Growth Inhibition of *Toxoplasma gondii* in Cell Cultures Treated with Murine Type II Interferon

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ABSTRACT. In vitro assessment was made on the effect of murine Type II interferon (IFN) on the multiplication of *Toxoplasma (T.) gondii* in mouse cell cultures. Macrophages and L cell cultures treated with Type II IFNs prepared specifically from the spleen cell cultures of *Toxoplasma*- or BCG-infected mice markedly suppressed the growth of *T. gondii*. The enhanced antitoxoplasma activities of these IFN-treated cells were nonspecific and of identical degree, and dependent on both IFN concentration and period of incubation in IFN preparations. Pretreatment with 500 U of Toxo-IFN for 24 hr before infection with *Toxoplasma* resulted in a significant decrease in percentage of infected cells and number of intracellular parasites in 1 and 2 hr postinfection. However, these IFNs conferred an enhanced antitoxoplasma activity on macrophages more effectively than in L cell cultures, and the activity of the activated macrophages and that of L cells seemed to be somewhat different each other; the growth inhibitory effect of the activated macrophages was microbicidal, whereas that of L cells was only microbiostatic and transient.

Three classes of murine interferon (IFN) have been described on the bases of the physicochemical properties and antigenic specificity. In 1966, Rytel and Jones [10] and Freshman et al. [2] first reported the induction and partial characterization of circulating IFN in the mice infected with virulent RH strain of *Toxoplasma gondii*. Since then, a considerable number of in vitro experiments have been made on the relationship between human and animal fibroblast type (Type I) IFN and this obligate intracellular parasite [1, 9, 11, 14]. However, little information is available about Type II (immune type) IFN and *Toxoplasma*.

In a previous study [14], we briefly showed that Type II IFN contained in lymphokine(s) prepared from spleen cell cultures of *Toxoplasma*-infected mice significantly inhibited multiplication of *Toxo-*

plasma within mouse macrophages and L cell cultures. In the present investigation, we attempted to analyze further the interaction between *Toxoplasma* and murine Type II IFN, with special reference to the IFN-dose dependency and the period of incubation for cell activation leading to killing or inhibition of multiplication of *Toxoplasma*.

MATERIALS AND METHODS

Mice: Female Swiss ICR mice (Japan Clea Co., Tokyo), 8–10 weeks old, were used for infection with *Toxoplasma gondii* and *Mycobacterium bovis* strain BCG.

***Toxoplasma* lysate antigen (TLA):** *Toxoplasma* tachyzoites of the RH strain were collected from the peritoneal cavities of 3-day infected mice and washed twice with 0.01 M phosphate-buffered saline (PBS, pH 7.4). After washing, 10 volumes of sterile distilled water was added to the centrifuged sediment. The resulting suspension was sonicated in ultrasonic vibrator (Kubota Insonator, Model 200, 100 W, 5 min) and kept at 4°C for 24 hr. The

extracted antigen was mixed with an equal volume of a sterile 1.7% NaCl solution and centrifuged at 12000 rpm for 60 min. The supernatant was then used as TLA.

Type II interferon: Two Type II IFNs were prepared from the spleen cells of *Toxoplasma*- and BCG-inoculated mice cultured with the specific antigen. TLA-induced IFN (Toxo-IFN) was prepared as described previously [14]. Tuberculin-induced IFN (BCG-IFN) was prepared from spleen cell cultures of the BCG-sensitized mice according to Sonnenfeld et al. [16], except for that 50 μ g of PPD instead of old tuberculin was injected into each mice. These IFN preparations were dialyzed against PBS and chromatographed on a Sephadex G-100 column (2.6 $\times$ 100 cm). The fractions corresponding to molecular weights of 35,000 to 65,000 daltons were pooled, concentrated by pressure ultrafiltration (Ultrafilter UK-10, Toyo Kagaku Co., Tokyo) to 1/20 of the original volume and stored at -80°C .

