Inhibition of *chlamydia trachomatis* growth by human interferon- α : mechanisms and synergistic effect with interferon- γ and tumor necrosis factor- α

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ABSTRACT

We have evaluated the effect of natural human interferon (IFN)- α on the growth of *chlamydia trachomatis* in human epithelial cells *in vitro* and revealed that IFN- α has reduced both growth and infectivity of *C. trachomatis*. The effect of IFN- α was reversed by the addition of exogenous L-tryptophan and iron to the culture medium, suggesting that antichlamydial effect of IFN- α was caused by depletion of intracellular tryptophan and iron, both of which are essential for chlamydial growth. When IFN- α was combined with another antichlamydial cytokines, IFN- γ and tumor necrosis factor (TNF)- α , the effect was synergistically enhanced. Therefore, IFN- α would act coordinately with other cytokines such as IFN- γ and TNF- α , and play an important role in host defense against infection and in the establishment of persistent chlamydial infection of host, in which the organism remains viable, but in a culture-negative state.

Chlamydia trachomatis is an obligate intracellular bacterium, which causes a wide spectrum of human diseases and is one of the most common sexually transmitted pathogens in the world. Upper genital tract infections with *C. trachomatis* in woman tend to have very serious repercussions, including the onset of pelvic inflammatory disease. This syndrome often results in severe and irreversible sequelae, such as infertility or ectopic pregnancy (5, 13).

Various cytokines have been demonstrated to restrict the growth of intracellular pathogens and are significant activators of host cell immune responses to infections. Interferon (IFN)- γ is required for resolution of chlamydial infections, and has been shown to inhibit the growth of chlamydia in cell culture (22). The mechanisms of the action of IFN- γ in cultured human cell lines have been elucidated and involve the induction of a cellular enzyme, indoleamine

2,3-dioxygenase (IDO), which causes a depletion of intracellular tryptophan. In addition to IFN- γ , two other cytokines such as tumor necrosis factor (TNF)- γ and interleukin (IL)-1 β have been shown to play a role in host defense against chlamydial infection (3, 24). Furthermore, it has been reported that these cytokines exerts the inhibitory effect synergistically when combined with each other (26).

IFN- α is a well recognized antiviral cytokine, in addition, with antiproliferative and immunomodulatory properties (1, 23). Beside its protective role against viral infection, IFN- α has been implicated in innate immunity against a variety of facultative and obligate intracellular non-viral pathogens such as *Leishmania donovani* and *Txoplasma gondii* (14, 16). Early works reported that IFN, reported to be IFN- α/β , showed to inhibit chlamydial growth (6, 20). However, the mechanisms of the action of IFN- α , and the interaction with other cytokines have not been intensively evaluated.

In this study, we examined the antichlamydial activity of natural IFN- α and evaluated the mechanisms of its action. Furthermore, since IFN- α is known to act synergistically with other cytokines

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such as IFN- γ and TNF- α , we also examined whether combination of IFN- α with other cytokines would exhibit more pronounced antichlamydial activity.

MATERIALS AND METHODS

Cytokines. Natural human IFN- α (sp. act. = 2×10^8 IU/mg), IFN- γ (sp. act. = 1×10^7 IU/mg) and TNF- α (sp. act. = 2×10^6 JRU/mg) were the products of Hayashibara Biochemical Laboratories Inc. (Okayama, Japan).

Cell lines and cell culture. HeLa (cervix epithelial cell) and A549 (airway epithelial cell) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in culture with Eagle's minimum essential medium (EMEM) (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), supplemented with 10% heat-inactivated fetal calf serum (FCS) (Thermo Trace Ltd, Melbourne, Australia) and 50 μ g/mL gentamycin at 37°C in a humidified atmosphere containing 5% CO₂.

Preparation of *C. trachomatis*. *C. trachomatis* serovar D/UW-3/Cx (ATCC VR-885) was employed in these studies. Chlamydial stocks for infecting epithelial monolayer were prepared in A549 cells by standard methods (11). Stocks were suspended in sucrose-phosphate-glutamate (SPG) medium (sucrose 75.0 g, KH₂PO₄ 0.52 g, Na₂HPO₄·12H₂O 3.07 g, glutamic acid 0.72 g/L) and frozen at -80°C until use.

