Effects of Gamma Interferon on Release of Tumor Necrosis Factor Alpha from Lipopolysaccharide-Tolerant Human Monocyte-Derived Macrophages

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After an initial stimulation of human monocyte-derived macrophages with bacterial lipopolysaccharide (LPS), which produces substantial release of tumor necrosis factor-α (TNF-α), a subsequent exposure to LPS results in about an order-of-magnitude reduction in the levels of TNF-α released. We have shown that macrophages which have been stimulated with LPS and then maintained in culture without LPS for as long as 2 weeks do not regain their original capacity to secrete TNF-α upon a second LPS challenge. After 2 to 4 days in adherent culture, monocyte-derived macrophages which were not pretreated with LPS also experience a measurable decline in their capacity to release TNF-α in response to an initial LPS stimulation. When compared with these previously nonstimulated cells, however, the levels of TNF-α released by LPS-pretreated cells in response to a second LPS challenge decline by over 90% after 8 to 9 days in culture. Unstimulated cells spontaneously release barely detectable levels of TNF-α. In contrast to the release of TNF-α, unstimulated cells release significant levels of prostaglandin E2 continuously over time, and these levels are variably increased by no more than a factor of two in response to a single LPS stimulation. Prostaglandin E2 levels released by LPS-pretreated cells in response to a second LPS stimulation are much closer to the levels released by unstimulated cells. We have also demonstrated that gamma interferon (IFN-γ) enhances TNF-α release from LPS-stimulated macrophages but not from phorbol myristate acetate-stimulated cells. Addition of IFN-γ to macrophages either during the initial stimulation or during a second stimulation with LPS enhances levels of TNF-α released after the second LPS challenge. The greatest enhancement is observed when IFN-γ is added during both exposures to LPS, but addition of IFN-γ during only the initial LPS stimulation still results in marked enhancement of TNF-α release in response to a second stimulation with LPS 24 h later. If an interval of 2 days of culture in medium alone separates the first and second 24-h LPS stimulations, IFN-γ enhances TNF-α release only when it is included during the second LPS exposure, indicating that, unlike the persistence of endotoxin tolerance, enhancement of TNF-α release by IFN-γ is transient.

Tumor necrosis factor-α (TNF-α) or cachectin was first described in 1975 by Carswell et al. (6) as an antitumor activity in the sera of mice immunized with Mycobacterium bovis bacillus Calmette-Guérin and challenged with bacterial lipopolysaccharide (LPS). TNF-α has been subsequently recognized to have pleomorphic effects in the host response to injury and infection (3). Blood monocytes and tissue macrophages will release TNF-α in response to a number of activating stimuli, including LPS from gram-negative bacteria. This released TNF-α is implicated as a major factor responsible for the pathophysiologic consequences of endotoxemia presenting as endotoxic shock (3).

Activation of monocytes and macrophages in vitro is facilitated significantly by the lymphokine gamma interferon (IFN-γ), which is generally regarded as the source of macrophage-activating factor/activity in vivo as well. The role of IFN-γ has been described as that of a priming signal for activation of mononuclear phagocytes by LPS (13), and it can lower the LPS dose required to bring monocytes and macrophages to a full state of activation (15).

IFN-γ has been shown to increase transcription of TNF-α mRNA in mouse peritoneal macrophages (8). In C3H/HeJ mice carrying the ipad mutation, which confers endotoxin resistance, treatment with IFN-γ overcomes this resistance and permits production of TNF-α in response to stimulation with LPS (4). In a recent report, IFN-γ has been demonstrated to inhibit the hyporesponsiveness to LPS induced by a prior LPS treatment in a transformed human mononcytic cell line in culture (12).