Assay for interferon: IFN was assayed by the plaque-reduction method in L-929 cells with vesicular stomatitis virus (VSV) as the challenge virus. L cells were cultured in plastic plates (12 flat-bottomed 24-mm diameter wells, Linbro Scientific, Hamden, CT) with Eagle's minimum essential medium containing 5% fetal bovine serum (5% EMEM). Serial 3-fold dilutions of IFN were made in 2% EMEM and 1 ml of each dilution was added to each of triplicate cultures of L cells. After incubation for 24 hr, the cultures were washed with EMEM and inoculated with approximately 40 plaques of VSV. One hour later, the cultures were overlaid with 1 ml of 2% EMEM containing 1.2% methylcellulose. After re-incubation for 48 hr, the wells were rinsed and fixed with 5% formol PBS and stained with 0.1% crystal violet. One unit in this assay was equivalent to 1.2 units (U) of the reference mouse IFN (Lot. No., G-002-904-511, National Institute of Health, Bethesda, MD).

Assessment of *Toxoplasma* growth inhibitory activity: The assay for the *Toxoplasma* growth inhibitory (Toxo-GIF) activity of Type II IFN preparations was essentially the same as that described previously [15]. Briefly, macrophages and L-cell cultures were incubated with various units of Type II IFN samples for 24 hr, washed twice with Hanks balanced salt solution (HBSS) and infected with 10^5 tachyzoites of virulent S-273 strain [13]. At various intervals thereafter, the cells were stained with May-Grünwald Giemsa stain [12] and examined by light microscopy. *Toxoplasma* per 100 cells of a total of 500 cells on each coverslip

was enumerated and the percentage of the infected cells was calculated. The Student's *t* test was used to analyze the mean number of *Toxoplasma* per 100 cells and percentage of the infected cells.

RESULTS

1. Time course of *Toxoplasma* killing by macrophages incubated with Type II IFNs.

An in vitro experiment was made to estimate the time course of *Toxoplasma* killing by macrophages treated with a constant unit of Toxo-IFN or BCG-IFN. As shown in Fig. 1A, the intracellular parasites in

Fig. 1. Time course of *Toxoplasma* killing in macrophages incubated with Type II IFN. Macrophages were incubated with 250 U of Toxo-IFN (●), BCG-IFN (○) or Medium alone (■) for 24 hr, washed and infected with *Toxoplasma*. Each plot is a mean from two experiments.

both Toxo-IFN- and BCG-IFN-treated macrophages decreased in number gradually to 50% of the initial population in 12 hr post-infection. Moreover, most intracellular parasites were killed and digested by the activated macrophages within 24 hr post-infection. This was apparent in the gradual decrease in number of the infected macrophages. The percentage of the infected cells was reduced to about half by 12 hr, and at the end of the incubation period, macrophages with *Toxoplasma* were negligible (Fig. 1B). In contrast, cells incubated with the control medium alone allowed replication of *Toxoplasma* and were eventually destroyed by the overwhelming multiplication of the parasites. Thus, the role of Type II IFNs on the macrophage activation was clearly demonstrated.

2. Effect of Type II IFN on L cell activation

The effects of Type II IFN on L cell activation causing inhibition of the *Toxo-*

Fig. 2. Effect of Type II interferon on *Toxoplasma* within L-929 cells.
● : Toxo-IFN, ○ : BCG-IFN, ■ : 2% EMEM.
Each plot is a mean from two experiments.

Fig. 3. Effect of daily renewal of Type II interferon on *Toxoplasma* within L cells.
Cells were incubated 200 U of Type II IFNs for 24 hr and infected with *Toxoplasma*. One hour later, cells were washed and 200 U of new IFNs were added every day.
● : Toxo-IFN, ○ : BCG-IFN, ■ : 2% EMEM.
Each plot is a mean from two experiments.

plasma growth are shown in Fig. 2. When L cells were pre-incubated for 24 hr with 500 U of BCG- or Toxo-IFN and infected with *Toxoplasma*, the replication of the parasites was markedly inhibited for 48 hr postinfection. However, a slight but significant increase in number of the intracellular parasites was noted in 72 hr post-infection. In the next experiment, the effect of pre-incubation plus daily renewal of IFN on the L cell activation was investigated. As shown in Fig. 3, pre-incubation and daily replacement with fresh Type II IFN containing 200 U conferred a higher microbiostatic activity than did the pre-incubation only. In this case, multiplication of *Toxoplasma* was almost completely stopped for 96 hr postinfection. However, the number of the intracellular parasites remained constant during the incubation period and these parasites could not be distinguished morphologically from the live

Fig. 4. Effect of Toxo-IFN of various units on *Toxoplasma* multiplication within mouse macrophages and L cells.