Infection and treatment with cytokines. HeLa cells were plated at density of 2×10^4 cells per well in 96-well microtiter plates. Twenty-four hours later, the medium was replaced with EMEM containing 8% FCS and various concentrations of cytokines, and cells were incubated for further 24 h. The medium was removed and chlamydial stocks diluted in SPG were applied to develop 10^4 inclusions per well. After centrifugation at $700 \times g$ for 1 h, the cultures were resuspended in medium without cytokines and then incubated for 48 h. Cells were fixed with methanol and the amounts of chlamydia were assessed by ELISA.

Enzyme-linked immunosorbent assay (ELISA). Methanol-fixed HeLa cell monolayer in 96 well microplates were blocked with block Ace (Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) at room temperature for 1 h. After the wells were washed with PBS containing 0.05% Tween for three times, 50 μ L of FITC-conjugated mouse anti-chlamydial li-

popolysaccharide (LPS) antibody (1/50 dilution) (Denka Seiken Co., Ltd., Tokyo, Japan) was added to each well and the plates were incubated at room temperature for 2 h. After washing three times, 50 μ L of β -galactosidase-conjugated anti-mouse IgG antibody (1/2000 dilution) (American Qualex International, Inc., San Clemente, CA) was added to each well and the plates were incubated at room temperature for 2 h. The wells were washed three times and 100 μ L of 10 mM phosphate buffer (pH 7.8) containing 0.1 mg/mL 4-methyl-umbelliferyl β -D-galactopyranoside (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 1 mM MgCl₂ were added. After incubation at 37°C for 2 h, the fluorescence intensities (excitation at 355 nm, emission at 460 nm) were measured.

Assessment of chlamydia infectivity. HeLa cells were plated in 24 well culture plates at 1×10^5 cells/well and cultured for 24 h. Then, cells were treated with various doses of cytokines for 24 h and were infected with *C. trachomatis* to develop 5×10^4 inclusions per well. Forty-eight hours later, cells were washed once with medium, scraped into SPG and sonicated briefly. Serial dilutions of these suspensions were inoculated on fresh HeLa cell monolayer in 96 well microtiter plates as described above, and then incubated in the medium with 2 μ g/mL cycloheximide (Wako Pure Chemical Industries) for 48 h. Cells were fixed with methanol and the number of inclusions was assessed by staining with FITC-labeled mouse anti-chlamydia antibody (Denka Seiken).

Statistical analysis. Results were analyzed by Student's unpaired *t*-test and a *p* value of < 0.05 was taken to be significant. The 50% inhibitory concentrations (IC₅₀) were estimated by probit analysis. To assess the synergistic interaction, the combination index (CI) was calculated by median effect analysis using CalcuSyn Software (Biosoft Inc., Cambridge, U. K.), a computer software for automated dose-effect analysis (4). CalcuSyn analyzes the shape of the drug dose response curves for each drug or combination of drugs and calculates the combination index (CI) by median effect analysis described by Chou. CI values of < 0.9, 0.9–1.1, and > 1.1 indicate synergism, additive effect and antagonism, respectively.

RESULTS

Effect of IFN- α on chlamydial growth and infectivity
To assess the antichlamydial effect of test samples

more conveniently, we developed cell ELISA to measure the amounts of *C. trachomatis* in the cells. As shown in Fig. 1A, the fluorescence intensities in cell ELISA were well correlated with the number of inclusions in the same wells, indicating that our ELISA actually represents the amounts of chlamydial particles in cells.

We first examined the antichlamydial effect of human natural IFN- α derived from lymphoblastoid cell lines. HeLa cells were treated with various doses of IFN- α for 24 h and infected with *C. trachomatis* serovar D. Forty-eight hours later, chlamydial growth was measured by cell ELISA. As shown in Fig. 1B, the amounts of *C. trachomatis* were dose-dependently decreased by the treatment with IFN- α . IFN- α at 1000 IU/mL reduced the amounts of chlamydia by 52%. The infectivity of *C. trachomatis* was also reduced by IFN- α to the same extent as seen in cell ELISA. These results indicated that IFN- α actually inhibited chlamydial growth and reduced the number of infectious particles as reported previously (6, 20).

