Molecular mechanisms in down-regulation of tumor necrosis factor expression
(desensitization/lipopolysaccharide/prostaglandin E₂/protein kinase C/transcription factor NF-κB)

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ABSTRACT Excessive production of tumor necrosis factor (TNF) after stimulation by lipopolysaccharide (LPS) may result in fever, intravascular coagulation, and lethal shock. An efficient way of preventing the excessive TNF production is desensitization of monocytes/macrophages to LPS. We have analyzed the molecular mechanisms involved in the induction of desensitization and the mechanisms operative in the desensitized, LPS-refractory cells by employing the human monocytic cell line Mono-Mac-6. Similar to human blood monocytes, treatment of Mono-Mac-6 cells with LPS (1 μg/ml) results in a rapid and transient expression of TNF. When Mono-Mac-6 cells are precultured in medium containing low levels of LPS, they become refractory to subsequent LPS stimulation and show no or little secretion of TNF protein. Desensitization can be blocked by the inhibition of cyclooxygenase and protein kinase C; both prostaglandin E₂ (together with a second signal) and phorbol 12-myristate 13-acetate can mimic desensitization. By employing prostaglandin E₂ and low concentrations of phorbol 12-myristate 13-acetate, a synergism in the induction of desensitization can be demonstrated. Hence, our studies show that two distinct pathways are involved in the induction of hyporesponsiveness. In both LPS-responsive and LPS-desensitized Mono-Mac-6 cells, LPS was able to induce the transcription factor NF-κB in the nucleus. Still, the prevalence of TNF-specific mRNA was dramatically reduced in the desensitized cells. These data indicate that LPS-desensitized Mono-Mac-6 cells are able to activate initial steps of signal transduction up to the level of the NF-κB transcription factor. The absence of TNF transcripts, however, indicates that additional nuclear factors may be missing or that silencers may be active such that transcription of the TNF gene is prevented.

Tumor necrosis factor (TNF) was originally discovered by its ability to destroy malignant tumors in vitro and in vivo (1, 2). Later on, when molecularly cloned TNF became available, it was shown that TNF can activate endothelial cells, granulocytes, monocytes, T cells, and B cells (3). Thus TNF appears to be an important mediator of nonspecific and specific defenses against tumors (4) and infection (5). On the other hand, TNF has many toxic effects that result, for instance, in weight loss, intravascular coagulation, and shock (6–8). Hence, regulatory mechanisms that control excessive and prolonged production of TNF have to exist. One of the major stimuli for TNF production is bacterial lipopolysaccharide (LPS). Upon repeated administration of LPS the responsiveness of the host decreases, a process termed tolerance or desensitization (9, 10). A prototypic example for desensitization is desensitization to β-adrenergic drugs in various cell types. In this case, modulation of the respective receptor is discussed as a major mechanism (11). By contrast, little is known about the molecular mechanisms involved in desensitization to LPS. Recent studies in experimental animals indicate that desensitization to LPS occurs at the level of the monocyte (12), and this is supported by in vitro studies with peritoneal macrophages (13) and a monoblastic cell line (14). For our studies, we have employed the cloned human monocyte cell line Mono-Mac-6 (15). In response to LPS (1 μg/ml), this cell line produces TNF mRNA and protein in large amounts, but preincubation of the cell line with low doses of LPS (10 ng/ml) can efficiently suppress the TNF production. In analyzing the molecular mechanisms of desensitization in these cells, we find two distinct pathways of signal transduction (i.e., generation of prostaglandin E₂ (PGE₂) and activation of protein kinase C (PKC)) are required. This suggests that pathways known to be involved in triggering TNF production may at the same time be involved in its down-regulation. Furthermore, LPS-desensitized monocytes are still able to activate nuclear factor κB (NF-κB) in the nucleus, but transcription of the TNF gene does not occur. These data suggest that desensitization in this model is not operative at the level of cell surface receptors but that additional mechanisms of transcriptional control are active in LPS-desensitized monocytes.

MATERIALS AND METHODS

Reagents. Culture medium for Mono-Mac-6 cells contained RPMI 1640 (GIBCO) supplemented with 10% (vol/vol) fetal calf serum (Interchem, Munich, F.R.G.), 1 mM oxalacetate (Sigma), 1 mM pyruvate (Fluka), 1× nonessential amino acids solution, 2× penicillin/streptomycin solution, and 2 mM l-glutamine (GIBCO). Before the addition of fetal calf serum (Interchem), the medium was ultrafiltrated (16) through a Gambro 2000 column (Gambro, Hechingen, F.R.G.) to eliminate LPS. Phenol-extracted LPS from Salmonella minnesota, 1-(5-isounfolinylsulfonyl)-2-methylpiperezine (H7), indomethacin, PGE₂, and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma.