Cells were incubated with Toxo-IFN of various units for 24 hr, washed with HBSS and infected with *Toxoplasma*.

□: 500 U, ■: 100 U, △: 20 U, ▲: 4 U, ●: Medium alone.

parasites.

3. Dose response and time course of macrophage and L cell activation by Type II IFN.

Dose response of Type II IFN for macrophage and L cell activation was investigated with various units of Toxo-IFN. As

Fig. 5. Effect of preincubation of cells with Toxo-IFN prior to *Toxoplasma* infection.

Cells were incubated with Toxo-IFN of 500 U for various periods of time, washed with HBSS and infected with *Toxoplasma*.

○: 24 hr, ●: 12 hr, □: 6 hr, ■: 3 hr, △: Medium alone.

shown in Fig. 4, pre-incubation of macrophages with more than 100 U of Toxo-IFN resulted in marked destruction of the parasites within 30 hr postinfection. A slight inhibitory effect was also recognized in macrophages treated with only 20 U of Toxo-IFN. By contrast, multiplication of *Toxoplasma* in L cells was suppressed after treatment with 500 U of Toxo-IFN but not with less than 100 U. Then, the pre-incubation time required for initiation of the

Table 1. Effect of Toxo-IFN on the number of *Toxoplasma* infected into L cells and macrophage

Cells	IFN-treatment	Mean <i>Toxoplasma</i> /100 cells after :		Per cent cells infected after :	
		1 hr	2 hr	1 hr	2 hr
L cells	with ^{a)}	14.2 ± 7.8*	21.4 ± 14.3	10.5 ± 4.7*	15.4 ± 8.8*
	without ^{b)}	21.4 ± 9.8	31.3 ± 16.2	16.9 ± 7.4	22.2 ± 9.2
Macrophages	with	10.2 ± 5.2**	11.2 ± 7.1**	7.6 ± 4.1**	8.4 ± 4.7**
	without	25.7 ± 16.4	29.2 ± 13.9	17.1 ± 7.9	21.6 ± 8.9

Data are mean values from two experiments.

a): Cells were incubated previously with 500 U of Toxo-IFN for 24 hr and washed.

b): Cells were incubated previously with 10 % RPMI-1640 for 24 hr.

**: $P < 0.01$, *: < 0.05 ; significantly.

Toxo-IFN effect on macrophages and L cells was investigated. Macrophages were incubated with 500 U of Toxo-IFN for various periods of time prior to infection with *Toxoplasma*. Microbicidal activity was seen only after pre-incubation for 3 hr or longer (Fig. 5). On the contrary, L cells preincubated for 3 hr did not show any growth inhibitory effect and required a period of 6 hr or longer before it exhibited a tangible effect, which was only microbiostatic.

4. Effect of Type II IFN on the number of *Toxoplasma* infecting L cells and macrophages.

We often noted a decreased number of the intracellular parasites in the cells preincubated with Type II IFN for 1 hr postinfection (e.g. Figs. 3 and 5). Therefore, we investigated the effect of Type II IFN on the number *Toxoplasma* in 1 and 2 hr postinfection. Macrophages and I cells were incubated previously with 500 U of Toxo-IFN for 24 hr, washed twice with HBSS and infected with *Toxoplasma*. As shown in Table 1, the mean number of the intracellular parasites and the percentage of the infected cells 1 and 2 hr postinfection both decreased significantly by this treatment. In a preliminary experiment, *Toxoplasma* tachyzoites were incubated for 4 hr at 37°C with Toxo-IFN (250 U/ml) or control medium and inoculated into L cell cultures. However, this treatment did not affect the multiplication of *Toxoplasma* in L cell cultures (data not shown).

DISCUSSION

In a previous report [14], we showed that the antigen-stimulated *Toxoplasma* immune lymphocytes produced considerable amounts of Type II IFN and a *Toxoplasma* growth inhibitory factor (Toxo-

GIF), and that these two substances were indistinguishable each other in the molecular weight and isoelectric point. The present study demonstrated that partially purified Type II IFNs prepared from the spleen cell cultures of the *Toxoplasma*- or BCG-infected mice potentiated the activities of the nonimmune mouse macrophages to kill *Toxoplasma* and L cells to inhibit the growth of the organism in vitro. These IFNs, however, had no effect on *Toxoplasma* multiplication in rabbit kidney cells (RK-13 cells) or guinea pig macrophages (data not shown) suggesting that the activation of macrophages and L cells is species specific. Nagasawa et al. [6] also reported that murine lymphokine prepared from the *Toxoplasma*-immune mouse spleen cells cultured with the *Toxoplasma* lysate antigen activated not only macrophages but also mouse kidney cells and embryonic fibroblasts. From these data, it may be possible that the enhanced anti-toxoplasma activity of activated macrophages and L cells was mediated by Type II IFN rather than typical macrophage-affecting lymphokines such as MIF and MAF.

