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Tryptophan Reversal of Recombinant Human Gamma-interferon Inhibition of *Chlamydia trachomatis* Growth

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Abstract. Recombinant human gamma-interferon was shown to inhibit the growth of *Chlamydia trachomatis* (L_2/434/Bu) in HEp-2 cells. This inhibition could be reversed by the addition of tryptophan. The effect of tryptophan was dose dependent and determined by the interferon concentration. At low concentrations of interferon, the addition of tryptophan completely restored *C. trachomatis* infectivity, whereas at high concentrations (100–1000 IU/ml) the effect of interferon could not be totally reversed. The reversal effect of tryptophan could be achieved even when the addition was 48 h after infection. The probability that tryptophan degradation induced by gamma-interferon might be the mechanism involved in its antichlamydial activity is discussed.

Chlamydiae are obligate intracellular bacteria that parasitize the host cell for nutrients and energy [13]. In the course of infection, the endocytosed parasites convert from their infectious form, elementary bodies (EBs), to their replicating form, reticulate bodies (RBs). After a period of binary fission, the latter transform into EBs within the inclusion present in the host cytoplasm. There are two known species of chlamydiae: *Chlamydia trachomatis* and *Chlamydia psittaci*. Different serovars of *C. trachomatis* have been associated with clinically distinct infections ranging from hyperendemic trachoma to sexually transmitted infections and pneumonia. *Chlamydia psittaci,* the etiologic agent of psittacosis/ornithosis, has a broad host range, which includes avian and mammalian species as well as man, in whom it causes pneumonia with systemic involvement [10]. The lymphogranuloma venereum (LGV) serovar of *C. trachomatis* infects both epithelial and lymphoid tissue, while non-LGV serovars are thought to grow only in epithelial cells [15].

Chlamydia trachomatis infection can be persistent, and reinfection commonly occurs [9, 12]. The factors that might modulate *C. trachomatis* infection to become persistent in vivo are unknown. Persistent infection might be a result of an inadequate supply of essential host cell nutrients [1, 3, 4] or inappropriate antibiotic treatment. Moreover, evidence showing that reactivation of apparently resolved infection occurs under conditions of immunosuppression [16] implicates the involvement of the immune system in persistent infection.

Gamma-interferon is a lymphokine produced by T-lymphocytes in response to antigenic stimuli. In a previous study we have shown that growth of *C. trachomatis* in vitro is inhibited by human gamma-interferon (h. gamma-IFN) [14]. The mode of action of interferon on chlamydia is unknown. Pfefferkorn has recently reported [11] that h. gamma-IFN blocks the growth of *Toxoplasma gondii* in human fibroblasts by inducing the host cells to degrade tryptophan (tryp), probably through the induction of indolamine 2-3-dioxygenase by interferon [17]. In contrast, De la Maza et al. [8] have shown that the inhibition of *C. trachomatis* growth and cell proliferation by murine gamma IFN in McCoy cells was independent of tryptophan concentration.

The purpose of this study was to investigate the influence of tryptophan on the inhibition of *C. trachomatis* (L_2/434 serovar) growth induced by recombinant h. gamma-IFN.

Materials and Methods

Cells. HEp-2 cells, originating from a human carcinoma of the larynx, were used to carry out the gamma IFN assays [14]. The HEp-2 cells were grown in minimum essential medium (MEM) (Biological Industries, Israel), supplemented with 10% fetal calf

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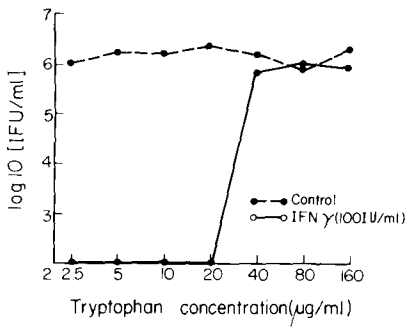

Fig. 1. The effect of tryptophan concentration on the inhibition of *Chlamydia trachomatis* growth in cells treated with gamma-interferon. The one-step growth experiment (*Materials and Methods*) was done on HEp-2 cultures pretreated with either control growth medium or medium containing 100 IU/ml recombinant h. gamma-IFN for 24 h. The cultures were then infected at MOI of 1. After 48 h, the cultures were harvested, frozen, and titrated on HEp-2 cells to determine the yield in IFU/ml. The results represent one of three experiments.

serum (FCS) (Biological Industries, Israel), glutamine, and antibiotics. BGM cells from monkey kidney cells were grown in RPMI medium 1640 supplemented as above.

