

# Human Macrophages Acquire a Hyporesponsive State of Tumor Necrosis Factor Alpha Production in Response to Successive *Mycobacterium avium* Serovar 4 Stimulation

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**Human macrophages (M $\phi$ ) from most donors respond to inoculation with *Mycobacterium avium* serovar 4 (*M. avium*) by tumor necrosis factor alpha (TNF- $\alpha$ ) production, which is of critical importance for proper defense against microorganisms. An initial infection of M $\phi$  with *M. avium* results in an incapacity to accumulate TNF- $\alpha$  mRNA after reinfection with *M. avium*, indicating adaptation to a hyporesponsive state by preexposure of the cells to *M. avium*. Adaptation to stimulation with *M. avium* is abrogated by the cyclooxygenase inhibitor indomethacin. In the presence of prostaglandin E<sub>2</sub>, indomethacin-exposed, *M. avium*-treated M $\phi$  remain unresponsive to a subsequent *M. avium* stimulus to increase steady-state TNF- $\alpha$  mRNA, suggesting that prostaglandin E<sub>2</sub> is instrumental for the adaptation to an *M. avium* challenge. TNF- $\alpha$  mRNA accumulation induced by a second *M. avium* stimulus in the presence of indomethacin is blocked by the protein tyrosine kinase inhibitor herbimycin. In contrast, the initial M $\phi$  response to *M. avium* is inhibited by staurosporin, an inhibitor of phospholipid Ca<sup>2+</sup>-dependent protein kinases, indicating that the initial and the successive TNF- $\alpha$  responses to *M. avium* are dependent on different mechanisms.**

*Mycobacterium avium* serovar 4 is an acid-fast bacillus pathogenic in long-term immunocompromised individuals, including human immunodeficiency virus type 1-infected patients. This virulent strain of *M. avium* (19) is generally found in the patients' macrophages (M $\phi$ ), important cells in the defense against microorganisms (39). Inoculation of M $\phi$  with *M. avium* induces production of several cytokines, including tumor necrosis factor alpha (TNF- $\alpha$ ) (32), which seems to be involved in killing of *M. avium* (1). Production of TNF- $\alpha$  is also associated with granuloma formation (21) and effective host resistance to a number of other infectious pathogens (17, 31, 38).

The increase of TNF- $\alpha$  mRNA levels following the initial infection of M $\phi$  with *M. avium* serovar 4 is transient, lasting only 1 to 4 h, thereby precluding an effective antimicrobial effect. In contrast, increased accumulation of interleukin-1 $\beta$  (IL-1 $\beta$ ) and IL-6 mRNA reaches its maximum 2 to 4 h after *M. avium* inoculation and lasts for several days (13).

The initial TNF- $\alpha$  response is abrogated by staurosporin, which inhibits protein kinases by blocking the ATP binding site (5), and TNF- $\alpha$  mRNA accumulated during the initial response is markedly unstable after staurosporin treatment (13). Destabilization of mRNA following treatment with staurosporin is also seen in similar systems (26).

In models of toxic shock, induction of TNF- $\alpha$  is tightly controlled as shown by the incapacity of lipopolysaccharide (LPS)-pretreated M $\phi$  to produce TNF- $\alpha$  after a subsequent LPS treatment (10, 29). This effect is referred to as adaptation (22). Therefore, the question of whether continuing exposure of M $\phi$  to *M. avium* during infection results in unresponsiveness of the cells to *M. avium* arose. In the present study we investigated the changes in TNF- $\alpha$  mRNA levels of M $\phi$  from healthy individuals in response to a reinfection with *M. avium* 24 h after

the initial challenge. We show that M $\phi$  inoculated with *M. avium* are markedly inhibited in their TNF- $\alpha$  production after a second inoculation with *M. avium*.