Cell Culture. The Mono-Mac-6 cell line (15) was cultured in LPS-free culture medium (less than 10 pg of LPS per ml as assayed by the Limulus test). Preculture of Mono-Mac-6 cells was carried out with or without LPS at 10–50 ng/ml for 3 days at a cell density of 2 × 10⁵ cells per ml unless indicated otherwise. Cells were pooled and washed in phosphate-buffered saline (PBS) and 2×10⁶ cells per ml were incubated for 3 hr in culture medium containing the indicated reagent. The supernatant was removed, centrifuged twice at 800 × g, and tested for cytotoxic activity.

Determination of TNF. Supernatants were tested for their cytotoxic activity in an ³²Cr-release assay with actinomycin D-pretreated WEHI 164 cells as targets. For the determination of TNF-α, the subclone WEHI 164S, which has a higher

Abbreviations: LPS, lipopolysaccharide; PGE₂, prostaglandin E₂; NF-κB, nuclear factor κB; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; TNF, tumor necrosis factor.

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sensitivity (1–10 pg of TNF-α per ml) than the original WEHI 164 cell line, was used. Experiments were carried out exactly as described (17), and specific release from WEHI 164 cells was calculated according to the following equation:

\[
\text{Experimental release} - \text{spontaneous release} = \frac{\text{Maximal release} - \text{spontaneous release}}{100}
\]

For determination of the TNF-α concentration, a standard of recombinant TNF with a specific activity of 1 × 10^7 units of TNF-α per ml (kindly provided by E. Schlick) was used.

**Northern Blot Analysis.** RNA was isolated by guanidine isothiocyanate lysis and CsCl density gradient centrifugation according to standard protocols (18). After electrophoresis of 20 μg of each sample in a 1% agarose gel containing 17.8% formaldehyde and blotting on a nylon membrane (Amerham), RNA was hybridized either with a 0.6-kilobase Xho I/HindIII fragment (kindly provided by E. Weiβ) derived from exon 4 of the TNF-α gene or with a synthetic 60-mer oligonucleotide that corresponds to amino acids 70–89 of TNF-α according to the published sequence data of Nedwin et al. (19). Control hybridizations were done with a 1.7-kilobase Pst I fragment derived from the human actin gene (20) or with a synthetic 52-mer oligonucleotide corresponding to amino acids 2–17 of the glyceraldehyde-3-phosphate dehydrogenase gene (21). The genomic DNA probes were labeled by random priming (22), and the oligonucleotides were labeled by poly(A)-tailing (23) with [a-32P]dATP (Amersham). The oligonucleotides were kindly synthesized by R. Mertz (Martinried, F.R.G.). Hybridizations with the control probes were done with the same blots after washing in 0.1% SDS (Sigma) at 75°C for 20 min.

**Electrophoretic Mobility Shift Assay.** Mono-Mac-6 cells were stimulated for 1 hr at 37°C with or without LPS at 1 μg/ml, followed by sonication. The nuclei were extracted according to Dignam et al. (24) in buffer C for 30 min on ice. After removal of nuclei, the extracts were diluted with an equal volume of buffer D containing 1% Nonidet P-40 (24).

Electrophoretic mobility shift assays were essentially performed as described (25). In brief, 2–3 μg of nuclear extract protein was admixed with 20 μg of bovine serum albumin, 2 μg of poly(dI-dC) (Pharmacia), and 10,000 cpm (Cerenkov counting) of the NF-κB oligonucleotide. The oligonucleotide used was a 34-mer with HindIII and the Sal I linker sites, containing 20 base pairs corresponding to the sequence of the mouse κ light-chain enhancer with the NF-κB binding site (25). The oligonucleotide was labeled with [α-32P]dCTP (Amersham) using the Klenow fragment of DNA polymerase (Boehringer Mannheim). Samples were run on a 4% native polyacrylamide gel as described (25).

