# HIGH CONCENTRATION OF RECOMBINANT MURINE INTERFERON-β ENHANCES THE GROWTH OF *ORIENTIA* (FORMERLY *RICKETTSIA*) *TSUTSUGAMUSHI* GILLIAM IN MOUSE L929 CELLS

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**Summary.** – We studied the effect of recombinant murine interferons (rMuIFNs) on the growth of *Orientia* (formerly *Rickettsia*) *tsutsugamushi* Gilliam in mouse L929 cells. Rickettsial growth was measured by flow cytometry. rMUIFN-γ inhibited the growth of *O. tsutsugamushi* at the concentrations of 100 IU/ml and 1,000 IU/ml in accord with previous reports. Relatively low concentrations (10 IU/ml and 100 IU/ml) of rMUIFN-β also inhibited the growth of *O. tsutsugamushi*. On the other hand, high concentrations (1,000 IU/ml and 10,000 IU/ml) of rMuIFN-β enhanced the growth of the rickettsia. This enhancement of rickettsial growth was blocked by anti-murine IFN-β monoclonal antibody (MoAb). rMuIFN-β also enhanced the growth of *Rickettsia sibirica* 246 in L929 cells to some extent.

**Key words:** rickettsia; *Orientia tsutsugamushi*; L929 cells; interferon

## Introduction

*Orientia* (formerly *Rickettsia*) *tsutsugamushi*, the causative agent of tsutsugamushi disease (scrub typhus), is an obligate intracellular bacterium which replicates within the cytoplasm of eukaryotic cells. The agent has been recently transferred from genus *Rickettsia* to a new genus, *Orientia* gen. nov., which belongs to the family *Rickettsiaceae* (Tamura *et al.*, 1995).

Attention has been paid to the effect of interferon (IFN) on the growth of rickettsiae because of their obligate intracellular parasitism. Mouse IFN inhibited the multiplication of *R. akari* in mouse L929 cells (Kazár *et al.*, 1971). IFN-γ inhibited the growth of *R. prowazekii* (Turco and Winkler, 1983, 1986), *R. conorii* (Jerrels *et al.*, 1986) and *O. tsutsugamushi* (Hanson, 1991a,b). Rickettsiae are able to induce acid-stable IFNs (IFN-α and/or IFN-β) in mice and in cultured cells. Acid-stable, IFN-like viral inhibitors were induced in mice infected with *R. prowazekii* (Kazár, 1966), and in cultured chick embryo cells infected with *R. typhii* (Kohno *et al.*, 1970). *R. prowazekii* also induced IFN-α/β in cultured mouse fibroblast cells (Turco and Winkler, 1990a). In *O. tsutsugamushi*-infected primary chick embryo cell culture, the appearance of an acid-stable, virus inhibitory protein followed an increase of infectious rickettsiae (Hanson, 1991a). The contribution of IFNs to the recovery from an acute infection or to the prevention of recrudescence of persisting rickettsiae is not entirely clear, but several studies have pointed to their potential importance (Hanson, 1991a; Li *et al.*, 1987; Turco and Winkler, 1991). Previous studies showed that the antirickettsial effect of IFN-α/β is less pronounced in general than that of IFN-γ (Turco and Winkler, 1990b). Therefore IFN-γ has been thought to play an important role in the host defense against a rickettsial infection. These observations raise a question about the role of IFN-α and/or IFN-β in *O. tsutsugamushi*

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**Abbreviations:** FCS = foetal calf serum; FITC = fluorescein isothiocyanate; IFN = interferon; MEM = Eagle's Minimal Essential Medium; MoAb = monoclonal antibody; PBS = phosphate-buffered saline; rMuIFN = recombinant murine interferon

infection. Recently, Hanson (1991b) reported the effect of IFN-γ and IFN-α/β on the growth of three strains of *O. tsutsugamushi* in two mouse fibroblast cell lines. In that study, a slight inhibitory effect on the growth of the Gilliam strain of *O. tsutsugamushi* in BALB/3T3 cells was shown by 300 to 450 IU of purified murine IFN-α/β per ml. But the effect was not very pronounced and was somewhat variable. In this study, we examined the effect of rMuIFN-β at various concentrations on the intracellular growth of *O. tsutsugamushi* assayed by flow cytometry. We report that a very high concentration of rMuIFN-β enhanced the growth of *O. tsutsugamushi* Gilliam in L929 cells. We also found that rMuIFN-β enhanced the growth of *R. sibirica* in L929 cells.