Studies in human M $\phi$ -like cell lines have demonstrated that prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), the major prostaglandin produced by M $\phi$  (9), is responsible for adaptation to LPS (15). In the present study we show that M $\phi$  hyporesponsiveness of the TNF- $\alpha$  response to reinfection with *M. avium* is abrogated by the cyclooxygenase inhibitor indomethacin and is restored by PGE<sub>2</sub>. This finding indicates that prostaglandins are involved in the development of hyporesponsiveness of human M $\phi$  after *M. avium* infection. In contrast, indomethacin has only a minor effect on TNF- $\alpha$  mRNA accumulation during the initial response. Furthermore, in contrast to the initial response, which is inhibited by staurosporin, TNF- $\alpha$  mRNA accumulation after a recurrent *M. avium* challenge in the presence of indomethacin is inhibited by the tyrosine kinase inhibitor herbimycin. Thus, hyporesponsiveness of human M $\phi$  to reinfection with *M. avium* is based on different mechanisms that prevent prolonged and excessive TNF- $\alpha$  production by the M $\phi$  following infection with the virulent *M. avium* serovar 4.

## MATERIALS AND METHODS

**Experimental design.** M $\phi$  (10<sup>6</sup> cells per ml) were incubated with 10 *M. avium* organisms per cell at time zero. The cells were then exposed to a second inoculum of *M. avium* at 24 h and harvested at 28 h. It should be noted that in these studies TNF- $\alpha$  mRNA accumulation 4 h after the initial inoculation with *M. avium* was determined by inoculating the cells with *M. avium* at 24 h and harvesting them at 28 h. After extraction and purification of the mRNA, TNF- $\alpha$  mRNA levels were measured by Northern analysis.

**Materials.** Staurosporin, genistein, and herbimycin were purchased from Calbiochem, La Jolla, Calif. Actinomycin D and indomethacin were obtained from Sigma, St. Louis, Mo. The activity of staurosporin was tested by determining its capacity to inhibit phorbol myristate acetate (10 ng/ml)-induced production of TNF- $\alpha$  in cultured M $\phi$ . One and 10  $\mu$ M staurosporin completely inhibited TNF- $\alpha$  production of M $\phi$  induced by phorbol myristate acetate.

**Cells.** Mononuclear leukocytes were obtained from peripheral blood of uninfected, healthy donors or from leukapheresis units from the Blood Bank of the New England Deaconess Hospital, Boston, Mass., and subsequent Ficoll-Hypaque gradient centrifugation. The cells were plated in Iscove's modified

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Dulbecco's medium (Hyclone Laboratories, Logan, Utah) with 10% pooled human male serum (Lampire) and 2 mM L-glutamine (Gibco). M $\phi$  were obtained by adherence purification of the mononuclear cells on plastic flasks or cluster plates (Costar, Cambridge, Mass.) overnight at 37°C with 5% CO<sub>2</sub>. Determination of the LPS content of all media and sera by the *Limulus* amoebocyte lysate assay (Whittaker Bioproducts, Walkersville, Md.) showed a concentration of less than 0.05 ng of LPS per ml.

M $\phi$  used for cytokine quantitation were cultured at  $2.0 \times 10^6$  mononuclear cells per well in 48-well cluster plates (Costar) or on Permax coverslips (Nunc, Naperville, Ill.) for microscopic analysis. M $\phi$  cultured for TNF- $\alpha$  mRNA analysis were plated at  $2.0 \times 10^7$  cells per 5 ml in tissue culture flasks (Costar). Cells from one flask were sufficient to perform Northern analysis. Cell populations were 97 to 99% M $\phi$  by nonspecific esterase staining.

**Growth and quantitation of *M. avium*.** *M. avium* serovar 4 isolated from the blood of an AIDS patient and typed by the Mycobacterial Culture Collection (National Jewish Hospital, Denver, Colo.) was used in these studies. The isolate was grown in Middlebrook 7H9 broth and albumin-dextrose-catalase enrichment (Difco, Detroit, Mich.) until in log phase, at which point it was harvested by centrifugation ( $2,000 \times g$ ). The bacteria were then washed twice in Hanks balanced salt solution (Gibco), resuspended in Middlebrook 7H9 broth, sonicated for 10 s at 500 W, aliquoted, and stored frozen at -70°C until use. The number of viable bacteria was determined by plate counting of an aliquot of the bacterial culture. These cultures yielded colonies of the smooth, transparent morphotype (30).

M $\phi$  were infected with *M. avium* in Iscove's modified Dulbecco's medium plus 2% pooled male human serum after culture for 5 to 7 days at 10 organisms per cell for various times at 37°C in a 5% CO<sub>2</sub> atmosphere. After 4 h the monolayers were washed twice with Hanks balanced salt solution to remove nonphagocytized bacteria, and fresh medium was added.

