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Inhibition of *Rickettsia conorii* Growth by Recombinant Tumor Necrosis Factor Alpha: Enhancement of Inhibition by Gamma Interferon†

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Purified human recombinant tumor necrosis factor alpha (rTNF-α) inhibited the growth of *Rickettsia conorii* (Casablanca strain) in HEp-2 cell culture. The effect was observed when the cells were pretreated with rTNF-α or when rTNF-α was added after adsorption of the rickettsiae. The inhibitory effect of rTNF-α on rickettsial growth was enhanced by gamma interferon. Cycloheximide had no effect on inhibition of the rickettsial yield, suggesting that de novo protein synthesis is not required for the inhibitory effect of rTNF-α. The addition of tryptophan partially abolished the inhibitory effect of rTNF-α and rTNF-α plus gamma interferon.

Rickettsiae are obligate intracellular gram-negative bacteria. They include three groups: the spotted fever, typhus, and scrub typhus groups (23). *Rickettsia conorii*, which belongs to the spotted fever group, has a wide geographic distribution and is transmitted to humans by bites from ticks (22). The rickettsiae are especially invasive for the endothelial cells of blood capillaries (21).

Tumor necrosis factor (TNF) is a macrophage-derived peptide that is known to have diverse effects on both physiologic and immunologic systems (1). It was initially identified as a serum factor that could exert antitumor activity in mice injected with bacterial lipopolysaccharide (2). TNF has been demonstrated to play a possible role in the host defense mechanism against several pathogens, including *Trypanosoma cruzi* (25), *Plasmodium falciparum* (16, 17), *Listeria monocytogenes* (5), and, more recently, *Chlamydia trachomatis* (24).

The purpose of this study was to investigate, in in vitro systems, the influence of human recombinant TNF alpha (rTNF-α) and the effect of both rTNF-α and gamma interferon (IFN-γ) on the yield of infective *R. conorii* in HEp-2 cells.

MATERIALS AND METHODS

Cells. BGM (an African green monkey kidney line) and HEp-2 cells (originating from human carcinoma of the larynx), both obtained from Flow Laboratories, were grown in RPMI 1640 and in minimal essential medium, respectively, with glutamine and antibiotics (Biological Industries, Beit Haemek, Israel) and 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.).

Rickettsial propagation. BGM cells were grown in 75-cm^2 flasks (Nunc, Roskilde, Denmark) in antibiotic-free RPMI 1640 medium supplemented with 10% fetal calf serum and 1% glutamine. After 48 h the cells were infected with *R. conorii* at an input multiplicity of infection of 1 to 2. After 2 h of rickettsial adsorption, medium containing 1 μg of cycloheximide per ml was added to the infected cells; 96 h later the cells were removed with beads, shaken by vortexing for 2 min, and sonicated in a Bransonic 12 for 30 s. The debris was pelleted by 10 min of centrifugation at 250 × *g*. The supernatant was centrifuged again for 10 min at 26,000 × *g* in a Sorvall centrifuge, and the pellet was suspended in buffer (0.01 M sodium phosphate [pH 7.2] containing 0.25 M sucrose and 5 mM L-glutamic acid) and stored at −70°C until used.

One-step growth-yield assay for rickettsiae. HEp-2 cells were grown in 96-well plates (Nunc) for 24 h (2 × 10^4 to 3 × 10^4 cells per well) in antibiotic-free RPMI 1640 supplemented with 10% fetal calf serum and 1% glutamine. When cells had reached confluency, the culture medium was decanted and the cells (triplicate wells) were treated with medium alone (control), with various dilutions of rTNF-α, with various concentrations of IFN-γ, or with both rTNF-α and IFN-γ together. After the cells were incubated for an additional 24 h, the medium was removed and the cells were infected with *R. conorii* (Casablanca strain) at a multiplicity of infection of 0.1. After 96 h, the cells were scraped from the wells and frozen at −70°C until titration. Treatment with rTNF-α or rTNF-α plus IFN-γ was also done after the rickettsial adsorption, up to 96 h postinfection.