The conditions required for Type II IFN-induced activation of macrophages were comparable with those for the microbicidal activity of lymphokine-treated macrophages against other intracellular pathogens, such as *Rickettsia tsutsugamushi* [5] and *Trypanosoma cruzi* [8]; killing *Toxoplasma* by the activated macrophages and growth inhibition of *Toxoplasma* in the IFN-treated L cells were dependent on both the IFN concentration and the period of pre-incubation of the cells with IFN.

Although the enhanced antitoxoplasma activity was clearly demonstrated in Type II IFN-treated macrophages and L cells, the resulting growth inhibitory effect

seemed to be somewhat different. The function of the activated macrophages was microbicidal, whereas that of the activated L cells was only microbiostatic and only transient. Employing mouse models, Nathan et al. [7] demonstrated that the peritoneal macrophages activated by incubation with the spleen cell factor (lymphokine) were capable of releasing a greatly increased amounts of H_2O_2 , and that the activities of releasing H_2O_2 and killing *Trypanosoma cruzi* were closely correlated each other. As in in vitro experiments [4, 7], the enhanced oxygen-dependent microbicidal activity may be responsible for the increased toxoplasmodicidal activity of Type II IFN-treated macrophages. Accordingly, the transient toxoplasmostatic activity of Type II IFN-treated L cells may be due to the absence of the phagolysosomal system [3] or of the oxygen-dependent microbicidal activity.

Nacy and Meltzer [5] showed a decreased infectivity of Rickettsiae for lymphokine-treated mouse macrophages. Our preliminary experiment showed that *Toxoplasma* incubated for 4 hr at 37°C in Toxo-IFN (250 U/ml) still retained its ability to penetrate and multiply in L cells as did those incubated with culture medium alone (data not shown). An interesting finding in this study was the fact that treating macrophages and L cells with Type II IFN before exposure to *Toxoplasma* resulted in a significant decrease in percentage of the infected cells and the number of intracellular parasites. Therefore, it could be speculated that the action of Toxo-IFN may be directed toward the cells, decreasing their susceptibility to *Toxoplasma* infection and increasing their activity of killing and/or inhibiting the growth of the intracellular organisms. The decreased infectivity of *Toxoplasma* for IFN-treated cells may be due to suppressed parasite penetra-

tion to the target cells. The mechanism, however, still needs further substantiation.

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要 約

マウスⅡ型インターフェロンによる培養細胞内トキソプラズマの増殖抑制: 白幡敏一, 清水亀平次 (帯広畜産大学獣医学科家畜微生物学教室)——トキソプラズマおよび BCG 感染マウスの脾細胞培養よりⅡ型インターフェロンを調製し, マウス培養細胞内でのトキソプラズマの増殖抑制効果について検討した。両 IFN サンプルともにマウス腹腔マクロファージや L 細胞内での本原虫の増殖を同様に抑制し, 特異性は認められなかった。Ⅱ型 IFN による原虫抑制効果は L 細胞よりもマクロファージに対しより効果的に作用した。Ⅱ型 IFN 処理細胞内におけるトキソプラズマの増殖抑制機序は供試細胞種により若干異なり, マクロファージ内では殺虫的に作用したが, L 細胞に対してはほんの静虫作用を賦与するのみで, 虫体の殺滅・消化は認められなかった。IFN 処理細胞による抗トキソプラズマ活性の発現は供試 IFN 量や処理時間に相関しており, 高単位の IFN で, より長時間処理された細胞ほど強い抑制効果を発揮した。また, Ⅱ型 IFN で24時間前処理後トキソプラズマを感染させた細胞では, 虫体接種1時間目と2時間目における細胞内虫体数や感染率が対照に比較して明らかに低下していた。