**Quantitative analysis of mRNA.** M $\phi$  were harvested for the determination of steady-state TNF- $\alpha$  mRNA by Northern blotting at 4 h after inoculation with *M. avium*. mRNA was extracted from the cells in 4 M guanidinium thiocyanate (Boehringer Mannheim, Indianapolis, Ind.) and 0.1 M 2-mercaptoethanol (Sigma) (6). The mRNA was purified by CsCl gradient ultracentrifugation and was resolved by electrophoresis in formaldehyde containing 1.2% agarose gels that were transferred to nylon membranes (American Bioanalytical, Meriden, Conn.) (35). The blotted mRNA was hybridized to an [ $\alpha$ -<sup>32</sup>P]dCTP-labeled 1.0-kb *Hind*III-*Bam*HI fragment of TNF- $\alpha$  genomic DNA (from Leo Lin, formerly of Cetus Corp., Emeryville, Calif.). The probe was radiolabeled by random priming with the large Klenow fragment of DNA polymerase I (Boehringer Mannheim) and [ $\alpha$ -<sup>32</sup>P]dCTP (11) and then visualized by autoradiography. The amount of TNF- $\alpha$  mRNA was determined by densitometry and expressed as density relative to the amount of total RNA applied for electrophoresis.

**Determination of mRNA stability.** M $\phi$  ( $5.0 \times 10^5$  cells per ml) inoculated with 10 *M. avium* organisms per cell in the presence or absence of 100  $\mu$ M indomethacin or with medium alone were incubated with 3.9  $\mu$ M actinomycin D. The total RNA collected at increasing time intervals thereafter was quantitated by Northern blotting and densitometry using a gel scanner (EC apparatus Corp., St. Petersburg, Fla.).

**Determination of PGE<sub>2</sub>.** PGE<sub>2</sub> was measured in the supernatants of M $\phi$  cultures at various times by a commercially available enzyme-linked immunosorbent assay (ELISA) (RPN 222) from Amersham International, Little Chalfont, England. PGE<sub>2</sub> was purified prior to the assay on Amprep C<sub>18</sub> columns as described elsewhere (20).

**Determination of TNF- $\alpha$  protein.** Supernatants from M $\phi$  treated with various regimens were harvested at various times after treatment for analysis of secreted TNF- $\alpha$ . The supernatants were stored at -70°C until assayed. TNF- $\alpha$  concentrations were measured by an ELISA (Genzyme, Cambridge, Mass.). The plates were read at 590 nm on an automated ELISA reader.

**Statistical analysis.** Statistical analysis of the data was performed by using the Student *t* test or the Kruskal-Wallis nonparametric one-way analysis of variance.

## RESULTS

**Preexposure of M $\phi$  to *M. avium* abrogates TNF- $\alpha$  mRNA accumulation to reinfection with *M. avium*.** Inoculation of M $\phi$  with *M. avium* (10 organisms per cell) at time zero results in a rapid increase of TNF- $\alpha$  mRNA levels at 4 h which reaches almost baseline levels at 6 h after inoculation in the absence of further challenge (13). Reinfection of the M $\phi$  with *M. avium* at 24 h fails to produce a significant upregulation of TNF- $\alpha$  mRNA levels over background levels when the cells are harvested 4 h after reinfection, indicating that the initial *M. avium* inoculation renders M $\phi$  hyporesponsive to a reinfection with *M. avium* (Fig. 1, 0 and 24 h, M).

Similar results were obtained with 1 and 20 *M. avium* organisms per cell (not shown). Hyporesponsiveness of TNF- $\alpha$

