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Inhibition of *Chlamydia trachomatis* Growth by Recombinant Tumor Necrosis Factor

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Purified human recombinant tumor necrosis factor (rTNF) alpha inhibited the growth of *Chlamydia trachomatis* (L_2/434/Bu) in HEp-2 cell cultures. The inhibition of *C. trachomatis* yield could be achieved even when the rTNF alpha (200 ng/ml) was added up to 12 h after infection. The effect of rTNF alpha on chlamydial infection was synergistic with that of gamma interferon.

Chlamydia trachomatis is an obligate intracellular bacterium which causes distinct infections ranging from hyperendemic trachoma to various sexually transmitted diseases (17). During the course of infection, the intracellular parasites evoke humoral and cell-mediated immune responses (6, 12, 16, 22). The activation of macrophages to a heightened virocidal or bacterocidal state by T-cell products such as gamma interferon (IFN-γ) (1, 28) is considered to be an important part of the cell-mediated immune response. Two broad mechanisms of macrophage antichlamydial activity have been described: intrinsic activity, in which chlamydial replication within the activated macrophage is aborted (19, 23), and extrinsic activity, in which chlamydial replication in other permissive cells is inhibited (27).

Microbial products and host factors activated by infection are effective in triggering the secretory repertoire of macrophages (28). Tumor necrosis factor (TNF) is a major mediator of the inflammatory response (20, 24) and can exert antitumor activity (7). It was initially discovered as a serum factor in mice injected with bacterial lipopolysaccharide. More recently, it has been shown that TNF production can also be elicited by pathogenic agents other than bacteria. It contributes in various ways to defense against such pathogenic agents (4, 21). It is produced in response to viruses (2) and has been found to selectively kill virus-infected cells (3, 15), to inhibit *Trypanosoma cruzi* in permissive cells (10), and to inhibit in vitro RNA and DNA virus replication (18, 32), although not necessarily through cytotoxic effects (18).

The purpose of this study was to investigate the influence of human recombinant TNF (rTNF) alpha on the development of the intracellular bacterium *C. trachomatis* in human laryngeal carcinoma cells (HEp-2).

C. trachomatis (L_2/434/Bu serovar) were harvested and purified as previously described (26). Human rTNF alpha (6×10^7 U per mg of protein; produced by Genentech Co., San Francisco, Calif.) was kindly provided by G. Adolf, Boehringer Inst., Austria. Human recombinant IFN-γ was a gift from Inter-Yeda Inc., Rehovot, Israel.

A one-step growth-yield assay was performed as follows. HEp-2 cells were seeded in 96-microwell plates at 3×10^4 cells per well. The following day, medium was changed to cell growth medium supplemented with 5% fetal calf serum, to which rTNF alpha at various concentrations was added. Cells were incubated at 37°C for 24 h. Purified *C. trachomatis* was diluted in *C. trachomatis* growth medium (minimal essential medium supplemented with 5% fetal calf serum, 10 μg of glutamine per ml, 0.01 ng of vancomycin per ml, 16 μg of gentamicin per ml, and 1% glucose). The HEp-2 cells were infected at a multiplicity of infection of 1. After 1 h of adsorption at 37°C, the inoculum was removed and 100 μl of *C. trachomatis* growth medium was added per well. Samples were assayed in triplicate. After an additional 48 h of incubation, the infected cells were harvested and frozen at −70°C as previously described (25). The titer of each sample was determined in triplicate on HEp-2 cells by immunoperoxidase assay, and the results of the titration were expressed as inclusion-forming units (IFU) per milliliter. Samples were prepared for electron microscopy as described by Biberfeld (5). Electron micrographs of the thin sections were taken with a Phillips 201C transmission electron microscope.

For cytotoxic tests, cell viability was determined by measurement of [^{3}H]uridine uptake of HEp-2 cells. HEp-2 cells were treated for 24 h with various concentrations of rTNF alpha either in the presence or in the absence of cycloheximide (40 μg/ml). At the end of this period, cytotoxicity was quantitated as described by Smith and Nicklin (29).

The effect of human rTNF alpha on the yield of *C. trachomatis* infectious particles was tested by one-step growth experiments. The yield of chlamydia (in IFU per milliliter) was determined for rTNF alpha-pretreated cells and for the control. In cells pretreated with different doses of rTNF alpha, chlamydial yield was reduced in a dose-dependent manner (Fig. 1).