## Materials and Methods

*Cell culture.* Mouse L929 cells were purchased from Flow Laboratories, Inc., McLean, VA, USA. African green monkey BSC-1 cells were obtained from Dr. N. Tachibana of Oita Prefectural Institute of Public Health, Oita, Japan. The cells were grown in Eagle's Minimal essential Medium (MEM) supplemented with 5% foetal calf serum (FCS) in a humidified atmosphere of 5% $CO_2$ in air at 35°C.

*Rickettsiae.* The Gilliam strain of *O. tsutsugamushi* was obtained from Dr. N. Kawabata of Miyazaki Prefectural Institute of Public Health and Environmental Science, Miyazaki, Japan. Strain 246 of *R. sibirica* was obtained from Dr. N. Tachibana of Oita Prefectural Institute of Public Health. Both of the rickettsiae were grown in L929 cells in MEM supplemented with 2% FCS (maintenance medium) in a humidified atmosphere of 5% $CO_2$ in air at 35°C. Rickettsial suspensions were prepared from heavily infected L929 cells by disrupting the cells with a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 700 x g for 5 mins and the supernatant was saved and stored at -80°C.

*IFNs.* rMuIFN-β (1 x $10^7$ IU/ml, Matsuda *et al.*, 1986) was supplied by Basic Research Laboratory, Toray Industries, Inc., Tokyo. rMuIFN-γ (1 x $10^5$ IU/ml) was purchased from Serotec, Oxford, UK. The titers of IFNs were determined by a cytopathic effect inhibition assay (Yamamoto and Kawade, 1980) using L929 cells and vesicular stomatitis virus.

*Antibodies.* Anti-*O. tsutsugamushi* serum was obtained from guinea pigs 28 days after inoculation with a single intracerebral injection (Tamura *et al.*, 1984) of Gilliam strain propagated in BSC-1 cells. The immune serum titer against *O. tsutsugamushi* Gilliam as determined by the indirect fluorescence antibody test was 1:640. The immune serum was used at a dilution of 1:100. Anti-IFN-β MoAb (rat IgG1) was purchased from Seikagaku-Kogyo Co., Ltd., Tokyo. This antibody neutralized 10 IU/ml mouse IFN-β to the final activity of 1 IU/ml at a dilution of 1:400,000 (Kawade, 1980). The antibody was used at a final dilution of 1:400.

*Interferon treatment.* Monolayers of L929 cells in 12-well plastic tissue culture plates (Corning Glass Works, Corning, NY, USA) received maintenance medium containing an appropriate amount of rMuIFN-β or IFN-γ and were incubated for 24 hrs. After aspiration of the medium, the cells were inoculated with the rickettsiae at a multiplicity of infection of approximately 0.5 rickettsial particle per cell and adsorbed for 1 hr at 35°C. Unadsorbed rickettsiae were aspirated off, and each culture was fed with 1 ml of maintenance medium containing rMuIFN (continuous IFN treatment). In some experiments, cultures were fed with maintenance medium without IFN after adsorption of rickettsiae (IFN pretreatment). Untreated control cultures were inoculated with rickettsiae and treated with maintenance medium alone. Cultures were incubated at 35°C and processed for flow cytometry at various intervals.