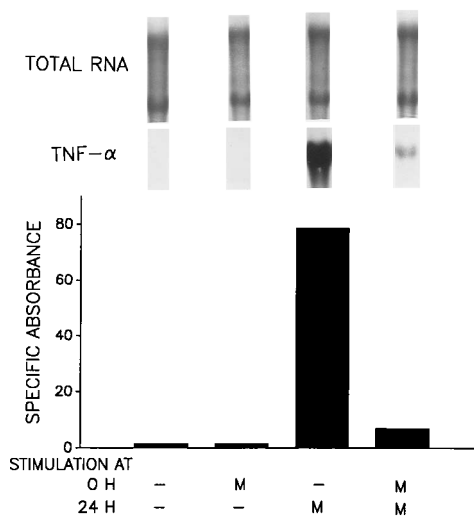


FIG. 1. Desensitization of TNF- $\alpha$  mRNA accumulation in human M $\phi$  to reinfection with *M. avium*. Northern blot analysis of TNF- $\alpha$  transcripts was performed with M $\phi$  incubated with *M. avium* (M, 10 organisms per cell) or in medium alone (-) at time zero and subsequently at 24 h at various combinations. mRNA was harvested 4 h after the second treatment, and 15  $\mu$ g of mRNA was analyzed for TNF- $\alpha$  transcripts. (Top) Total RNA; (middle) mRNA hybridized with a human TNF- $\alpha$  probe; (bottom) relative densities of the blots. The data are from a representative of four experiments.

mRNA accumulation was also seen when M $\phi$  were inoculated with *M. avium* (10 organisms per cell) at time points earlier than 24 h after initial exposure to *M. avium* (8, 12, and 16 h [not shown]).

**Hyporesponsiveness of TNF- $\alpha$  mRNA accumulation of M $\phi$  induced by *M. avium* is caused by prostaglandins.** PGE<sub>2</sub> is involved in suppression of TNF- $\alpha$  production by M $\phi$  and T cells (12, 24). We examined therefore whether the cyclooxygenase inhibitor indomethacin (which blocks PGE synthesis) abrogates *M. avium*-induced M $\phi$  hyporesponsiveness of TNF- $\alpha$  production to a second *M. avium* stimulus. Figure 2 shows that no TNF- $\alpha$  mRNA is present in nonstimulated cells (0 h - and 24 h - [which indicates that the cells were infected at neither 0 h nor 24 h]). Significant TNF- $\alpha$  mRNA accumulation can be seen in cells which were not infected at 0 h, received an *M. avium* inoculum at 24 h, and were harvested 4 h after the infection (Fig. 2, 0 h - and 24 h M). Immediately prior to reinfection, steady-state TNF- $\alpha$  mRNA levels of M $\phi$  initially infected at 0 h have almost returned to background levels (0 h M and 24 h -). Four hours after reinfection with *M. avium* at 24 h the TNF- $\alpha$  mRNA accumulation was largely reduced, indicating the development of a nonresponsive state of TNF- $\alpha$  mRNA accumulation (0 h M and 24 h M). In contrast, addition of 100 ng of LPS per ml 24 h after the initial inoculation with *M. avium* resulted in a full-blown response (0 h M and 24 h L). This finding shows that the adaptation is specific to *M. avium*.

Addition of 100  $\mu$ M indomethacin to the cells at the time of initial infection with *M. avium* abrogates the adaptation to *M. avium* and results in significant accumulation of TNF- $\alpha$  mRNA when the cells are harvested 4 h after reinfection (Fig. 2, 0 h M/I and 24 h M). This finding indicates that prostaglandin production after the initial *M. avium* inoculation prevents TNF- $\alpha$  mRNA accumulation in response to reinfection with *M. avium*. Fifty micromolar indomethacin yields a marginal effect (not shown). The specificity of the effect of indomethacin is demonstrated by addition of exogenous PGE<sub>2</sub> (30  $\mu$ M) to indomethacin-treated, *M. avium*-inoculated M $\phi$  at the time of