The effect of cycloheximide on the rTNF-α inhibition of rickettsial growth was examined by the addition of 10 μg of cycloheximide per ml to the HEp-2 cells treated with rTNF-α 24 h before infection with *R. conorii*; cycloheximide was added again after adsorption up to 96 h postinfection.

To examine the influence of tryptophan, we added an excess of tryptophan (100 μg/ml; Biological Industries, Beit Haemek, Israel) to RPMI 1640 medium after the rickettsial adsorption.

Plaque assay for rickettsiae. HEp-2 cells were grown in 96-well plates (Nunc) (2 × 10^4 to 3 × 10^4 cells per well). After 48 h, triplicate 50-μl samples of serial 10-fold dilutions of rickettsial inoculum were added. After 2 h, 100 μl of medium containing 0.5% agarose and 1 μg of cycloheximide per ml was added to each well. Four days later 0.01% neutral red in Hanks balanced salt solution was added, and after 6 to 12 h the plaques were counted. The final titration results were expressed as PFU per milliliter.

Cytotoxicity test. For the cytotoxicity test, cell viability was determined by measurement of [^{3}H]uridine uptake by

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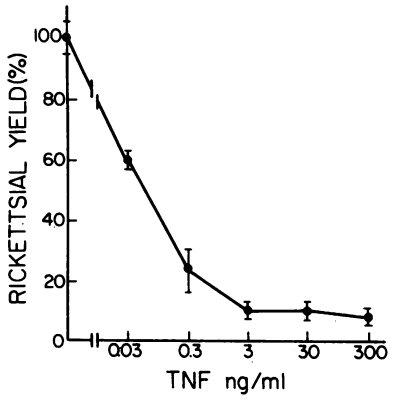

FIG. 1. *R. conorii* inhibition by rTNF-α as measured by the plaque assay. After 24 h of HEp-2 cell exposure to growth medium containing rTNF-α at various concentrations, the cells were infected with *R. conorii* at a multiplicity of infection of 0.1; at 96 h postinfection, samples of the infected cells were collected and sonicated, and titers were determined for each of the concentrations. Data are from one of five experiments.

HEp-2 cells as described by Smith and Nicklin (13). The percentage of cytotoxicity in the infected cells was calculated with reference to uridine uptake in the uninfected cells undergoing parallel treatment.

IFN. Human IFN-γ was a gift from Inter-Yeda, Inc., Rehovot, Israel.

TNF. Human rTNF-α (300 μg of protein per ml; produced by Cetus Co., Emeryville, Calif.) was kindly provided by A. Izental, National Cancer Institute, Bethesda, Md.

Rickettsial strain. *R. conorii* (FB Casablanca strain) was kindly provided by C. Wisseman, Microbiology Department, University of Maryland, Baltimore.

RESULTS

The effect of human rTNF-α on the yield of *R. conorii* infectious particles was determined. rTNF-α reduced the yield of infectious *R. conorii* in a dose-dependent manner (Fig. 1). The 50% effective dose of rTNF-α was 0.035 ng/ml. Inhibitions of 80 and 90% could be achieved by treatment with rTNF-α in concentrations of 30 and 300 ng/ml, respectively. Figure 2 shows the kinetics of rTNF-α (300 ng/ml) inhibition of rickettsial growth in HEp-2 cells. No difference in the rickettsial infectious yield was observed whether the cells were pretreated for 24 h before infection or treated with rTNF-α after absorption of *R. conorii*. rTNF-α at a concentration of 300 ng/ml did not affect the viability of HEp-2 cells as determined by the [^{3}H]uridine uptake assay (Fig. 3). The addition of cycloheximide (10 μg/ml) to the rTNF-α-treated, *R. conorii*-infected HEp-2 cells did not affect the rickettsial yield. Treatment of HEp-2 cells with rTNF-α and IFN-γ together resulted in an increase of the inhibition of the rickettsial yield (Fig. 4) and a decrease in cellular uridine uptake (Fig. 3). The synergistic effect was most pronounced at an IFN-γ concentration of 100 U/ml and at rTNF-α concentrations of 3 and 30 ng/ml. The additional decrease of the rickettsial yield was 0.6 and 0.8 log units, respectively, at these concentrations.