***Chlamydia trachomatis* growth and purification.** The *Chlamydia trachomatis* serovar, lymphogranuloma venereum (L_2/434/Bu), was grown in BGM cells in RPMI supplemented with 5% FCS, 1% glucose, 0.15% bicarbonate, 100 μg/ml streptomycin, 10 μg/ml glutamine, 10 μg/ml fungizone, and 1 μg/ml cycloheximide. The chlamydiae were harvested and purified as previously described [14].

Chlamydial growth in medium lacking tryptophan. MEM was prepared without tryptophan, with a "Select Amine" kit (Gibco) supplemented with L-tryptophan (Biological Industries, Israel) at the appropriate concentrations, 5% FCS, 10 μg/ml glutamine, 100 μg/ml streptomycin, 1% glucose, and 0.15% bicarbonate.

Interferon. Human recombinant gamma-interferon was a gift from Inter-Yeda, Israel. It was stored at 4°C, diluted in distilled water to 3×10^6 international units per milliliter (IU/ml).

One-step growth–yield assay. HEp-2 cells were seeded in 96-microwell plates at 2×10^4 cells/well. The following day, medium was changed to cell growth medium (MEM without tryptophan) supplemented with 5% FCS, streptomycin, and glutamine, to which interferon and tryptophan at various concentrations were added. Cells were incubated at 37°C for 24 h. Purified *C. trachomatis* was diluted in *C. trachomatis* growth medium (as above) without cycloheximide. The HEp-2 cells were infected at a multiplicity of infection (MOI) of 1. After 1 h adsorption at 37°C the inoculum was removed, cells were washed with 300 μl of medium, and 150 μl/well of *C. trachomatis* growth medium with the appropriate tryptophan concentration was added. Samples were run in triplicate. After an additional 48 h of incubation, the infected cells in the microwell plate were scratched into the medium inside the well. Triplicate wells were pooled, and the samples were frozen at −70°C. Each sample was titrated on HEp-2 cells in the presence of *C. trachomatis* growth medium containing cycloheximide as previously described [14]. After 48 h, the cells were fixed for 10 min in absolute ethanol, and an immunoperoxidase assay (IPA) was performed on the plate. The final results of the titration were expressed as inclusion-forming units per millimeter (IFU/ml). Figures 1–3 demonstrate representative experiments and not aggregate results.

Electron microscopy (EM). Samples were prepared according to the method of Biberfeld [5]. HEp-2-infected cells were fixed in 2% glutaraldehyde in cacodylate buffer for 60 min and washed twice with cacodylate buffer. Postfixation was done with 1% OsO_4 for 60 min followed by dehydration in alcohol and three washes with propylene oxide. Embedding was done in Araldite 502. The blocks were sectioned and stained with uranyl acetate and lead citrate. Electron micrographs of the thin sections were taken with a Phillips 201C transmission electron microscope.

Results

The influence of tryptophan on *Chlamydia trachomatis* inhibition by h. gamma-IFN was tested. A concentration of 100 IU/ml of gamma-IFN that was previously shown to completely block the *C. trachomatis* infection in the standard growth medium [14] was used, and tryptophan concentrations ranging between 2.5 and 160 μg/ml were added. The yield of chlamydia (IFU/ml) was determined by a one-step growth experiment (see *Materials and Methods*). In the presence of increasing concentrations of tryptophan, the inhibitory effect of 100 IU/ml of gamma IFN on chlamydial growth was reversed (Fig. 1). A minimal tryptophan concentration of 40 μg/ml was required to achieve almost complete *C. trachomatis* recovery from interferon inhibition.

Progressively more gamma-IFN was required to inhibit the chlamydia as the tryptophan concentration in the medium was raised (Fig. 2); i.e., at 5 μg/ml of tryptophan complete inhibition of *C. trachomatis* by interferon occurred at 36 IU/ml, whereas at 40 μg/ml the same effect was obtained with 100 IU/ml. The ED_{50}'s (the dose of interferon required to reduce the yield by 50%) for the recombinant h. gamma-IFN were elevated as the tryptophan present in the reaction medium was raised. In the presence of 5 and 10 μg/ml tryptophan, the ED_{50} was 4.6–5.0 IU/ml; in the presence of 20 and 40 μg/ml tryptophan, the ED_{50} was elevated to 23 and 33 IU/ml, respectively.