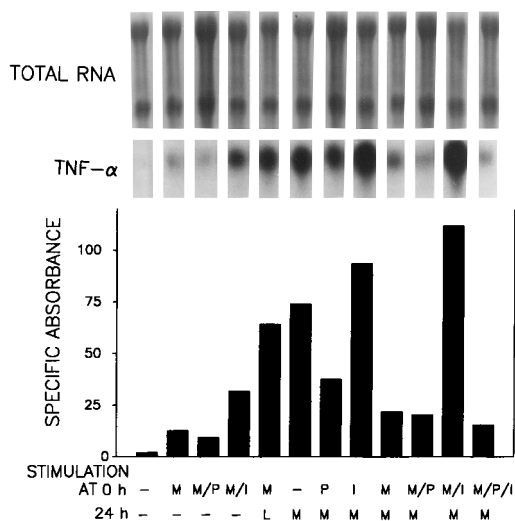


FIG. 2. Northern blot analysis of the effect of indomethacin on the TNF- $\alpha$  mRNA accumulation after reinfection with *M. avium*. M $\phi$  were incubated with *M. avium* (M, 10 organisms per cell), with medium alone (-), with *M. avium* in the presence of 30  $\mu$ M PGE<sub>2</sub> (M/P), with *M. avium* in the presence of 100  $\mu$ M indomethacin (M/I), with 30  $\mu$ M PGE<sub>2</sub> (P), with 100  $\mu$ M indomethacin (I), and with *M. avium* in the presence of 30  $\mu$ M PGE<sub>2</sub> and 100  $\mu$ M indomethacin (M/P/I) at time zero and subsequently at 24 h with medium alone (-), 100 ng of LPS (L), or 10 *M. avium* organisms per cell (M) in the combinations indicated below the graph. mRNA from the cells incubated under these various conditions was harvested 4 h after the second treatment, and 15  $\mu$ g of RNA was analyzed for TNF- $\alpha$  transcripts by Northern blotting. (Top) Total RNA; (middle) mRNA hybridized with a human TNF- $\alpha$  probe; (bottom) relative densities of the blots. The data are from a representative of three experiments.

the initial infection, which overcomes the effect of indomethacin on TNF- $\alpha$  mRNA accumulation 4 h after reinfection (Fig. 2, 0 h M/P/I and 24 h M). Addition of PGE<sub>2</sub> at the time of the initial infection (0 h) and subsequent infection with *M. avium* (0 h P and 24 h M) decreases TNF- $\alpha$  mRNA accumulation in comparison with that in M $\phi$  not pretreated with PGE<sub>2</sub>, while addition of PGE<sub>2</sub> to the other controls (0 h M/P and 24 h -; 0 h M/P and 24 h M) has no effect. Marginal increases of TNF- $\alpha$  mRNA accumulation are also seen if indomethacin is added together with *M. avium* at the time of initial infection and the cells are harvested 4 h after reinfection (0 h M/I and 24 h -) and if M $\phi$  incubated with indomethacin at the time of initial infection are stimulated with *M. avium* at the time of reinfection (0 h I and 24 h M).

The presence of indomethacin increases also the concentration of TNF- $\alpha$  protein in M $\phi$  supernatants after reinfection with *M. avium*. Production of TNF- $\alpha$  by M $\phi$  inoculated with *M. avium* (10 organisms per cell) at 0 h in the presence or absence of 100  $\mu$ M indomethacin and reinfected either 24, 48, or 72 h after the initial infection with *M. avium* (10 organisms per cell) was determined after harvesting the supernatants at 4 h following the respective reinfections. TNF- $\alpha$  concentrations in supernatants of *M. avium*-infected M $\phi$  incubated with indomethacin are significantly increased in comparison with those in *M. avium*-infected cells incubated in the absence of indomethacin under all conditions (Table 1). These findings indicate that inhibition of prostaglandin production by indomethacin results in increased TNF- $\alpha$  secretion and that prostaglandin-dependent suppression of PGE<sub>2</sub> secretion as shown by decreased TNF- $\alpha$  concentration in the cell supernatants is still apparent when the cells are reinfected 72 h after the initial infection.

***M. avium*-inoculated M $\phi$  produce PGE<sub>2</sub>.** The finding that

TABLE 1. TNF- $\alpha$  concentration in supernatants of human M $\phi$  initially infected with *M. avium* at 0 h in the presence or absence of indomethacin and reinfected with *M. avium*

Time of reinfection (h) after initial infection	TNF- $\alpha$ concn (pg/ml $\pm$ SE) <sup>a</sup>		% Increase
	Without indomethacin	With indomethacin	
24	565 $\pm$ 8	795 $\pm$ 57	41
48	99 $\pm$ 28	511 $\pm$ 35	416
72	125 $\pm$ 77	285 $\pm$ 35	128

<sup>a</sup> One of three experiments is presented. Standard errors are based on triplicate samples from different wells.