The 50% effective dose of TNF was 0.13 ng/ml, which is about 10-fold the concentration at which rTNF alpha exerts a toxic effect on rTNF alpha-sensitive L-929 cells. An inhibition of 99% could be achieved with 200 ng of rTNF alpha per ml. The antichlamydial effect of rTNF alpha could be neutralized with a monoclonal antibody to rTNF alpha. rTNF alpha alone (250 ng/ml) reduced the chlamydial yield from $7.2 \times 10^7 \pm 1.2 \times 10^7$ IFU/ml (mock-treated cells) to $2.5 \times 10^6 \pm 3 \times 10^6$ IFU/ml, while in cultures treated with rTNF alpha-specific antibody at 37°C for 1 h, the yield was $5.7 \times 10^7 \pm 1.5 \times 10^7$ IFU/ml.

rTNF alpha at a concentration of 200 ng/ml did not affect HEp-2 cell viability as determined by the [^{3}H]uridine incorporation assay. However, in agreement with results of Mestan et al. (18), HEp-2 cells were effectively killed in the presence of cycloheximide.

IFNs have been shown to enhance the cytotoxic activity of TNFs (1, 3, 30, 31). Therefore, titration of TNF in the

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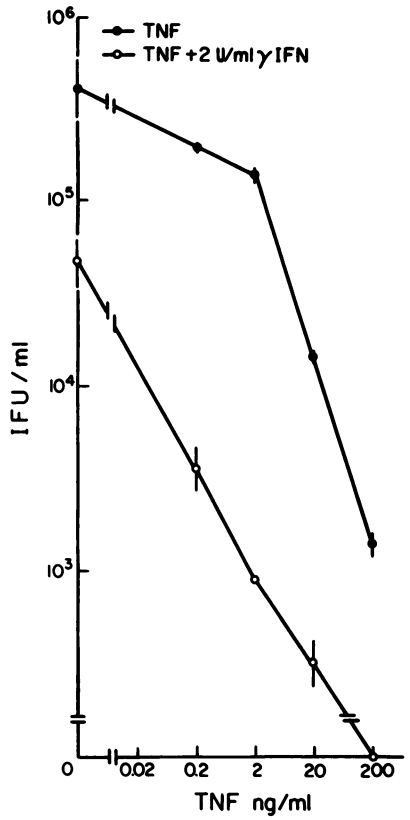

FIG. 1. Synergistic effect of rTNF alpha and IFN-γ on chlamydial yield in HEp-2 cells. Cells were treated 24 h before infection with serial dilutions of TNF either in the presence or in the absence of IFN-γ. The open circle at 0 ng of TNF per ml represents the effect of 2 U of IFN-γ per ml alone on chlamydial yield. The figure shows results from one of three similar experiments.

presence of 2 U of IFN-γ was carried out and compared with the effect of TNF alone. TNF acted synergistically with IFN-γ in the induction of resistance to chlamydial infection (Fig. 1). rTNF alpha alone at a concentration of 0.2 ng/ml reduced the logarithm of the yield of chlamydia by one-third. IFN-γ at 2 U/ml reduced the logarithm of the chlamydia titer by one-half. The two combined produced a 100-fold reduction in chlamydial titer. These results are reminiscent of those of Wong and Goeddel, who reported that both TNFs alpha and beta acted synergistically with IFNs in the induction of resistance to vesicular stomatitis virus, encephalomyocarditis virus, adenovirus type 2, and herpes simplex virus type 2 infection in diverse cell types (32).

To determine the kinetics of rTNF alpha induction of an antichlamydial state, HEp-2 cell monolayers were treated with 200 ng of rTNF alpha per ml for various time periods before and after infection. Chlamydial yield was examined at 24, 48, and 72 h after infection.

The inhibition of *C. trachomatis* yield was achieved even when the rTNF alpha was added up to 12 h after infection, although not to the same extent as with rTNF alpha pretreatment, continuous exposure to rTNF alpha, or addition of rTNF alpha immediately after infection (Fig. 2).

The effect of rTNF alpha at 200 ng/ml on chlamydial development was not readily reversible. Removal of rTNF alpha from the medium prior to infection did not result in a rise in chlamydial yield for up to 4 days after infection (Fig. 2).

The development of chlamydia in rTNF alpha-treated HEp-2 cells was examined by electron microscopy 48 h after infection. Infected cells not treated with rTNF alpha exhibited typical large inclusion bodies that contained mostly elementary bodies (Fig. 3A). Cells which were treated with rTNF alpha had relatively smaller inclusion bodies in which irregular forms of the chlamydial developmental cycle and enlarged reticulate bodies (Fig. 3B) could be seen. Very few elementary bodies were observed.