To determine the effectiveness of tryptophan in the reversal of gamma-interferon inhibition of *C. trachomatis* at different points during infection, HEp-2 cell cultures were treated with 100 IU/ml of gamma-IFN in the presence of 5 μg/ml tryptophan

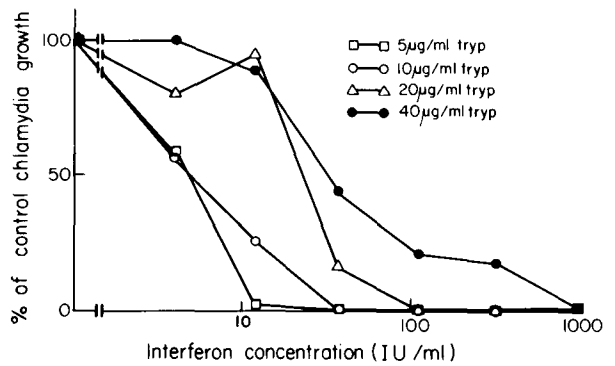

Fig. 2. Dose–response curve for gamma-interferon in media with four tryptophan concentrations. The one-step growth experiment (*Materials and Methods*) was carried out on HEp-2 cell cultures treated with three-fold dilutions of h. gamma-IFN in four media that differed only in tryptophan content. The results are expressed as percentage of IFU/ml observed in control cultures not treated with interferon. The results represent one of three experiments.

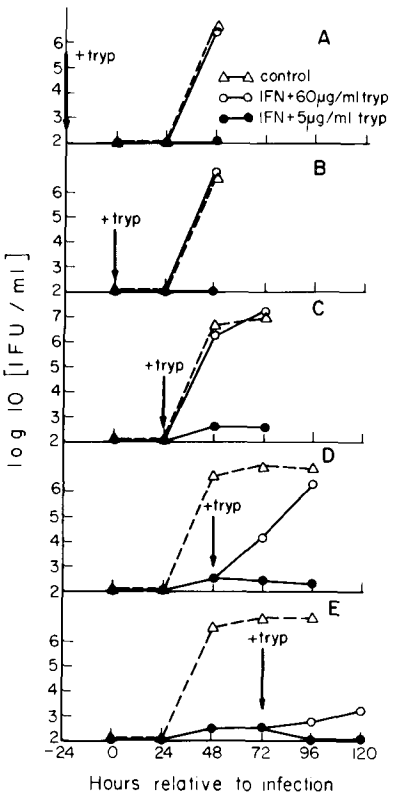

Fig. 3. Time course of tryptophan reversion of *Chlamydia trachomatis* inhibition by gamma-interferon in HEp-2 cells expressed as titer of chlamydia produced in the culture. Cultures were pretreated with 100 IU/ml gamma-IFN in the presence of 5 μg/ml tryptophan for 24 h and infected at an MOI of 1. Cells not treated with interferon served as control. At various times cultures (control and gamma-IFN pretreated) received an additional 5 or 60 μg/ml tryptophan (final concentration) as follows: (A) 24 h before infection, (B) on the same day as infection, (C) 24 h after infection, (D) 48 h after infection, and (E) 72 h after infection. The results represent one of three experiments.

and infected with *C. trachomatis* at a MOI of 1. Cells that were not treated with interferon served as a control. At various times after infection, tryptophan was added so that the final concentration in the medium was either 5 or 60 μg/ml. The effect of tryptophan was followed by determining the yield (IFU/ml) 24 and 48 h after its addition. Tryptophan was added 1 day prior to infection (the day of IFN treatment: −24 h), on the day of infection (day 0), or 24, 48, and 72 h after infection.

Inhibition of *C. trachomatis* by gamma-IFN could be fully reversed even when the tryptophan was added up to 24 h after infection (Fig. 3A–C). When tryptophan was added 48 h after infection, *C. trachomatis* could be recovered to the extent of 30% of optimal yield (Fig. 3D). When the tryptophan was added 72 h after infection, the reversion of *C. trachomatis* infection was less than 1% (Fig. 3E).