Assessment of the mechanisms of antichlamydial activity of IFN- α

Early studies have shown that, like IFN- γ , IFN- α also induces IDO expression, resulting the depletion of the cellular tryptophan (17). To investigate whether IDO is involved in the antichlamydial ac-

tivity of IFN- α , we examined the ability of excess L-tryptophan to reverse the effect of IFN- α . As shown in Fig. 2, the addition of excess L-tryptophan to the cell cultures partially but significantly reversed the inhibition of chlamydial growth mediated by IFN- α ($p < 0.05$). However, even at concentrations greater than 400 $\mu\text{g/mL}$, L-tryptophan could not completely suppress the effect of IFN- α (data not shown).

It has been shown that IFN- α induces depletion of intracellular iron content (2), and intracellular iron levels affect chlamydial growth in the host cells (19). Therefore, we next examined whether IFN- α would exert antichlamydial activity by inducing intracellular iron depletion. When excess FeCl_3 was added to the cultures, IFN- α -induced inhibition of chlamydial growth was significantly reversed ($p < 0.005$). Again, this effect was only partial and higher concentrations of FeCl_3 could not completely suppress the effect of IFN- α . When both L-tryptophan and FeCl_3 were added to IFN- α -treated cultures, the effect of IFN- α was almost completely suppressed ($p < 0.001$). These results suggested that depletion of both L-tryptophan and iron was implicated in the antichlamydial activity of IFN- α .

Combined antichlamydial activity of IFN- α with either IFN- γ or TNF- α

It has been reported that IFN- α shows synergistic

Fig. 1 Effect of IFN- α on chlamydial growth. (A) Comparison of cell ELISA with the number of Inclusions. (B) Antichlamydial activity of IFN- α . HeLa cells were treated with IFN- α for 24 h and infected with *C. trachomatis*. Forty-eight hours later, chlamydial growth was measured by cell ELISA (black bar) or by measuring infectivity (white bar). Data represents mean $\pm$ SD from three independent experiments.

Fig. 2 Effect of L-tryptophan and iron on IFN- α -induced inhibition of chlamydial growth. HeLa cells were treated with IFN- α in the presence or absence of L-tryptophan (400 μ g/mL) or FeCl₃ (200 μ M) for 24 h and infected with *C. trachomatis*. Forty-eight hours later, chlamydial growth was measured by cell ELISA. Data represents mean $\pm$ SD from seven independent experiments. * p < 0.05, ** p < 0.01, *** p < 0.005 compared with IFN- α alone.

antiviral and antiproliferative effects when combined with IFN- γ or TNF- α (8, 12, 27). Therefore, we next examined the effect of IFN- α combined with IFN- γ or TNF- α on chlamydial growth. As shown in Fig. 3, both IFN- α plus IFN- γ and IFN- α plus TNF- α combinations showed more pronounced activity than those of each cytokine alone. The IC₅₀ values of IFN- α , IFN- γ and TNF- α alone were 1800 IU/mL, 40 IU/mL, and 210 JRU/mL, respectively (Table 1). On the other hand, when combined with two cytokines at ratio 1 : 1, the IC₅₀ values of each combination decreased more than 10 times less than those of each cytokine alone. Thus, when combined with 2.2 IU/mL IFN- α and 2.2 IU/mL IFN- γ or 11 IU/mL IFN- α and 11 JRU/mL TNF- α , 50% inhibition was achieved. To determine whether these combined effects were synergistic, CI values were calculated by median effect analysis using CalcuSyn software. CI values of < 0.9, 0.9–1.1, and > 1.1 indicate synergism, additive effect and antagonism, respectively. The CI values at IC₅₀ for IFN- α + IFN- γ and IFN- α + TNF- α combinations were 0.056 and 0.060, respectively, indicating strong synergy. Thus, IFN- α acted synergistically with IFN- γ or TNF- α in inhibiting chlamydial growth.

DISCUSSION

IFN- α plays an essential role in many antiviral immune responses. The role of IFN- α in the defense against non-viral agents is considerably less studied, and functional relationships have not been issued. The antichlamydial activity of IFN- α was first reported 30 years ago by using IFN- α preparation induced by duck hepatitis virus in chicken embryos (25). However, the precise roles of IFN- α against chlamydial infection still remain unclear. In this study, we examined antichlamydial activity of natural IFN- α and revealed that IFN- α inhibited chlamydial growth as reported previously.