*Preparation of samples for flow cytometry.* The fixation and staining of the rickettsia-infected cells were carried out basically according to the method of Laffin and Lehman (1990). Briefly, cells infected for 4 to 10 days were washed with phosphate-buffered saline (PBS), dispersed with 0.25% trypsin-0.1% ethylene diamine tetraacetate-PBS (warmend to 37°C), and washed with washing solution (PBS containing 10% FCS, 0.1% sodium azide and 0.002% Triton X-100). The cell suspensions were centrifuged at 5,000 rpm for 20 secs and the cell pellets were resuspended in PBS to approximately 1 x $10^6$ cells/ml. After centrifugation at 5,000 rpm for 15 secs, the cells were resuspended in 100 µl of cold PBS. Then 900 µl of -30°C cold methanol was added to the cell suspension, which was fixed by exposure to -30°C for a minimum of 15 mins. The fixed cells suspended in 90% methanol were stored at -30°C until staining. Rickettsial particles were stained by the indirect immunofluorescent antibody method, using an anti-*O. tsutsugamushi* guinea pig serum and fluorescein isothiocyanate (FITC)-conjugated anti-guinea pig IgG goat globulin (Cappel Laboratories, Cochranville, PA, USA) diluted 1:80 in PBS. Finally, stained cells were suspended in PBS and subjected to flow cytometry.

*Flow cytometry.* The green fluorescence of rickettsial particles within the host cells was measured by flow cytometry on an EPICS Elite flow cytometer (Coulter Corp., Hialeah, FL, USA). For each of the fluorescence profiles, 20,000 cells were analyzed. The excitation wave length was 488 nm. Data are presented as fluorescence profiles, with the cell frequency plotted as a function of the log of fluorescence intensity.

*Evaluation of the effect of rMuIFN-β on the growth of L929 cells.* 5.1 x $10^4$ L929 cells in 1 ml was seeded in each well of 12-well plastic tissue culture plates. rMuIFN-β was added to the wells at final concentrations of 10 – 1,000 IU/ml. The plates were then incubated at 35°C for 1 to 10 days. Each culture was fed every 4 days with 1 ml of fresh medium containing rMuIFN-β. At appropriate intervals, the cells were removed with 0.25% trypsin in PBS and counted by flow cytometry without staining.

## Results

### *Determination of the growth of O. tsutsugamushi by flow cytometry*

The normal growth pattern of *O. tsutsugamushi* in L929 cells was determined by flow cytometry (Fig. 1). The fluorescence intensity in the histograms represents both the num-

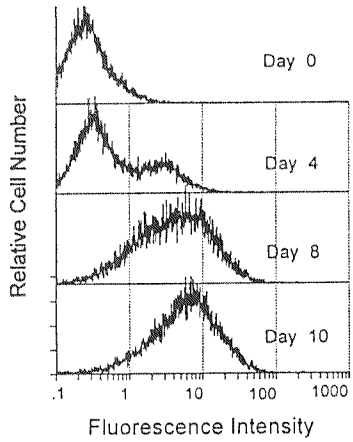


Fig. 1
**Profiles of normal growth of *O. tsutsugamushi* Giliam in L929 cells obtained by flow cytometry**
Cells were infected with *O. tsutsugamushi* and incubated for 4 to 10 days. Infected cells were fixed with cold methanol and stained by the indirect immunofluorescent antibody technique using the anti-Gilliam guinea pig serum and FITC-conjugated anti-guine pig IgG goat globulin.

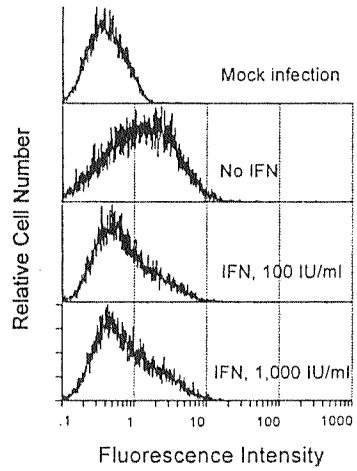


Fig. 2
**Effect of rMuIFN-γ on the growth of *O. tsutsugamushi***
L929 cells were treated with rMuIFN-γ for 24 hrs and then infected with *O. tsutsugamushi*. The infected cells were incubated for 6 days with rMuIFN-γ in the maintenance medium.

