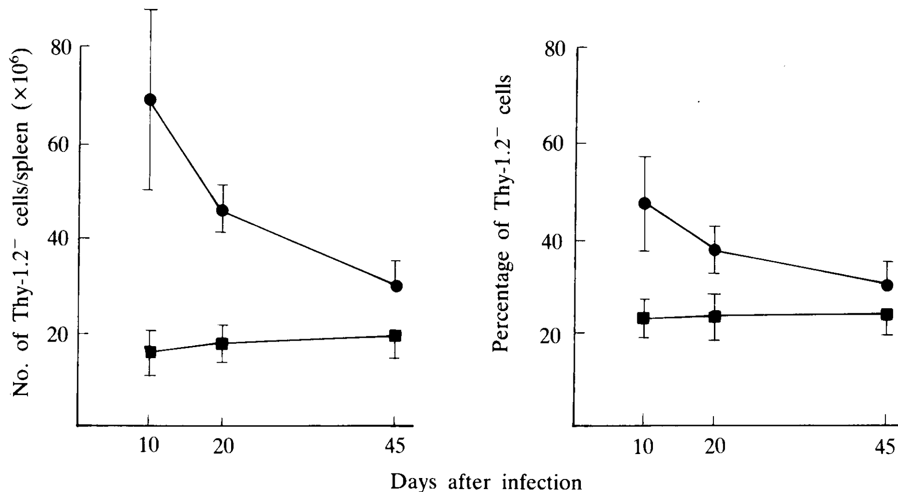

Fig. 4. Changes in Thy-1.2$^+$ cell population in the spleens of C57BL/6 mice during Toxoplasma infection. The numbers of Thy-1.2$^+$ cells were calculated by cytotoxicity technique as described in MATERIALS AND METHODS. Each point represents mean ± S.D. from 5 infected (●) or 5 noninfected (■) mice. For the differences in total numbers and percentages of Thy-1.2$^+$ cells between infected and noninfected mice, P values were as follows: <0.01 and <0.02 at 10days and <0.01 and <0.02 at 20 days after infection, respectively.

the induction of IFN-γ by LPS was not observed in mice infected with Toxoplasma 21 days previously. Thus, the IFN-γ producibility induced by LPS was also pronounced in the acute stage of Toxoplasma infection. This is in contrast to the previous observation wherein it was demonstrated that considerable amounts of IFN-γ production could be induced in Toxoplasma-infected mice by stimulation with Toxoplasma lysate antigen (TLA) from 7th to 35th day after infection [12–14]. These results suggest that the cellular mechanism of IFN-γ production induced by LPS might be somewhat different from that induced by TLA. At present, however, the available data are not sufficient to analyze the mechanism underlying in the sequential production of IFN-α/β and IFN-γ induced by LPS in Toxoplasma-infected mice.