Antichlamydial effect of IFN- α was reversed by the addition of excess L-tryptophan. This result suggests that IFN- α exerts antichlamydial activity by inducing IDO, which causes a depletion of intracellular tryptophan as reported previously (17). However, the suppressive effect of L-tryptophan on the action of IFN- α was only partial, suggesting that another mechanism would be implicated in the action of IFN- α . We found that the addition of iron, which is also essential for chlamydial growth (19), partially reversed the effect of IFN- α . Furthermore, the addition of both L-tryptophan and iron resulted in complete suppression on the effect of IFN- α . It has been reported that *C. trachomatis* requires intracellular iron for its growth, and limitation of host cell iron contents causes a reduction in chlamydial growth (19). Furthermore, stimulation with the mixture of IFN- γ , TNF- α , IL-1 and LPS inhibits chlamydial growth mediated by at least in part depleting intracellular iron in epithelial cells (10). Since it has been reported that IFN- α induces depletion of intracellular iron in human epidermoid cancer cells (2), our result suggests that one of the mechanisms of the action of IFN- α is to deplete intracellular iron and restrict iron availability of chlamydia growing in the host cells. The precise mechanisms by which IFN- α reduces intracellular iron remain unclear. It has been shown that IFN- γ reduces transferrin receptor expression and decreases cellular iron store, which appears to account for antimicrobial activity of IFN- γ against *L. pneumophila* and *H. capsulatum* in macrophages (15). However, we did not detect any changes in transferrin receptor levels in IFN- α -treated HeLa cells (unpublished data). Further study must be done to identify the mechanisms by which IFN- α modulates intracellular iron levels.

Synergistic interactions between cytokines have been shown to contribute to the inhibition of pathogen: the combination of IFN- γ with TNF- α synergis-

Fig. 3 Antichlamydial effects of IFN- α combined with IFN- γ (A) or TNF- α (B). HeLa cells were treated with IFN- α combined with IFN- γ or TNF- α at ratio 1:1 and infected with *C. trachomatis*. Forty-eight hours later, chlamydial growth was measured by cell ELISA. Data represent mean $\pm$ SD from three independent experiments.

Table 1 Analysis of IC_{50} and CI for IFN- α , IFN- γ , TNF- α and their combinations

Combinations	IC_{50} [IU or JRU/mL]	CI
IFN- α	1800	–
IFN- γ	40	–
TNF- α	210	–
IFN- α + IFN- γ (ratio 1 : 1)	2.2	0.056
IFN- α + TNF- α (ratio 1 : 1)	11	0.060

tically inhibits *C. pneumoniae* growth (26), and IL-1 enhances antichlamydial effect of IFNs by increasing the amount of IDO activity (3). In this study, we

demonstrated that combination of IFN- α with either IFN- γ or TNF- α showed more pronounced antichlamydial activity than that of each cytokine alone. Furthermore, we assessed the synergism between IFN- α and two cytokines by applying the IC_{50} values to CalcuSyn Software analysis. We found that combination of IFN- α with either IFN- γ or TNF- α had a CI values below 1, indicating that there was strong synergy between IFN- α and IFN- γ or TNF- α .

It is unclear whether IFN- α actually has an important role in host immune response to chlamydial infection, since there is no report to assess the *in vivo* role of IFN- α during chlamydial infection.

However, early *in vitro* works reported that various cells such as fibroblast and macrophages produce IFN- α in response to chlamydial infection to modulate host cell physiology (7, 9, 21). Furthermore, in addition to IFN- α , infection of *C. trachomatis* induces the secretion of an array of cytokines including IL-1 α , IL-6, IL-8, IL-18 and TNF- α in epithelial cells (18, 28), suggesting that synergistic interaction between IFN- α and other cytokines is likely to occur within the infectious environment. Therefore, together with our results, we suppose that during the course of chlamydial infection, IFN- α produced by infected cells would act coordinately with other cytokines such as IFN- γ and TNF- α , and play an important role in the immunological persistence such as host defense against infection and in the establishment of persistent chlamydial infection of host. Further investigations on the role of IFN- α may lead to the development of new therapeutic approaches toward the treatment of many sexually transmitted diseases caused by *C. trachomatis* infection.

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