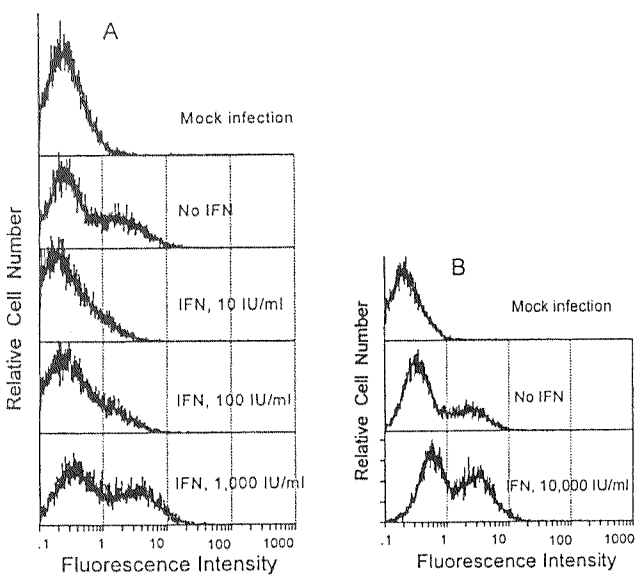


Fig. 3
**Effect of various concentrations of rMuIFN-β on the growth of *O. tsutsugamushi***
L929 cells were treated with 10 to 1,000 IU/ml (A), or 10,000 IU/ml (B) rMuIFN-β for 24 hrs, and then infected with *O. tsutsugamushi*. The infected cells were incubated for 6 dyas with the maintenance medium containing rMuIFN-β at the same concentration as that used for the pretreatment.

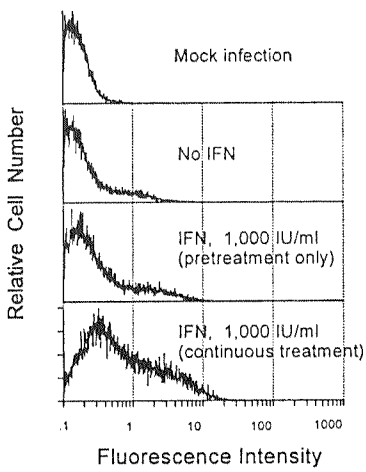


Fig. 4
**Effect of different treatments of L929 cells with rMuIFN-β on the growth of *O. tsutsugamushi***
Cells were treated with 1,000 IU/ml rMuIFN-β for 24 hrs, and then infected with *O. tsutsugamushi*. The infected cells were incubated for 6 dyas with or without rMuIFN-β (continuous IFN treatment and IFN pretreatment only, respectively).

ber of rickettsial particles in the cells and the relative number of infected cells. The fluorescence intensity gradually increased from the 4th to the 10th day of infection. The growth of *O. tsutsugamushi* was confirmed by visual fluorescence microscopy of the same samples.

### *Effect of rMuIFN-γ and rMuIFN-β on the growth of O. tsutsugamushi*

Fig. 2 shows the effect of rMuIFN-γ on the growth of *O. tsutsugamushi* in L929 cells. An observation on the 6th day of infection revealed that a treatment of the cells with rMuIFN-γ at the concentrations of 100 IU/ml and 1,000 IU/ml inhibited the growth of *O. tsutsugamushi*. A similar treatment of the cells with IFN-β at the concen-

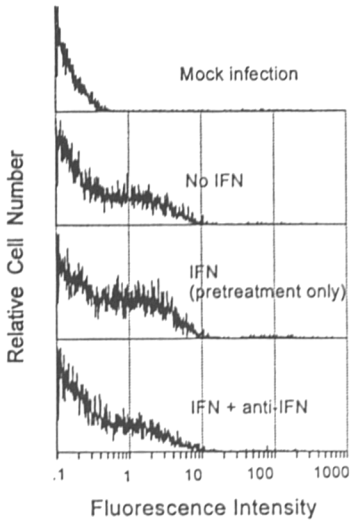


**Fig. 5**
**Effect of anti-IFN-β antibody on the growth enhancement of *O. tsutsugamushi* by rMuIFN-β**
L929 cells were treated with 1,000 IU/ml rMuIFN-β in the presence or absence of anti-IFN-β MoAb for 24 hrs, and then infected with *O. tsutsugamushi*. The infected cells were incubated for 6 days without rMuIFN-β.