REFERENCES

1. Black, C. M., Catteral, J. R., and Remington, J. S. 1987. *In vitro* and *in vivo* activation of alveolar macrophages by recombinant interferon-γ. *J. Immunol.* 138: 491–495.
2. Budd, R. C., Cerottin, J., and MacDonald, H. R. 1987. Selectively increased production of interferon-γ by subsets of Lyt-2^+ and L3T4^+ T cells identified by expression of Pgp-1. *J. Immunol.* 138: 3583–3586.
3. Hanaki, T., Sato, U., Nishimura, Y., and Nobuto, K. 1961. Studies of toxoplasmosis in domestic animals. III. Relationship between T. S. C. skin test and detection of parasites in asymptomatic toxoplasmosis in swine. *Jpn. J. Vet. Sci.* 23: 509 (in Japanese).
4. Havell, E. A., Spitalny, G. L., and Patel, P. J. 1982. Enhanced production of murine interferon-γ by T cells generated in response to bacterial infection. *J. Exp. Med.* 156: 112–127.
5. Hirt, H. M., Becker, H., and Kirchner, H. 1978. Induction of interferon production in mouse spleen cell cultures by *Corynebacterium parvum*. *Cell. Immunol.* 38: 168–175.
6. Macario, A. J. L., Stahl, W., and Miller, R. 1980. Lymphocyte subpopulations and function in chronic murine toxoplasmosis. I. Thy-1^+ cells in lymphoid tissues. *Clin. Exp. Immunol.* 41: 415–422.
7. McCabe, R. E., Luft, B. J., and Remington, J. S. 1984. Effect of murine interferon gamma on murine toxoplasmosis. *J. Infect. Dis.* 150: 961–962.
8. Murray, H. W., Spitalny, G. L., and Nathan, C. F. 1985. Activation of mouse peritoneal macrophages *in vitro* and *in vivo* by interferon-γ. *J. Immunol.* 134: 1619–1622.
9. Nakane, A. and Minagawa, T. 1985. Sequential production of alpha and beta interferons and gamma interferon in the circulation of *Listeria monocytogenes*-infected mice after stimulation with bacterial lipopolysaccharide. *Microbiol. Immunol.* 29: 659–669.
10. Okamura, H., Wada, M., Nagata, K., Tamura, T., and Shoji, K. 1987. Induction of murine gamma interferon produced by lipopolysaccharide and interleukin-2 in *Propionibacterium acnes-induced* peritoneal exudate cells. *Infect. Immun.* 55: 335–341.
11. Omata, Y., Sakurai, H., Izumo, M., Saito, A., and Suzuki, N. 1984. Interferon production in non-immune and immune mice after Toxoplasma infection. *Jpn. J. Vet. Sci.* 46: 155–165.
12. Sakurai, H., Takei, Y., Omata, Y., and Suzuki, N. 1981. Production and properties of Toxoplasma growth inhibitory factor and interferon in the lymphokines and the circulation of Toxoplasma immune mice. *Zbl. Bakt. Hyg.*, I. Abt. Org. A 251: 134–143.
13. Shirahata, T., Mori, A., Ishikawa, H., and Goto, H. 1986. Strain differences of interferon-generating capacity and resistance in Toxoplasma-infected mice. *Microbiol. Immunol.* 30: 1307–1316.
14. Shirahata, T. and Shimizu, K. 1980. Production and properties of immune interferon from spleen cell cultures of Toxoplasma-infected mice. *Microbiol. Immunol.* 24: 1109–1120.
15. Suzuki, Y., Orellana, N. A., Schreiber, R. D., and Remington, J. S. 1988. Interferon-γ: The major mediator of resistance against *Toxoplasma gondii*. *Science* 240: 516–517.
16. Torres, B. A., Yamamoto, J. K., and Johnson, H. M. 1982. Cellular regulation of gamma interferon production: Lyt phenotype of the suppressor cell. *Infect. Immun.* 35: 770–776.
17. Wada, M., Okamura, H., Nagata, K., Shimoyama, T., and Kawade, Y. 1985. Cellular mechanisms *in vivo* production of gamma interferon induced by lipopolysaccharide in mice infected with *Mycobacterium bovis* BCG. *J. Interferon Res.* 5: 431–443.
18. Yamaguchi, T., Kuroda, Y., Yokoyama, T., Saito, M., Ebina, T., Hoshino, F., and Ishida, N. 1984. Participation of macrophages in enhanced *in vitro* immune interferon (IFN-γ) production with mouse spleen cells. *Microbiol. Immunol.* 29: 1031–1040.

要　　約

トキソプラズマ感染マウスにおけるガンマ・インターフェロンの産生増大：白幡敏一，小島英理，石川　潤[1)]，後藤　仁（帯広畜産大学獣医学科家畜微生物学教室，[1)]附属家畜病院）――トキソプラズマ（Tp）感染マウスの脾細胞をコンカナバリンA（Con A）や溶連菌製剤（OK-432）で刺激培養すると，非感染マウスに比較し，高力価のIFN-γが誘発・産生された．脾細胞培養系におけるIFN-γの産生増大は感染早期において顕著に認められ，脾内Thy-1陽性細胞数の推移に一致する傾向がみられた．IFN-γの主たる産生細胞は$CD4^+$サブセットに属するT細胞亜集団であるが，その産生にはマクロファージの関与が必須であった．さらにIFN-α/βの誘発剤として知られる大腸菌内毒素（LPS）をTp感染10日目のマウスに静脈内注射すると，IFN-α/βの産生に引き続き高力価のIFN-γが血中に誘発・産生された．