IFN-γ alone (10, 100, or 1,000 U/ml) did not affect the rickettsial yield even though it was somewhat cytotoxic for HEp-2 cells at 100 and 1,000 U/ml (7 to 12% decrease in uridine uptake relative to that in untreated cells) and more so for infected cells (Fig. 3).

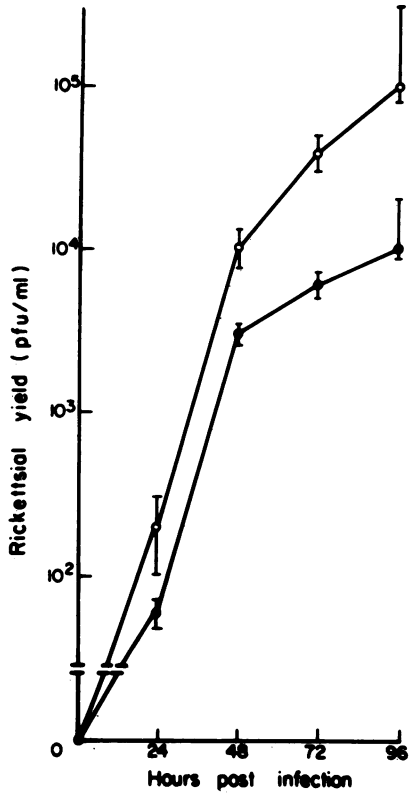

FIG. 2. *R. conorii* inhibition by rTNF-α as measured by plaque assay. After 24 h of exposure to growth medium containing 300 ng of rTNF-α per ml, the HEp-2 cells were infected with *R. conorii* at a multiplicity of infection of 0.1 at various time intervals (0 time was 2 h postinfection). Samples of the infected cells were collected and frozen at −70°C. The titers were determined for each sample. Symbols: ○, infected HEp-2 cells without rTNF-α treatment; ●, infected HEp-2 cells with rTNF-α treatment. Data are from one of three experiments.

The influence of tryptophan on *R. conorii* inhibition by rTNF-α or both rTNF-α and IFN-γ was determined by increasing the tryptophan concentration to 100 μg/ml as compared with 5 μg/ml in RPMI 1640. After 96 h the samples were harvested, and titers were determined. In the presence of tryptophan, the inhibitory effect of rTNF-α alone or both rTNF-α and IFN-γ was partially reversed (Fig. 5). Tryptophan also caused a partial reversion of the cytotoxic effect of rTNF-α- and IFN-γ-treated HEp-2 cells infected with *R. conorii* (Fig. 6).

DISCUSSION

The host defense mechanisms against rickettsial diseases are not clearly understood, but studies have indicated that both humoral and cell-mediated immunity are involved (7, 20).

Recently, rTNF-α was found to inhibit the growth of a wide variety of pathogens. Inhibitory activity of TNF has been shown against a number of viruses, such as vesicular stomatitis virus, herpes simplex virus, encephalomyocarditis virus (9), and cytomegalovirus (6), against *Trypanosoma cruzi* (4), and against the obligate intracellular bacterium *Chlamydia trachomatis* (8, 11). The present study is the first demonstration that rTNF-α inhibits the growth of *R. conorii* in an in vitro system.

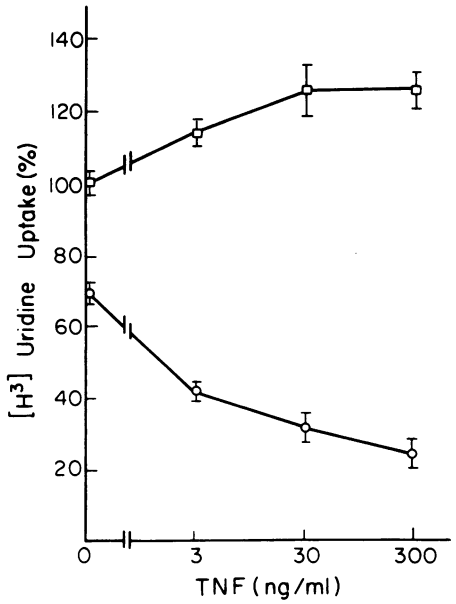

FIG. 3. Synergistic effect of rTNF-α and IFN-γ in cytotoxicity on HEp-2 cells infected with *R. conorii*, as measured by [^{3}H]uridine uptake. Cells were treated 24 h before infection with serial dilutions of rTNF-α in the presence or absence of IFN-γ. Ninety-six hours later [^{3}H]uridine uptake was measured. Symbols: □, treatment with rTNF-α alone; ○, treatment with rTNF-α and 100 U of IFN-γ per ml. Data are from one of three experiments.