The stagnant state of *C. trachomatis* in interferon-treated cells 24 and 48 h after infection (Fig. 3C and D) differed from that of *C. trachomatis* in newly infected cells, as demonstrated by the shortening of the time of production of infective particles. The yield after 24 h following tryptophan addition was already 2×10^6 IFU/ml (Fig. 3C) while, at 24 h after regular infection, almost no infectivity of *C. trachomatis* was detected (Fig. 3A and B).

The development of chlamydia in the presence of gamma-IFN and varying concentrations of tryptophan was followed by electron microscopy. HEp-2 cells were pretreated for 24 h with 100 IU/ml of recombinant gamma-IFN in the presence of either 10 or 60 μg/ml tryptophan and infected with *C. trachomatis* at MOI of 1. The infected cells were examined by electron microscopy at 24 and 48 h after infection. The control cultures received 10 or 60 μg/ml tryptophan without gamma-IFN. At 24 h, 70% of the control cells had inclusion bodies which contained RBs and EBs in an approximate ratio of 8 : 1 (Fig. 4C). In most of the cells treated with gamma-IFN (100 IU/ml) and 10 μg/ml tryptophan, infection could not be detected by EM, although occasionally a reticulate body (Fig. 4A) or, more rarely, an elementary body could be found. In contrast, when the cells were pretreated with 100 IU/ml gamma-IFN and 60 μg/ml of tryptophan (Fig. 4B), 50% contained inclusion bodies with an RB/EB ratio of

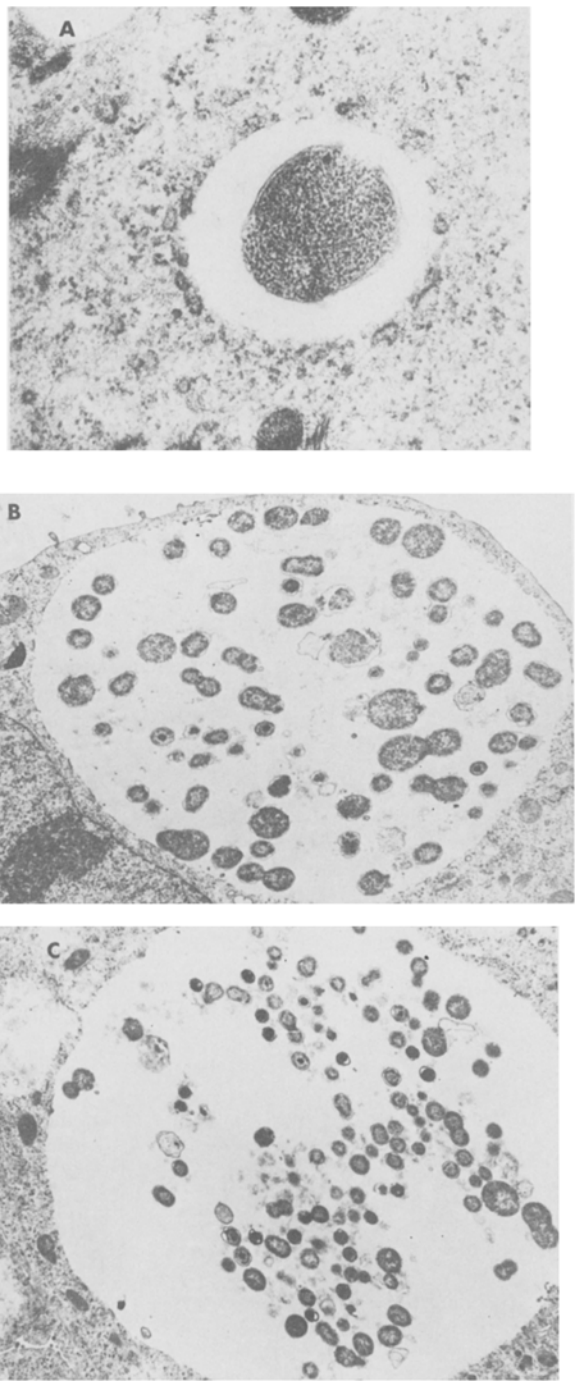

Fig. 4. Electron micrograph of HEp-2 cells infected with *Chlamydia trachomatis* 24 h after infection: (A) Treatment with tryptophan (10 μg/ml) and h. gamma-IFN (100 IU/ml). ×33,750. (B) Treatment with tryptophan (60 μg/ml) and h. gamma-IFN (100 IU/ml). ×5250. (C) Control, no interferon treatment, tryptophan 10 μg/ml. ×5250.