Hyporesponsiveness to multiple LPS stimulations, or endotoxin tolerance, has been demonstrated both in vitro and in vivo in several model systems (1, 11, 12, 16, 27). We have recently described several features of endotoxin tolerance in human monocytes and macrophages as reflected by depressed release of TNF-α upon consecutive LPS stimulations and have shown that the magnitude of the reduction in TNF-α release is directly correlated with the LPS concentration during the initial stimulation but is inversely correlated with the LPS level during the second stimulation (21). Questions which arose from these studies included how long the hyporesponsiveness to a second endotoxin exposure might persist and whether physiological mechanisms which would lead to an apparent reversal of such hyporesponsiveness might exist. In this report, we show that human monocyte-derived macrophages which have been stimulated once with LPS do not release TNF-α in response to a second LPS stimulation at levels which are comparable to the levels released in response to the initial stimulation, even when an interval of 2 weeks is introduced between the consecutive stimulations. We also show that the release of TNF-α can be markedly enhanced by exposure of the macrophages to
IFN-γ. This enhancement of TNF-α release can effectively counterbalance LPS-induced hyporesponsiveness. However, the enhancement induced by IFN-γ is transient, unlike LPS-induced endotoxin tolerance, and after an interval of 2 days between consecutive LPS stimulations, a second exposure to IFN-γ at the time of the second stimulation with LPS may be required to reverse the depression in TNF-α release.

**MATERIALS AND METHODS**

**Chemicals.** LPS (Escherichia coli serotype O55:B5 [phenol extracted]) and phorbolmyristic acid (PMA) were obtained from Sigma (St. Louis, Mo.). Recombinant human IFN-γ was obtained from Amgen (Thousand Oaks, Calif.).

**Cell isolation and culture.** Leukocyte concentrations were obtained from healthy donors, and human peripheral blood mononuclear cells were isolated by gradient centrifugation over Isopaque (Nycomed, Oslo, Norway) by the method of Boyum (5). Monocytes were separated from lymphocytes by adherence to plastic coated with human serum (Normalcera-plus, NABI, Miami, Fla.) and detachment with Puck’s saline supplemented with 2.5% human serum albumin and 5 mM EDTA by the method of Levy and Edgington (19). The monocytes obtained by this procedure are routinely >95% pure, as judged by morphologies of cytocentrifuge preparations. To obtain monocyte-derived macrophages, the cells were cultured under nonadherent conditions in Teflon beakers for 1 week in RPMI 1640 supplemented with 10% human serum and buffered with 22 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid). In other studies in our laboratory, we have shown that by 1 week of culture, the monocytes express surface marker levels characteristic of macrophages. All media and reagents used in isolation and culture were checked to ensure that endogenous endotoxin levels were below 0.5 EU/ml. Teflon beakers were routinely washed with an endotoxin-removing detergent solution (Pyroclean, Alerchef, Portland, Maine) before final pyrogen-free water rinses were performed. For assays of released TNF-α, the monocyte-derived macrophages were either allowed to adhere to 24-well plates (Costar, Cambridge, Mass.) or were maintained under nonadherent conditions in 5-ml Teflon beakers (Scientific Specialties Service, Ralaland, Md.). In both cases, cells were employed at a density of 4 × 10^7/ml.

Assays. Macrophages were stimulated with LPS doses ranging from 0.1 to 10 μg/ml or with 12 ng of PMA per ml in the presence or absence of 10 U of IFN-γ (specific activity, 10^8 U/mg) per ml for the times indicated. Cells from a single donor were used in any one experiment. Supernatants were removed and frozen at −70°C prior to performance of an assay. TNF-α was assayed by enzyme-linked immunosorbent assay (ELISA) kits (Olympus, Lake Success, N.Y., and Endogen, Boston, Mass.) with reported lower limits of detection of 5 to 10 pg/ml. The variation among replicate samples of cells in any one experiment was low, with a standard error of 5% of the mean TNF-α value. Comparison between experiments performed with cells from different donors showed much larger variability on a quantitative level, as had been previously described for macrophage responsiveness to LPS (31), but all replicate experiments gave qualitatively similar results. PGE2 was assayed by an H radioimmunoassay (Advanced Magnetics, Cambridge, Mass.) with a reported lower limit of detection of 82 pg/ml. The standard error of the mean PGE2 value for replicate samples in any one experiment was about 9%, with larger donor-to-donor variability as discussed above. Replicate experiments gave qualitatively similar results. Endogenous endotoxin levels in all media were assayed by an ELISA (QCL-1000, Whittaker, Walkersville, Md.) with a reported lower limit of detection of 0.01 endotoxin unit (EU)/ml. Viability of nonadherent macrophages was determined after collection of supernatants by exclusion of Trypan blue. Viability of macrophages adherent to 24-well plates was determined by measuring the conversion of the tetrazolium salt MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] to its formazan by the method of Mosmann (23).