adaptation of the *M. avium*-dependent TNF- $\alpha$  response is mediated by PGE<sub>2</sub> suggests that the initial inoculation of M $\phi$  with *M. avium* upregulates production of prostaglandin, which inhibits the response to reinfection with *M. avium*. To investigate whether PGE<sub>2</sub> production is upregulated by *M. avium* inoculation, we measured PGE<sub>2</sub> levels at various times after the initial *M. avium* inoculation in the absence and presence of 100  $\mu$ M indomethacin. Figure 3 demonstrates that PGE<sub>2</sub> concentrations in M $\phi$  supernatants following *M. avium* inoculation reach 25 pg/ml 4 h after inoculation and 30 pg/ml 12 and 24 h after inoculation. These values are reduced to less than 10 pg/ml, respectively, in the presence of indomethacin and in control supernatants from M $\phi$  not challenged with *M. avium*. It is of note that prostaglandin levels in supernatants from *M. avium*-infected M $\phi$  are higher than prostaglandin levels in supernatants from indomethacin-treated M $\phi$  inoculated with *M. avium*.

**Different mechanisms are involved in the TNF- $\alpha$  response of M $\phi$  to a second *M. avium* stimulus in the presence of indomethacin and in the initial response.** The different durations of the initial *M. avium* response and the response after reinfection suggest different mechanisms for the two responses. We therefore examined the kinase dependency of the response to a second *M. avium* stimulus in the presence of indomethacin. The nonspecific protein kinase inhibitor staurosporin and the protein tyrosine kinase (PTK) inhibitors herbimycin and genistein were tested for their capacity to inhibit upregulation

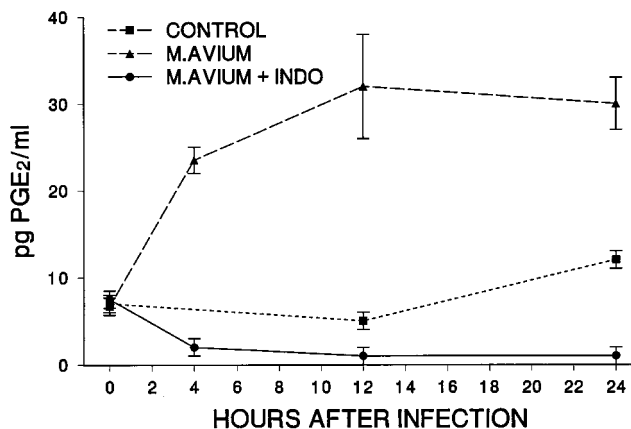


FIG. 3. Time response of PGE<sub>2</sub> accumulation in supernatants from unstimulated M $\phi$  (CONTROL), from M $\phi$  inoculated with *M. avium* serovar 4, and from M $\phi$  inoculated with *M. avium* in the presence of 100  $\mu$ M indomethacin (INDO). The abscissa shows the incubation time of the M $\phi$  after inoculation with *M. avium*, and the ordinate shows the concentration of PGE<sub>2</sub> in the M $\phi$  supernatants. The standard error of the mean of triplicate determinations of one typical experiment of four is shown.

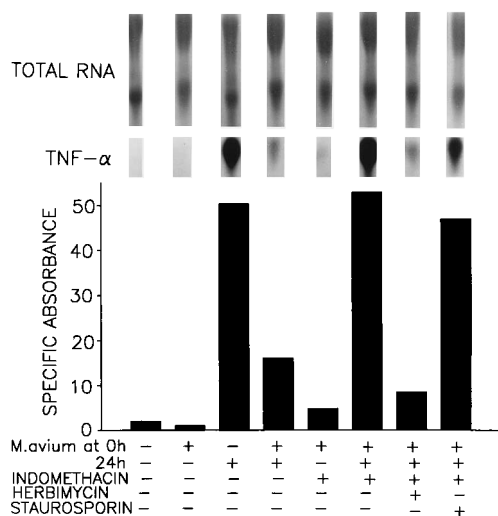


FIG. 4. TNF- $\alpha$  mRNA accumulation after reinfection with *M. avium* in the presence of indomethacin is inhibited by the PTK inhibitor herbimycin. M $\phi$  inoculated with *M. avium* at time zero in the presence (+) or absence (-) of indomethacin were subsequently inoculated with *M. avium* at 24 h in the presence or absence of staurosporin (1  $\mu$ M) and herbimycin (10  $\mu$ M). The cells were harvested 4 h after reinfection, and the mRNA was isolated and analyzed for TNF- $\alpha$  transcripts by Northern blot analysis. (Top) Total RNA; (middle) mRNA hybridized with a human TNF- $\alpha$  probe; (bottom) relative densities of the blots. The data are from one representative of three experiments.

of TNF- $\alpha$  mRNA levels. Staurosporin inhibits phorbol myristate acetate-induced secretion of TNF- $\alpha$  in cultured monocytes (13). It is therefore possible that its target in the M $\phi$  is protein kinase.