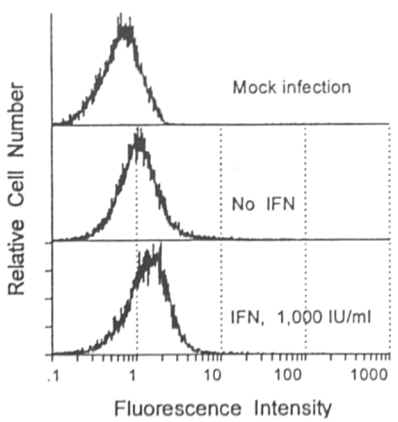


**Fig. 6**
**Effect of rMuIFN-β on the growth of *R. sibirica* in L929 cells**
Cells were treated with 1,000 IU/ml rMuIFN-β for 24 hrs, and then infected with *R. sibirica* strain 246. The infected cells were incubated for 6 days with the maintenance medium containing rMuIFN-β.

trations of 10 IU/ml and 100 IU/ml also inhibited the growth of the rickettsiae (Fig. 3A). On the other hand, 1,000 IU/ml rMuIFN-β enhanced the growth of *O. tsutsugamushi* (Fig. 3A). A similar enhancement was also observed even with 10,000 IU/ml rMuIFN-β (Fig. 3B). This growth enhancement was more pronounced in the case of continuous IFN treatment (see Materials and Methods) than in that of IFN pretreatment only (Fig. 4).

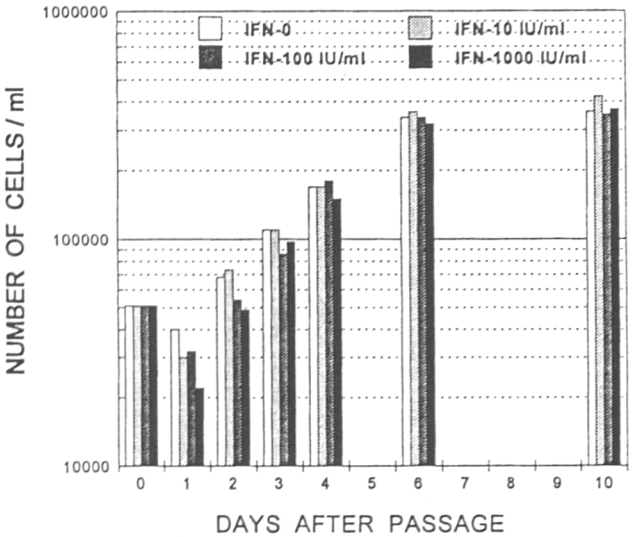


**Fig. 7**
**Effect of rMuIFN-β on the growth of L929 cells**
Cells were grown in the presence of 10 to 1,000 IU/ml rMuIFN-β.

*Effect of anti-IFN-β antibody on the growth enhancement by rMuIFN-β*

To confirm that the enhancement of rickettsial growth was actually mediated by IFN-β, the anti-IFN-β MoAb was added to rMuIFN-β-pretreated L929 cells. Then the cells were infected with *O. tsutsugamushi*, and the rickettsial growth was measured by flow cytometry. The enhancement of the growth of *O. tsutsugamushi* was blocked in the presence of the anti-IFN-β MoAb (Fig. 5).

*Effect of rMuIFN-β on the growth of R. sibirica*

The effect of IFN-β on the growth of *R. sibirica* in L929 cells was examined. A little enhancement of rickettsial growth was observed in the presence of 1,000 IU/ml rMuIFN-β (Fig. 6).

*Effect of rMuIFN-β on the growth of L929 cells*

The effect of rMuIFN-β on the growth of L929 cells was investigated. Uninfected L929 cells treated with 10 to 1,000 IU/ml rMuIFN-β grew in almost the same manner as the untreated cells (Fig. 7).