**RESULTS**

Human monocyte-derived macrophages were exposed for 24 h to 10.0 μg of LPS per ml as an initial stimulus at the time of adherence to 24-well polystyrene plates. Control cells were plated in the absence of LPS. After removal of the supernatants and addition of fresh medium, which had been stimulated at the time of adherence were either restimulated immediately for an additional 24 h with 10 μg of LPS per ml or were restimulated with this concentration of LPS after intervals of 24, 48, 72, 96, 120, 144, or 168 h in LPS-free medium, which was changed daily. Separate wells of macrophages which had been plated and maintained in the absence of LPS received their initial LPS stimulation at the same time that the cells which had been stimulated initially at the time of adherence were receiving their second stimulation. As a further control, one set of cells was stimulated at the time of plating, and then, after replacement of medium after 24 h, the cells were incubated for an additional 24 h in the fresh medium in the absence of further stimulation. Two representative sets of data are shown for this experiment (Fig. 1) to illustrate the reproducibility of qualitative trends even when absolute values of TNF-α release varied from donor to donor, as was previously described by others (31). As can be seen from Fig. 1, after a single stimulus with LPS at the time of adherence (0 h), macrophages stimulated with 10 μg of LPS per ml released levels of TNF-α in the nanograms-per-milliliter range. If the cells were allowed to adhere for periods ranging from 24 to 72 h prior to their first stimulation with LPS, the level of released TNF-α remained in this elevated range. If the cells were restimulated for 24 h immediately after the first LPS exposure, the release of TNF-α was reduced to only one-fourth of the levels released by macrophages which had been stimulated only immediately after plating or even 24 h after plating. Inserting an interval of 24 h in LPS-free medium between the first and second stimuli reduced the levels of TNF released to 100 to 200 pg/ml. Further extension of the interval between the first and second stimulations to as long as 168 h had little further effect on the level of TNF-α released after the second stimulus. The levels of TNF-α released by cells which received their first 24-h stimulation with LPS more than 1 day after adherence continued to fall from the high levels seen when the initiation of the stimulus had occurred 24 to 72 h after adherence, and then gradually began to increase. These levels were generally greater than the levels released by cells which had been stimulated twice with LPS by the eighth day of adherence, the differential response of macrophages which had been stimulated for the first time was 14 to 16 times greater than that of cells which had been stimulated for the second time on that day. Minimally detectable amounts of TNF-α were found in the supernatants of cells which were not stimulated with LPS but which were simply cultured in fresh LPS-free medium for 24 or 48 h. Similarly, the levels of TNF-α released by cells which were not
restimulated following a single 24-h exposure to LPS, but which were simply maintained in LPS-free medium for 24 h thereafter, were close to the lower limit of detection. The results shown here were obtained with cells from two different donors. In a separate experiment with cells from a third donor, levels of TNF-α released by macrophages stimulated with either 0.1, 1.0, or 10.0 μg of LPS per ml at the time of adherence were 2,490, 2,200, and 2,516 pg, respectively. Since these levels did not differ appreciably over the range of LPS concentrations used in these studies, further experiments were conducted with the highest dose of LPS as a stimulus, primarily to minimize the relative contribution from any residual LPS contamination between successive stimulations and to ensure that the maximum response of the cells to each stimulus was obtained.

In an effort to determine the time of recovery from hyporesponsiveness to a second stimulation with endotoxin, we increased the period between the first and second exposures to LPS by up to 2 weeks (Table 1). After 1 week in culture following the initial stimulus, levels of TNF-α released after a second stimulus were lower than those released from cells stimulated a second time immediately after the first 24-h exposure, confirming the results obtained with macrophages from the two different donors shown in Fig. 1. A 2-week interval between the first and second exposures resulted in even lower levels of released TNF-α. Since the macrophages still displayed normal morphology as determined by light microscopy and since they retained their capacity to metabolize MTT to its formazan (data not shown), we conclude that monocyte-derived macrophages lose some of their capacity to release TNF-α in response to an initial LPS stimulus as they continue their differentiation in adherent culture but that they retain their hyporesponsiveness to a second stimulation with LPS for at least 2 weeks. By this time, the difference between the responses of previously unstimulated cells and cells which had been stimulated initially at the time of adherence had actually increased from that seen 2 weeks earlier.