21 : 1. At 48 h after infection, the control cells exhibited inclusion bodies that occupied most of the cell and had RB/EB ratios of 1 : 3. In IFN-treated cells that received 10 μg/ml tryptophan, no infection could be demonstrated, and only a few cells had inclusions that contained a reticulate body. In contrast, cells treated with 100 IU/ml of interferon and 60 μg/ml tryptophan showed large inclusion bodies with RB/EB ratios of 2 : 3.

Discussion

The present study demonstrates that recombinant human gamma-interferon inhibited the growth of *Chlamydia trachomatis* L_2/434 serovar to the same extent as h. gamma-IFN purified from human lymphocytes as previously described [14].

The antiviral and growth-modulating activities of the alpha-, beta-, and gamma-interferons appear to be mediated by different sets of cellular mechanisms [7]. As for chlamydia, the question arises whether the intracellular parasite, which is separated from the hostile cell environment by the chlamydial wall, outer membrane, and cytoplasmic inclusion membrane, is inhibited by the enzymes that accumulate during the establishment of antiviral activity. It may be related to the deprivation of necessary metabolites caused by h. gamma-IFN, which has been shown to cause tryptophan degradation in human fibroblasts [11]. Inhibition of chlamydial growth as a result of the deprivation of necessary metabolites, such as certain amino acids, has been previously described and shown to be serovar-specific [2].

In the present study, we have found that growth of the L_2/434 serovar in HEp-2 cells require a minimal concentration of 2.5–5.0 μg/ml tryptophan to achieve optimal yield (data not shown). Inhibition of *C. trachomatis* L_2/434 serovar by recombinant h. gamma-IFN could be overcome by the addition of tryptophan when the HEp-2 cells were pretreated with low concentrations of gamma-IFN (Figs. 1 and 2). Furthermore, the reversal effect of tryptophan could be achieved even when the addition was 48 h after infection (Fig. 3).

These results differ from those of De la Maza et al. [8], which have shown that the antiproliferative activity and antichlamydial activity of recombinant murine gamma interferon was independent of tryptophan concentration. However, their work was carried out under different conditions, which included the *C. trachomatis* L_1 serovar and murine gamma IFN in McCoy cells, whereas the present study was performed with human gamma IFN and L_2(434/bu) in HEp-2 cells. The possibility that tryptophan degradation may be related to the mechanism of gamma-IFN inhibition of chlamydia, as was

shown in the present study, is further supported by the recent communication of Byrne [6], who demonstrated that the mechanism of human gamma-IFN-mediated inhibition of *C. psittaci* growth in T24 cells is reversed by tryptophan. Further studies are required to explore the significance of different factors, such as chlamydial strain, cells used, and the interferon origin, on the tryptophan reversal of gamma-interferon inhibition.

The reversal effect of tryptophan on *C. trachomatis* replication in cells pretreated with gamma-IFN reveals a number of features that require discussion. When tryptophan was added 24 h after infection, the recovery of the *C. trachomatis* infectivity was faster, as compared with the addition of tryptophan on the day of infection or on the day of interferon pretreatment (Fig. 3). This might imply that the inhibition by gamma-IFN is not limited to the EB that had entered the cells, but to a later stage in the *C. trachomatis* developmental cycle. This is further supported by our electron microscopy data. We have detected reticulate bodies more frequently than elementary bodies with both recombinant and lymphocyte IFN-treated cells 24 and 48 h after infection [14].

When the cells were pretreated with a relatively high concentration of gamma-IFN (100–1000 IU/ml), its inhibitory effect could not be completely reversed with tryptophan (Fig. 2). The latter results may be explained by the possibility that at high concentrations of gamma-interferon, the phagosome-enclosed chlamydia may be harmed, thereby permitting phagolysosome fusion and the destruction of the parasite. A second possibility is that in cells pretreated with high concentrations of gamma-interferon, an irreversible inhibitory effect on cell metabolism results in inhibition of *C. trachomatis* replication. Experiments are in progress to differentiate between these two possibilities.

Tryptophan reversal of recombinant human gamma-interferon inhibition of *C. trachomatis* growth might be of importance in clarifying the delicate relationship between chlamydia and their hosts. Furthermore, knowledge of the mechanisms by which an irreversible inhibition of chlamydia can be obtained by gamma-IFN may be useful in the development of an efficient chlamydiacidic drug.

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