Treatment of HEp-2 cells with rTNF-α alone, either before or after adsorption of *R. conorii*, resulted in a decrease of the infective yield of *R. conorii* in a dose-dependent manner (Fig. 1). rTNF-α at a concentration of 300 ng/ml caused a 90% reduction in the infective yield compared with that in untreated cells. At this concentration of rTNF-α, no cytotoxicity was observed in *R. conorii*-infected or noninfected HEp-2 cells (Fig. 3).

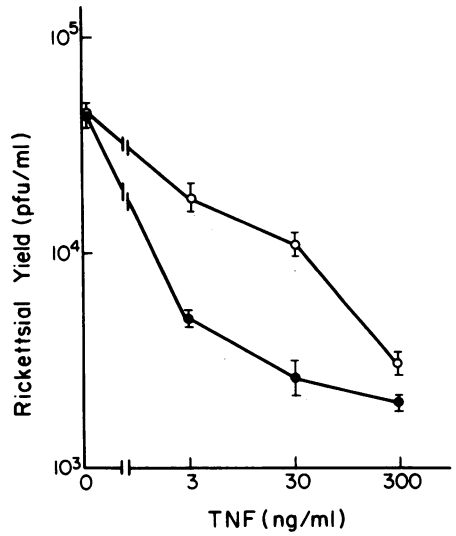

FIG. 4. Synergistic effect of rTNF-α and IFN-γ on rickettsial yield in HEp-2 cells. Cells were treated 24 h before infection with serial dilutions of rTNF-α in the presence or absence of IFN-γ (100 U/ml). Symbols: ○, rTNF-α; ●, IFN-γ and rTNF-α. Data are from one of five experiments.

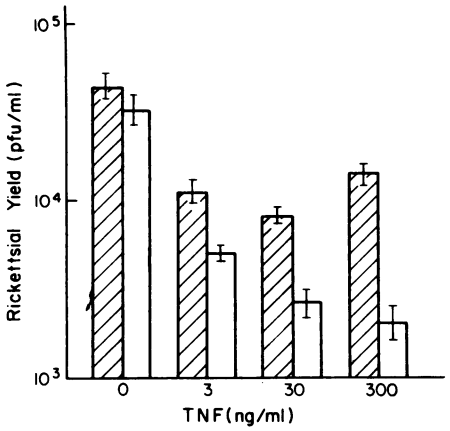

FIG. 5. Effect of tryptophan (100 μg/ml) on the inhibition of *R. conorii* growth in cells treated with various concentrations of rTNF-α and IFN-γ (100 U/ml). The one-step growth yield assay (Materials and Methods) was done on HEp-2 cultures pretreated with either control growth medium or medium containing 100 U of recombinant IFN-γ and rTNF-α at various concentrations for 24 h. The cultures were then infected with *R. conorii* at a multiplicity of infection of 0.1. After 96 h, the cultures were harvested and frozen, and titers of *R. conorii* cells were determined on HEp-2 cells. Symbols: ▨, IFN-γ and tryptophan; ▭, IFN-γ alone. Data are from one of three experiments.

At present very little is known about the mechanism(s) of rTNF-α inhibition of *R. conorii* growth. Blocking the synthesis of cellular proteins with cycloheximide did not alter the rickettsial yield inhibition caused by rTNF-α. These findings suggest that the effect of rTNF-α on rickettsial growth did not require de novo protein synthesis. In this