In contrast to the pattern of TNF-α release following either a single stimulus or multiple stimuli with LPS, the levels of PGE2 released by macrophages during 192 h in culture were relatively invariant (Fig. 2). A single 24-h stimulation with 10 μg of LPS per ml, either at the time of adherence or after several days of maintenance under adherent conditions in LPS-free medium, increased the levels of PGE2 released during the stimulation period by no more than a factor of two over the levels released by unstimulated cells, which were constitutively maintained at a fairly constant rate over the course of the experiment. After the initial stimulation, the cells became minimally responsive to further stimulations with LPS and continued to release PGE2 at roughly the same levels that unstimulated cells did. Separate aliquots of the same cell-free supernatants were used to determine TNF-α and PGE2 levels.

IFN-γ increased levels of TNF-α released from monocyte-derived macrophages receiving a single stimulation with LPS

![Graph](http://iai.asm.org/)

**FIG. 1.** Release of TNF-α by human monocyte-derived macrophages isolated from two different donors after one or two stimulations with LPS. A total of $4 \times 10^5$ cells in 1 ml of RPMI medium–10% human serum were added to each well of a 24-well microplate. Cells in one set of wells were stimulated once with 10 μg of LPS per ml at the times after plating indicated on the abscissa, and incubation was continued for an additional 24 h before collection of the supernatants for ELISA ( ). Cells in a second set of wells were stimulated initially with 10 μg of LPS per ml at the time of plating and were restimulated again at the times indicated on the abscissa. Incubation was continued for 24 h before collection of supernatants for the TNF-α assay ( ). Cells in a third set of wells were incubated in medium alone for 24 or 48 h after plating before supernatants were collected for the TNF-α assay ( ). Cells in a fourth set of wells were stimulated for 24 h beginning at the time of adherence, and after replacement of medium were incubated in the absence of further stimuli for an additional 24 h before collection of supernatant for the TNF-α assay ( ). The medium in all wells was changed every 24 h. The concentration of released TNF-α is indicated on the ordinate.

**TABLE 1.** TNF-α release by macrophages after two separated LPS stimulations

<table>
<thead>
<tr>
<th>Stimulation protocol</th>
<th>TNF-α concn (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 0 only</td>
<td>3,514</td>
</tr>
<tr>
<td>Day 0 and day 1</td>
<td>421</td>
</tr>
<tr>
<td>Day 0 and day 7</td>
<td>244</td>
</tr>
<tr>
<td>Day 0 and day 16</td>
<td>128</td>
</tr>
</tbody>
</table>

* Monocyte-derived macrophages were applied to 24-well plates and stimulated for 24 h with 10 μg of LPS per ml (day 0). Medium was removed and replaced with fresh medium containing 10 μg of LPS per ml for 24 h (day 1) or with LPS-free medium. LPS-free medium was replaced every 24 h until day 7, when some wells received medium containing 10 μg of LPS for 24 h. Other wells continued to receive LPS-free medium, which was changed daily until day 16, when medium containing 10 μg of LPS per ml was added for 24 h. The supernatants were removed at the end of the second LPS stimulations and were assayed for TNF-α as described in the text. The results shown are from one of two replicate experiments with monocyte-derived macrophages from different donors, both of which gave qualitatively similar results.
FIG. 2. Release of PGE₂ from human monocyte-derived macrophages after one or two stimulations with LPS. A total of $4 \times 10^5$ cells in 1 ml of RPMI medium–10% human serum were added to each well of a 24-well microplate. Cells in one set of wells were stimulated once with 10 μg of LPS per ml at the time after plating indicated on the abscissa, and incubation was continued for an additional 24 h before collection of the supernatants for radioimmunoassay for PGE₂ ([ ]). Cells in a second set of wells were stimulated initially with 10 μg of LPS per ml at the time of plating and were restimulated at the times indicated on the abscissa, and incubation was continued for 24 h prior to collection of supernatants for PGE₂ assay ([ ]). Cells in a third set of wells were incubated in medium alone for 24 h after plating before supernatants were collected for PGE₂ assay ([ ]). The medium in all wells was changed every 24 h. Concentration of released PGE₂ is indicated on the ordinate. These results were obtained with separate aliquots of supernatants of cells from the same donor as were the results in Fig. 1b.

but not from cells stimulated with PMA, as seen in Fig. 3. Preincubation of macrophages with IFN-γ for 3 h followed by stimulation with LPS for the next 24 h resulted in increased TNF-α release compared with levels of TNF-α released by cells stimulated with LPS alone. A 3-h preincubation with IFN-γ had no significant effect, however, on TNF-α release in response to PMA stimulation. Incubation of macrophages with IFN-γ for 24 h in the absence of other exogenously added agents resulted in only a modest stimulation of TNF-α release compared with the levels released by the cells in the absence of any additions at all to the culture medium, which were below the level of detection. The stimulatory effect of IFN-γ alone was only about 27% of the effect of LPS or PMA.