**RESULTS**

**TNF Expression in Mono-Mac-6 Cells.** Unstimulated Mono-Mac-6 cells cultured in LPS-free medium express neither TNF bioactivity nor TNF mRNA (Fig. 1). After stimulation for 3 hr with as little as 1 ng of LPS per ml, TNF bioactivity is detectable in the WEHI 164/actinomycin D assay, and maximum levels are already obtained at an LPS concentration of 10 ng/ml (Fig. 1a). Time course studies reveal that after stimulation with LPS TNF protein is detectable in supernatants of Mono-Mac-6 cells within 1 hr and remains at this high level over the 6-hr observation period (Fig. 1b). In contrast, TNF-specific mRNA, which is first detectable at 1 hr and is still present at 3 hr, has completely disappeared at 6 hr. In experiments not shown, TNF mRNA was already detectable after 0.5 hr of stimulation with LPS.

**Desensitization of Mono-Mac-6 Cells by LPS Pretreatment.** When Mono-Mac-6 cells are pretreated with low amounts of LPS, they become refractory to a subsequent stimulation with LPS. This desensitization of Mono-Mac-6 cells is dependent on the time of LPS pretreatment. Whereas short times of up to 6 hr are insufficient to lead to a significant reduction in TNF production, a significant decrease of TNF production can be observed at 24 hr of LPS pretreatment, and after 48 hr a reduction by more than 3 logarithms is achieved (Fig. 2a). Dose–response analysis of Mono-Mac-6 cells pretreated with LPS for 3 days shows that LPS at 10 ng/ml leads to a marked reduction of TNF release. After preculturing cells with LPS at 100 ng/ml, almost no TNF production was found upon subsequent stimulation with 1 LPS at 1 μg/ml (Fig. 2b).

**Mechanisms in Induction of Desensitization to LPS.** Next we asked whether signal transduction pathways known to be used in the induction of TNF expression (i.e., prostaglandins and PKC) may also be involved in the down-regulation of TNF expression during desensitization. For this purpose we have analyzed whether inhibitors could block and stimulators could mimic LPS-induced desensitization.
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preculture experiments using desensitization is PKC inhibitor and hr, washed with PBS, When stimulated with LPS, is able to PKC in desensitization. Mono-Mac-6 cells were tested with PBS, and stimulated with LPS at 1 µg/ml for 3 hr. Supernatants were tested for TNF bioactivity in a 51Cr-release assay with actinomycin-pre-treated WEHI 164 target cells. (b) Dose-dependent suppression of TNF bioactivity in LPS-induced desensitization. Mono-Mac-6 cells were precultured with the indicated amounts of LPS for 3 days, washed with PBS, and stimulated with LPS at 1 µg/ml for 3 hr, and supernatants were tested for TNF bioactivity.

When Mono-Mac-6 cells are precultured with LPS and the PKC inhibitor H7 is added at 100 µM, the LPS-induced desensitization is partially overcome in that TNF production is increased about 10-fold (Fig. 3a). This is confirmed by experiments using the PKC activator PMA during a 3-day preculture period, which also results in a decrease of TNF expression (Fig. 3b). In repeated experiments over a wide dose range, we could not achieve a complete blockade by H7, indicating that in this long culture period the inhibitor is not sufficiently stable or that additional mechanisms are operative. To investigate this issue, we asked whether other signal transduction pathways act in concert with PKC. In fact, cyclooxygenase inhibitors like indomethacin or ibuprofen are also able to inhibit desensitization. Addition of indomethacin to LPS precultures of Mono-Mac-6 can partially overcome desensitization at 10 µM and near completely at 100 µM (Fig. 4a). Conversely, addition of PGE2 to such cultures containing LPS and indomethacin is able again to reduce the subsequent TNF production by Mono-Mac-6 (Fig. 4b). In contrast to PMA, PGE2 on its own was completely inactive in three experiments with concentrations ranging from 1 nM to 100 µM (including daily addition of PGE2 to cultures), indicating that prostaglandins alone without a second signal do not modulate the cytokine expression. Since PKC might provide such a signal that acts in concert with PGE2, we asked whether PMA synergizes with PGE2 in the induction of hyporesponsiveness. As demonstrated in Fig. 5, PMA at a dose of 48 nM moderately reduced TNF production after subsequent LPS stimulation. The combination of both reagents (PMA and PGE2), however, effectively reduced LPS induction of TNF. Hence, it appears that desensitization to LPS involves the cooperation of at least two different signal transduction pathways.

Molecular Mechanisms in Desensitized, LPS-Refractory Cells. Next we have asked at what level the expression of TNF is prevented in the LPS-refractory cells. Since the CD18 cell surface molecule is assumed to be the LPS receptor, we have studied CD18 expression by immunofluorescence. No significant difference was found in that 69.8% ± 17.6% of the Mono-Mac-6 cells without LPS preculture and 53.6% ± 4.5% with LPS preculture were positive with an anti-CD18 monoclonal antibody (n = 3).