Since chlamydiae both induce and are inhibited by rTNF alpha and IFN-γ (27), it is possible that these two cytokines play a role in the host immune response to chlamydial infection. Furthermore, chlamydial infection has been associated with the etiology of arthritis and other rheumatic diseases (8) by a mechanism which is not well understood (for a review, see reference 14). Recently, Keat et al. (13) showed with fluorescent antibodies to *C. trachomatis* the presence of noninfectious chlamydial particles in joint material from five of eight patients with sexually acquired reactive arthritis. The presence of chlamydial antigen in

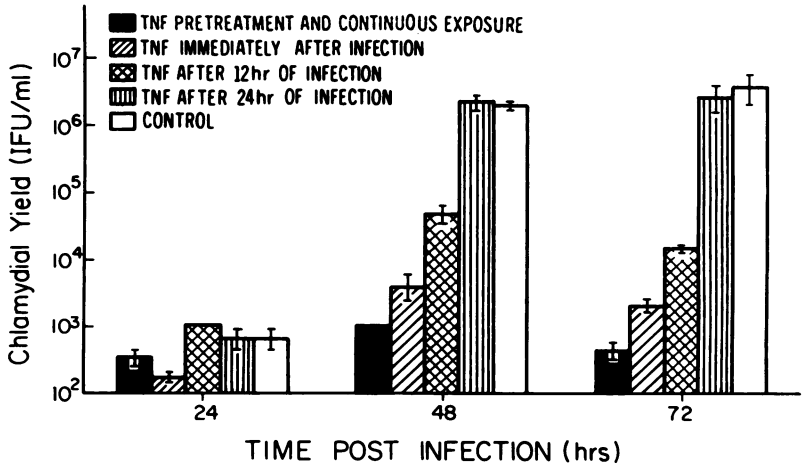

FIG. 2. Kinetics of rTNF alpha induction of an antichlamydial state. rTNF alpha (200 ng/ml) was added to HEp-2 cells starting at various times relative to infection, as indicated on the figure. Chlamydial yield (IFU/ml) was determined at 24, 48, and 72 h after infection (one-step growth-yield assay). The results shown are from one of three similar experiments.

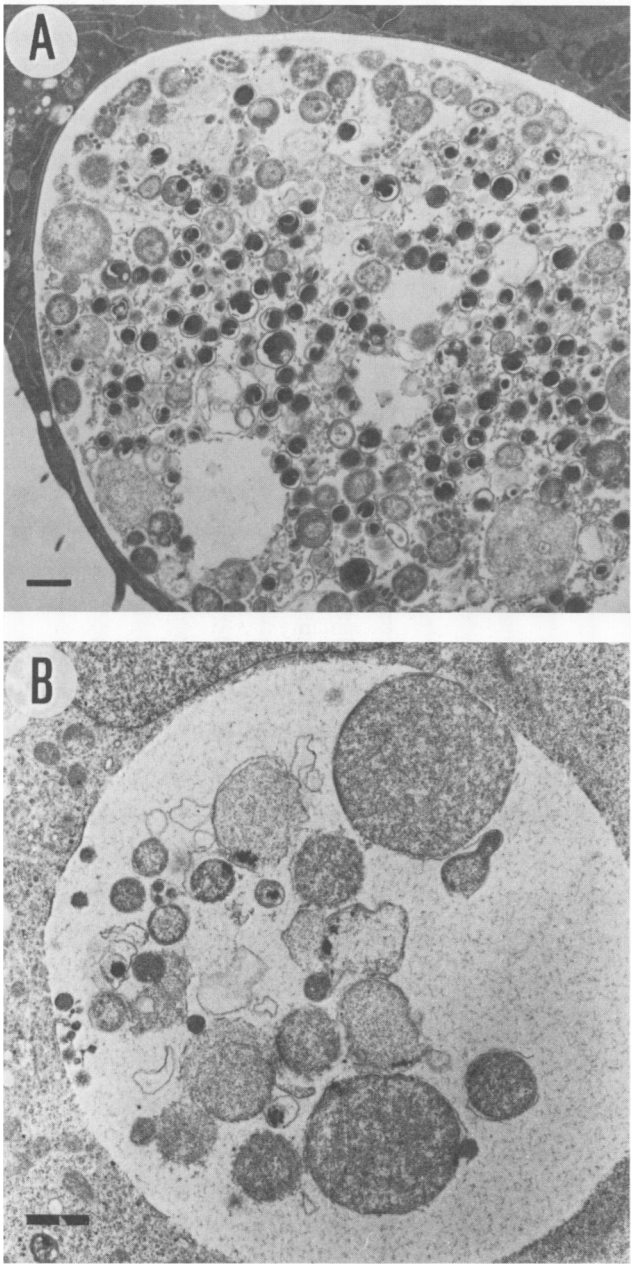

FIG. 3. Electron micrograph of *C. trachomatis* development in HEp-2 cells 48 h after infection. (A) Control, no rTNF alpha treatment. Magnification, ×11,200. (B) rTNF alpha treatment (200 ng/ml). Magnification, ×16,200. The bar in each micrograph represents 0.5 μm.

synovial tissue might initiate local inflammation (11), with resultant macrophage attraction and TNF secretion (Manor and Sarov, submitted for publication). TNF has been shown to stimulate collagenase and prostaglandin E_2 production in synovial cells (9); this could contribute to the cartilage destruction observed in rheumatic diseases. Further studies are required to delineate the role which TNF may have in the pathogenesis of chlamydial infection.

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