The capacity of IFN-γ to boost TNF-α levels could be seen from analysis of supernatants collected after 24 h of stimulation with LPS in the presence of IFN-γ, as shown in Fig. 3, but was even apparent on examination of supernatants collected 4 h after initial stimulation. After 24 h of stimulation of macrophages from another donor in the presence of IFN-γ, TNF-α levels reached 8 ng/ml, more than twice the level of 3 ng/ml reached after stimulated with LPS only. In a separate experiment, cells released 19% more TNF-α in 4 h when stimulated with LPS in the presence of IFN-γ than they did when stimulated with LPS alone. When IFN-γ was present along with PMA, however, the cells released 11% less TNF-α than they did when stimulated with PMA alone.

The potentiating effect of IFN-γ on LPS-stimulated TNF-α release was also evidenced by its capacity to reduce the magnitude of the downregulation of TNF-α release seen after a second stimulation of macrophages with LPS. Figure 4 illustrates the levels of TNF-α released by macrophages which were pretreated with LPS for 24 h either in the presence or in the absence of IFN-γ and then, after replacement of the medium, immediately restimulated for another 24 h with LPS, again either in the presence or in the absence of IFN-γ. The levels of TNF-α released during the second 24 h, when IFN-γ was present during both LPS stimulations, were about three times as high as the control levels released when IFN-γ was absent during both stimulations. Addition of IFN-γ to the LPS, either during the first or second stimulation, had a smaller enhancing effect (about twofold).
FIG. 5. Effect of introduction of a 2-day interval between two 24-h LPS stimulations on the enhancement of TNF-α release by IFN-γ. In all of the experiments, the cells were stimulated initially with 10 μg of LPS per ml for 24 h in the presence or absence of 10 U of IFN-γ per ml. After 2 days in LPS-free medium, the cells were restimulated for 24 h with 10 μg of LPS per ml in the presence or absence of 10 U of IFN-γ per ml at the beginning of the fourth day of culture. Supernatants were removed at the end of the second stimulation for TNF-α assay. Results shown are from one of two replicate experiments with macrophages from different donors, both of which gave qualitatively similar results. LPS1/LPS4, cells were stimulated initially with LPS alone and, after a 2-day interval in LPS-free medium, were restimulated on the fourth day with LPS alone. IFN+LPS1/LPS4, cells were stimulated initially with LPS plus IFN-γ and were restimulated on the fourth day with LPS alone. LPS1/IFN+LPS4, cells were stimulated initially with LPS alone and were restimulated on the fourth day with LPS plus IFN-γ. Medium was replaced daily during this experiment.

The results in Fig. 4 show clearly that macrophages which had been stimulated initially with LPS plus IFN-γ release more TNF-α upon a second stimulation with LPS alone than do cells which had been stimulated twice with LPS only. In order to determine whether the potentiating effect of IFN-γ on TNF-α release could be sustained for extended periods after removal of the IFN-γ, we first stimulated macrophages for 24 h with LPS in the presence of IFN-γ and then, after replacement of the medium, we maintained the cells in culture for an additional 2 days in the absence of exogenously added agents. A second 24-h stimulation with LPS alone after this 48-h intervening period in medium alone no longer resulted in increased secretion of TNF-α when compared with levels released by control macrophages stimulated by two separated exposures to LPS alone, as shown in Fig. 5. However, when we introduced a 2-day interval between 24-h LPS stimulations and added IFN-γ only during the second LPS treatment, the lymphokine still enhanced the levels of TNF-α released during the second LPS stimulation by a factor of two over those released by the control cells, which received no IFN-γ. We conclude from these results that the enhancing effect of IFN-γ on TNF-α release is transient and may persist for no more than 24 h, unlike the acquisition of endotoxin tolerance, which persists for weeks. However, cells which have become hyperresponsive to a second stimulation with LPS are still capable of responding to a costimulation with IFN-γ at the time of the second LPS stimulus by releasing increased levels of TNF-α.