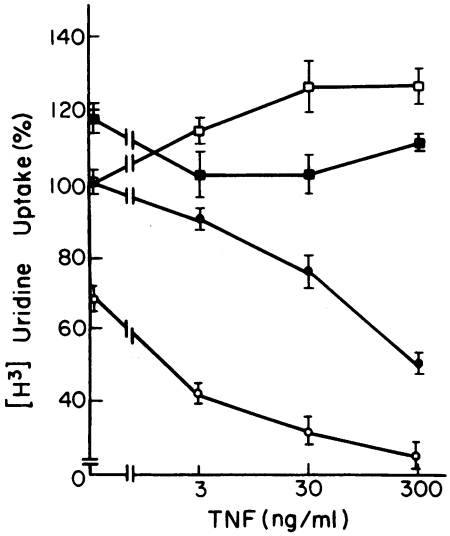

FIG. 6. Effect of tryptophan on the rTNF-α- and IFN-γ-mediated cytotoxicity for HEp-2 cells infected with *R. conorii* in HEp-2 cells treated with various concentrations of rTNF-α and IFN-γ (100 U/ml). Cells were treated 24 h before infection with serial dilutions of rTNF-α in the presence or absence of IFN-γ. Cells were infected; after 2 h of rickettsial absorption, media were removed and replaced with fresh media with or without tryptophan (100 μg/ml), as indicated. After 96 h, [^{3}H]uridine uptake was measured. Symbols: □, rTNF-α; ■, rTNF-α and tryptophan; ○, IFN-γ; ●, IFN-γ and tryptophan.

respect the mechanism of inhibition of *R. conorii* growth by rTNF-α appears to differ from that of chlamydial growth inhibition; we have recently shown that cycloheximide abolished the inhibition of *C. trachomatis* growth in HEp-2 cells caused by rTNF-α. This was related to induction of the enzyme indoleamide 2,3-dioxygenase, which degraded tryptophan, which is essential for chlamydial growth (12). The mechanism by which tryptophan partially reverses (Fig. 5) the inhibition of *R. conorii* by rTNF-α is presently under investigation.

The synergistic effect of IFN-γ and rTNF-α reported in this study has also been observed with both RNA and DNA viruses (26) and the obligate intracellular parasite *C. trachomatis* (11). It should be noted that IFN-γ alone (10 to 1,000 U/ml) had no effect on the rickettsial yield. These results are different from those reported by Turco and Winkler, who studied the effect of IFN-γ on the replication of *Rickettsia prowazekii* in mouse-macrophage-like RAW 264.7 cells (19) and in L929 cells (18). They showed that IFN-γ can cause a decrease in the percentage of infected cells and in the average number of rickettsiae present in infected cells. The difference in the effects of IFN-γ in these studies might be due to different rickettsial species or target cells or to the different methods used to monitor rickettsial replication. Whereas Turco and Winkler (18, 19) determined the number of rickettsial morphological particles, we detected the number of infectious particles.

In the present study we have shown that treatment of HEp-2 cells with IFN-γ resulted in a cytotoxic effect (Fig. 3) which was further enhanced after infection of the cells with *R. conorii*. Similar results have been obtained by Turco and Winkler in *R. prowazekii*-infected L929 cells and human fibroblast cells treated with IFN-γ (18). A cytotoxic effect was also found in the present study when *R. conorii*-infected HEp-2 cells were treated with IFN-γ and rTNF-α. The effect was partially reversed by the addition of tryptophan. Recently we have shown (Yuhas et al., unpublished data) that tryptophan can partially reverse the toxic effect of rTNF-α on L929 cells. The mechanism by which tryptophan reversed the cytotoxic effect on *R. conorii*-infected cells of rTNF-α and IFN-γ requires further study.

In summary, the present study demonstrates that rTNF-α inhibits *R. conorii* growth and that the inhibition is further enhanced by IFN-γ, which suggests that rTNF-α might play a role in the host defense mechanism in rickettsial infections. rTNF-α has been reported to protect mice against *Klebsiella pneumoniae* and *L. monocytogenes* (10) and against different species of plasmodia (3, 16). On the other hand, rTNF-α was shown to have numerous deleterious effects, such as a direct cytotoxic effect on vascular endothelial cells, inducing the expression of procoagulant activity (14, 15), which might play a role in the pathogenicity of rickettsial infections. Therefore, the use of rTNF-α antibody at certain stages of infection may be of therapeutic value in rickettsial diseases. Further studies in animal models are required to explore this possibility.

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