While staurosporin (1  $\mu$ M) had no effect, the PTK inhibitor herbimycin (10  $\mu$ M) inhibited TNF- $\alpha$  mRNA accumulation induced by reinfection with *M. avium* (Fig. 4). Genistein (20  $\mu$ M), a second PTK inhibitor, also inhibited TNF- $\alpha$  mRNA accumulation induced by reinfection with *M. avium*. No conclusions could be drawn from the genistein experiments, because this agent also inhibits protein kinases and S6 kinase (18, 28). Thus, TNF- $\alpha$  accumulation induced by reinfection with *M. avium* in the presence of indomethacin is PTK dependent, in contrast to the TNF- $\alpha$  accumulation after the initial infection (13).

We then compared the stability of the TNF- $\alpha$  mRNA transcripts accumulated during the initial infection and the reinfection with *M. avium*. To exclude a nonspecific effect of indomethacin, we examined transcript stability during the initial infection and found similar TNF- $\alpha$  transcript stability during the initial infection in the absence and presence of indomethacin (not shown). In contrast, the half-life of TNF- $\alpha$  transcripts accumulated during the initial infection, 160 min, was greater than the half-life of TNF- $\alpha$  mRNA induced in the presence of indomethacin by reinfection with *M. avium*, 40 min (Fig. 5). The increased lability of the TNF- $\alpha$  transcripts accumulated in response to reinfection in the presence of indomethacin in comparison to the lability of TNF- $\alpha$  transcripts accumulated in response to the initial infection with *M. avium* is thus another characteristic which differentiates the response to an initial infection from the response to a reinfection with *M. avium*.

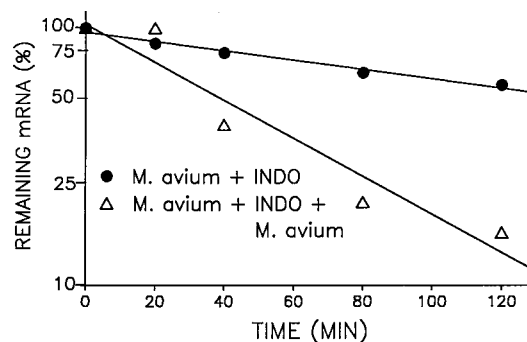


FIG. 5. Stability of TNF- $\alpha$  transcripts in either M $\phi$  stimulated with *M. avium* (10 microorganisms per cell) at 0 h in the presence of indomethacin (INDO) and harvested after 4 h (circles) or M $\phi$  stimulated with *M. avium* at 0 h in the presence of indomethacin, reinfected with *M. avium* (10 microorganisms per cell) at 24 h, and harvested at 28 h (triangles). M $\phi$  ( $5 \times 10^5$  cells per 0.5 ml) incubated as indicated above were treated with actinomycin D as described in Materials and Methods. The cells were then harvested at various time points as indicated on the abscissa, and the total RNA was isolated. Northern blots were performed with 20  $\mu$ g of total RNA per lane and hybridized with a human TNF- $\alpha$  probe. The relative absorbance indicated on the ordinate was normalized relative to the amount of corresponding total RNA by scanning, and the half-life was determined on a semilogarithmic plot fitted by linear regression. Similar results were obtained by two other experiments.

## DISCUSSION

Inoculation of human M $\phi$  from healthy individuals with *M. avium* serovar 4 results in an initial TNF- $\alpha$  response abating after 12 h and renders the cells hyporesponsive to reinfection with *M. avium*. The adaptation to *M. avium* observed in M $\phi$  from healthy donors is abrogated by indomethacin, an inhibitor of prostaglandin synthesis, suggesting that prostaglandins mediate the hyporesponsiveness. This assumption is supported by the presence of high PGE<sub>2</sub> levels in the supernatants of M $\phi$  following the initial *M. avium* inoculation and also by the capacity of PGE<sub>2</sub> to overcome the inhibitory effect of indomethacin on TNF- $\alpha$  mRNA accumulation. Cumulatively, these results show that by upregulation of PGE<sub>2</sub> levels in M $\phi$  *M. avium* is capable of effectively limiting the long-term production of TNF- $\alpha$ , a cytokine essential for defense against intracellular pathogens (3, 27, 38).