**DISCUSSION**

Although the release of TNF-α associated with inflammatory reactions may have beneficial consequences for immune stimulation and for tumor cell cytotoxicity, this monokine is known to mediate most of the pathologic effects induced by LPS in endotoxic shock. It is not surprising, therefore, that synthesis and release of TNF-α are under stringent control. Endotoxin tolerance is a well-established phenomenon (7) which may be considered a reflection of the control mechanisms regulating TNF-α release. It has been demonstrated both in vivo and in vitro, using rabbits, mice, and a human monocytic cell line, that production of TNF-α is reduced after a second stimulation with LPS (12, 16, 20, 27). We have previously shown that human monocytes release barely detectable levels of TNF-α after a second LPS stimulation if the cells are treated initially with a high dose of LPS (21). Although monocytes have been estimated to remain in the circulation for only 18 to 54 h prior to their migration into the interstitium (32), once the extravasated cells take up residence within the interstitium, they may survive as macrophages for months to years. It is therefore likely that in an individual exposed to multiple elevations in endotoxin levels, a significant fraction of the mononuclear phagocytes may have experienced repeated stimulations by LPS. Our studies in this report were designed to address some of the additional factors which might modulate the expression of endotoxin tolerance in vivo compared with the phenomenon as expressed by monocytes in culture receiving two consecutive treatments with LPS spaced 24 h apart. Two of these factors which we conclude might have significant modulating effects on endotoxin tolerance in vivo are the presence of IFN-γ, a product of activated lymphocytes which is likely to be produced in an inflammatory response, and the interval of time which separates two consecutive exposures to LPS.

Our results reported here show that after a total duration of 3 weeks in culture from the time of isolation from peripheral blood, monocyte-derived macrophages release lower levels of TNF-α in response to a single stimulation with LPS than do monocyte-derived macrophages which are stimulated for the first time 1 week after isolation. This decline in TNF-α production in vitro parallels the reported decreased cytotoxicity displayed by monocyte-derived macrophages after 2 weeks in culture (9). The decline we observe may be partly due to adherence to plastic, but it still takes 2 to 4 days after plating to develop. Moreover, reduced levels of TNF-α are also released from monocyte-derived macrophages cultured under nonadherent conditions for periods in excess of 1 week (data not shown). Even though the TNF-α levels released from previously unstimulated 3-week-old macrophages are lower than levels released from 1-week-old cells, the macrophages also release even less TNF-α after a second stimulation with LPS, displaying the same pattern of endotoxin tolerance in vitro as monocytes.

There is no significant decline in the release of PGE₂ by previously unstimulated monocyte-derived macrophages if they are maintained in adherent culture for 1-week prior to a single LPS stimulation. Rather, PGE₂ is released at invariant levels by the cells in culture, even in the absence of an LPS stimulus. This PGE₂ would be most likely to accumulate in cultures of cells in which medium was not changed, and such cultures do tend to release lower levels of TNF-α (data not shown). We have previously shown that exogenously added PGE₂ can downregulate the levels of TNF-α released by monocytes (21), but the role of endogenously produced PGE₂ in contributing to declining TNF-α levels in monocyte-derived macrophages after a single stimulus remains speculative.

The hyporesponsiveness of monocyte-derived macrophages to a second exposure to LPS is noteworthy for its persistence, which was clearly evidenced in cells which had received their first exposure to LPS 2 weeks prior to a
second LPS challenge. Since macrophages may be exposed to a number of other cytokines in the interstitial fluid in vivo, we investigated the effect of IFN-γ, a macrophage-activating factor of major physiological importance, on recovery from endotoxin tolerance. IFN-γ has been well recognized as a factor which enhances the expression of a variety of functional properties, the release of a number of soluble mediators, and the appearance of several membrane surface marker proteins, all of which are considered features of monocyte and macrophage activation (29). It has been demonstrated that at least some of these effects of IFN-γ are mediated by binding of the lymphokine to receptors (2, 10, 26). We selected an IFN-γ level of 10 U/ml, which has been previously shown to enhance responsiveness of mononuclear phagocytes and which still lies within a physiologically attainable range (1, 13, 28). The low but significant levels of TNF-α released by our cultured cells in the presence of IFN-γ alone probably reflect the potentiating effect of the lymphokine on the trace levels of endotoxin present in our culture medium, which contains 10% human serum, another factor shown to potentiate responsiveness to LPS. These trace levels of endotoxin in the serum-containing medium alone were insufficient to stimulate the cells to release TNF-α above background levels, but they may have been adequate to stimulate the cells in the presence of IFN-γ, which has been reported to lower the minimum dose requirement of LPS for activation (15).