## Discussion

Several studies on the effect of IFN-γ, IFN-α and/or IFN-β on the growth of rickettsiae have been reported (Hanson, 1991a,b; Park and Rikihisa, 1991; Turco and Winkler, 1986,

1990b, 1991). Previous studies showed consistently that murine IFN-γ inhibits the growth of rickettsiae in mouse cells. On the other hand, the results of investigations of the effect of IFN-α/β on the growth of rickettsiae were variable and the effect was less pronounced than that of IFN-γ. In the present study, we found that high concentrations (1,000 IU/ml and 10,000 IU/ml) of rMuIFN-β enhanced the growth of *O. tsutsugamushi* Gilliam in mouse L929 cells. We demonstrated that the growth enhancement was IFN-β-mediated by means of the antibody to IFN-β. Hanson (1991b) reported recently that 300 to 450 IU of IFN-α/β per ml partially inhibited the growth of *O. tsutsugamushi* Gilliam in BALB/c mouse cells. Our finding is not contradictory to that because an apparent inhibition of rickettsial growth was observed with 10 IU/ml to 100 IU/ml rMuIFN-β.

In the studies on the growth of rickettsiae, an irradiation, and colchicine and daunomycin treatments have been used to inhibit the host cell growth to facilitate the observation of rickettsial replication (Hanson, 1987; Turco and Winkler, 1984). However, we used untreated L929 cells for the testing of the rickettsial growth inhibition in this study. Our experiment showed that the uninfected L929 cells treated with 10 IU/ml to 1,000 IU/ml rMuIFN-β replicated in almost the same manner as the untreated cells. Although we do not have any own data on the effect of rMuIFN-γ on the replication of L929 cells, there is a report that a treatment of uninfected L929 cells with 10 IU of cloned mouse IFN-γ per ml was not toxic (Turco and Winkler, 1984). These authors also reported that a treatment with cloned mouse IFN-γ and an infection with *R. prowazekii* led to killing of more than half of RAW267.3 cells (macrophage-like cells), but such cytotoxicity was not observed with L929 cells (Turco and Winkler, 1984). Therefore a little increase of the number of rMuIFN-treated L929 cells after inoculation of *O. tsutsugamushi* was considered almost of the same extent as that of rMuIFNs-untreated L929 cells.

It is very difficult to explain the mechanism(s) by which a high concentration of rMuIFN-β enhanced rickettsial growth in L929 cells. The mechanism of action of IFNs on intracellular replication of rickettsiae has not been studied extensively. Kazár *et al.* (1971) showed that mouse IFN had an inhibitory effect on the multiplication of *R. akari* in homologous L929 cells, and that the effect was mediated by the host cells. What concerns IFN-γ, it is almost sure that it does not affect the rickettsiae directly because murine IFN-γ does not induce an antirickettsial activity in human cells and the antirickettsial activity of IFN-γ is partially alleviated by cycloheximide (Gao *et al.*, 1993). Therefore IFN-γ probably interacts with specific plasma membrane receptors and triggers a series of intracellular events that lead to the appearance of IFN-γ induced gene products. These gene products may inhibit rickettsial growth by their direct action on the rickettsiae and/or by nutritional deprivation (Gao *et al.*, 1993; Winkler *et al.*, 1993). A similar process may be induced also by IFN-β. The nutritional state of the cell might be changed to become advantageous for rickettsial growth rather than to become worsened as a consequence of intracellular events tirggered by IFN-β at high concentration. The significance of this unexpected effect of IFN-β is at present unknown.

The flow cytometry is widely used in studies on the interaction between rickettsiae and host cells (Li and Walker, 1992; Rikihisa and Messick, 1993). In the present study, we used flow cytometry to measure the growth of *O. tsutsugamushi* and obtained good results. Usually, the growth of *O. tsutsugamushi* is measured either by an *in vitro* plaque assay, infected cell ratio assay, or direct particle count under a microscope. These methods, however, are laborious and time consuming, and sometimes result in variable data. The flow cytometry method avoids the weak points of these methods and enables to detect delicate differences in the growth status with good reproducibility.

High concentrations of rMuIFN-β enhanced the growth of *R. sibirica* as well. Therefore further studies should be carried out to determine whether this phenomenon occurs also in other intracellular bacteria besides rickettsiae.

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