It could be argued that the abrogated response of M $\phi$  to reinfection with *M. avium* is caused by a reduced stimulus due to the impaired ability of the M $\phi$  to ingest mycobacteria. However, in BACTEC analysis of the ingestion of *M. avium* by M $\phi$  after the initial challenge and a second challenge, no difference in bacterial uptake could be observed (data not shown). These experiments suggest that the adaptation of M $\phi$  to *M. avium* is not based on diminished ingestion of mycobacteria.

Cyclooxygenase products have been shown to be potent inhibitors of immune reactions. PGE<sub>2</sub>, the prostaglandin species produced by M $\phi$  (9), inhibits IL-2 and gamma interferon production by human T cells (16) and IL-1 production by M $\phi$  (23) but not production of the Th2 cytokines IL-4 and IL-5 (2). It also suppresses NK cell activity (14). On the other hand, the cyclooxygenase inhibitor indomethacin inhibits the gamma interferon-dependent growth inhibition of *M. avium* LR114F in human monocytes (36), indicating that prostaglandins might also play a role in the induction of the M $\phi$ 's hyporesponsiveness to gamma interferon. We have, however, found no evidence that indomethacin increases the growth of *M. avium* serovar 4 in M $\phi$  (data not shown). PGE<sub>2</sub> also has stimulatory properties and increases at low concentrations TNF- $\alpha$  protein

secretion (34) and the production of immunoglobulin E and immunoglobulin G1 (33).

PGE<sub>2</sub> is known to be involved in the desensitization of a variety of cell types. Exposure of NIH 3T3 cells to vasopressin causes hyporesponsiveness of the cells to platelet-derived growth factor with regard to arachidonic acid and PGE<sub>2</sub> production (8), and pretreatment of mouse peritoneal M $\phi$  with PGE<sub>2</sub> abrogates the PGE<sub>2</sub>-dependent inhibition of TNF- $\alpha$  production (37). Desensitization to LPS is partially blocked by inhibition of cyclooxygenase activity in Mono-Mac-6 cells, a human monocytic cell line (15). In contrast, hyporesponsiveness of thioglycolate-elicited murine BALB/c peritoneal M $\phi$  to LPS is not associated with down regulation of TNF- $\alpha$  mRNA accumulation and seems to be a posttranslational process (40). In the present study, human M $\phi$  inoculated with *M. avium* were desensitized to reinfection with *M. avium* by a mechanism involving PGE<sub>2</sub>, suggesting that desensitization in different systems is based on different mechanisms.

PGE<sub>2</sub> inhibits LPS-induced synthesis of TNF- $\alpha$  in murine M $\phi$  (2, 24). In our studies, after an initial *M. avium* inoculation TNF- $\alpha$  mRNA accumulation was also inhibited to some extent by PGE<sub>2</sub> (Fig. 2, 0 h P and 24 h M), but indomethacin did not significantly enhance initial TNF- $\alpha$  mRNA accumulation (Fig. 2, 0 h I and 24 h M). The significance of this finding is not clear.

Interestingly, TNF- $\alpha$  stimulates prostaglandin synthesis in human and murine M $\phi$ . Induction of PGE<sub>2</sub> synthesis varies from 4 to 6 h after TNF- $\alpha$  application in murine M $\phi$  (25) to 18 to 72 h in human M $\phi$  and human synovial cells (4, 7). We have no evidence that induction of PGE<sub>2</sub> by TNF- $\alpha$  occurs in our system.

We conclude that the virulent *M. avium* serovar 4 suppresses long-term TNF- $\alpha$  production of human M $\phi$  by a prostaglandin-dependent mechanism which inhibits the nonspecific immune defense against this pathogen. It will be of interest to examine whether other less pathogenic strains of *M. avium*, such as serovar 2, are comparable or are different in their capacity to evade a strong TNF- $\alpha$  response.

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