IFN-γ was capable of potentiating the response of macrophages to a single exposure to LPS, inducing increased levels of released TNF-α as soon as 4 h after stimulation and sustaining the increased levels for up to 24 h after stimulation. The maximum efficacy of this single exposure to IFN-γ was achieved when the cells were reincubated with the lymphokine prior to exposure to endotoxin. Our results obtained with these incubation protocols are consistent with the observations of others, which have led to the conclusion that IFN-γ functions as a priming signal rather than as an activator per se (17, 18, 24).

PMA has been shown to exert many of its effects through activation of protein kinase C (25). IFN-γ also appears to exert its effect on macrophages through protein kinase C (PKC) activation (14) and in fact has been shown to be two to four times more potent in stimulating murine macrophage PKC-catalyzed protein phosphorylation than diacyl glycerol (30). Clearly, since IFN-γ alone is a relatively weak stimulator of TNF-α release and PMA is a strong stimulator, the effects of these two agents cannot be precisely identical, even though they may share a common pathway through which they act. The failure of IFN-γ to augment release of TNF-α by macrophages stimulated with PMA suggests that the effects of the lymphokine and the phorbol ester on macrophages involve a shared pathway rather than completely independent parallel pathways. In recent experiments in our laboratory, we have found that specific inhibitors of PKC are effective in blocking release of TNF-α from monocytes in response to stimulation with LPS, implicating a PKC-dependent signal transduction pathway in the regulation of TNF-α release (22). We have also already reported that monocytes previously stimulated with LPS can still release significant quantities of TNF-α in response to stimulation with PMA, even when only minimal levels of TNF-α are released in response to a second stimulation with LPS (21). These findings suggest that PMA and IFN-γ may both oppose the expression of endotoxin tolerance in cells through their action on a PKC-dependent signal transduction pathway.

Our observations on the effects of IFN-γ on in vitro endotoxin tolerance are compatible with the findings of Haas et al. on in vitro inhibition of LPS-induced desensitization by IFN-γ in a transformed human monocyte cell line (11). The augmented enhancement generated by two consecutive exposures to IFN-γ and the transience of the enhancing effect seen when an interval separates the exposures to IFN-γ both support our contention that IFN-γ does not directly block the mechanisms underlying endotoxin tolerance but rather enhances TNF-α release sufficiently to counterbalance the downregulation of the response to a second exposure to LPS. While we have shown that the effects of IFN-γ on enhancement of TNF-α release are transient, we do not have evidence as to whether this transience is also reflected in any of the other features associated with enhancement of activation by IFN-γ.

While the detailed mechanism of endotoxin tolerance at a cellular level still requires much further study, our results do eliminate some possible factors from consideration. The actual level of TNF-α released during the first stimulation with LPS does not seem to control the magnitude of depression of TNF-α release after a second LPS stimulation, since cells stimulated initially with LPS plus IFN-γ release very high levels of TNF-α after the first exposure but do not show more hyporesponsiveness to a second LPS treatment than do macrophages stimulated initially with LPS alone. These findings are consistent with earlier observations that unresponsiveness to LPS is not directly induced by TNF-α or by other products released when macrophages are exposed to exogenous TNF-α (20-22).

These results point to a potentially significant modulating role of an important inflammatory lymphokine, IFN-γ, in regulating the response of macrophages to repeated exposure to LPS. From our findings, we expect that levels of TNF-α will be elevated and the magnitude of the loss of responsiveness to repeated endotoxin exposure will be significantly reduced if tissue macrophages have been repeatedly primed by IFN-γ, but hyporesponsiveness may still be apparent for some time after an initial endotoxin exposure if the IFN-γ levels are not sustained during repeated endotoxinemic episodes. These considerations may be of especially great importance in the management of patients with chronic infections involving gram-negative pathogens and, eventually, in the design of pharmacologic interventions for therapy.

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REFERENCES