Nuclear factors are involved in the control of inducible genes (26), and for the TNF-α gene, NF-κB has been identified as an important DNA binding protein that initiates transcription (27, 28). Although there appears to be a low-level constitutive activity of NF-κB in the nuclei of Mono-Mac-6 cells, stimulation for 1 hr results in a strong induction of NF-κB as demonstrated by electrophoretic mobility shift assay (Fig. 6, lane C). When Mono-Mac-6 cells were precultured with LPS at 50 ng/ml, a subsequent stimulation with LPS will still induce

**Fig. 2.** Desensitization to LPS in the Mono-Mac-6 cell line. (a) Time course of LPS-induced desensitization. Mono-Mac-6 cells were precultured with LPS at 10 ng/ml for the indicated times, washed with PBS, and stimulated with LPS at 1 µg/ml for 3 hr. Supernatants were tested for TNF bioactivity in a 51Cr-release assay with actinomycin-pren treated WEHI 164 target cells. (b) Dose-dependent suppression of TNF bioactivity in LPS-induced desensitization. Mono-Mac-6 cells were precultured with the indicated amounts of LPS for 3 days, washed with PBS, and stimulated with LPS at 1 µg/ml for 3 hr, and supernatants were tested for TNF bioactivity.

**Fig. 3.** Involvement of PKC in desensitization. Mono-Mac-6 cells were precultured for 3 days without any additive (φ), with LPS at 10 ng/ml, with LPS at 10 ng/ml plus 100 µM H7 (α), or with 16 nM PMA (β). After this preculture, cells were washed and stimulated with LPS at 1 µg/ml for 3 hr, and TNF was assayed in the supernatant. In separate experiments, it had been shown that addition of H7 during the preculture period had no effect on TNF production. Results are representative of three experiments.

**Fig. 4.** Involvement of PGE2 in desensitization. (a) Mono-Mac-6 cells were precultured for 3 days without any additive (φ) or with LPS at 10 ng/ml or with LPS plus one of the two indomethacin (INDO) concentrations given. (b) Cells were precultured without any additive (φ), with 100 µM indomethacin, with 30 µM PGE2, with LPS at 10 ng/ml, or with combinations thereof. After this preculture, cells were washed with PBS and stimulated with LPS at 1 µg/ml for 3 hr, and TNF was assayed in the supernatant. Results are representative of three experiments.
NF-κB in the LPS-desensitized cells as efficiently as in Mono-Mac-6 cells without LPS preculture (Fig. 6, lane E). Parallel assays for production of TNF protein demonstrated a complete suppression of TNF protein production (preculture with LPS, 1.5% specific release; preculture with culture medium, 67.2%). The molecular identity of the DNA binding protein was demonstrated by comigration with purified human NF-κB (Fig. 6, lane A) and by competition of the NF-κB binding to the labeled oligonucleotide with an unlabeled NF-κB oligonucleotide containing the NF-κB binding site from the interleukin 2 receptor α-chain upstream promoter element (29) (Fig. 6, lanes F–I). In a series of three independent experiments we found that Mono-Mac-6 cells desensitized by preculture with PMA could still be activated by LPS to mobilize NF-κB as well (Fig. 6, lanes K and L).

Finally, we have asked whether the LPS-desensitized cells can synthesize TNF transcripts in response to LPS stimulation. Fig. 7 demonstrates that preculture with LPS at 10 ng/ml results in a complete suppression of TNF mRNA.

**DISCUSSION**

Desensitization to LPS is a phenomenon that mainly has been investigated in experimental animals in vivo (9, 10, 30, 31), but little is known about the molecular mechanisms controlling this phenomenon. LPS activates cells of the monocyte system, leading to the production of cytokines including TNF. TNF has been shown to be responsible for many of the toxic effects of LPS and, therefore, it appears reasonable to study TNF expression in these cells to understand the molecular mechanisms involved in desensitization. Since the recently established human mononuclear cell line Mono-Mac-6 (15) is able to express cytokines in a manner similar to human blood monocytes, we asked whether this cell line might serve as a model for LPS-induced desensitization with regard to cytokines like TNF. In fact, in this system preincubation of Mono-Mac-6 cells with low amounts of LPS (10–50 ng/ml) for at least 24 hr results in a refractory state for a subsequent stimulation with a high amount of LPS (1 μg/ml). Whereas in in vivo models repeated daily injections of LPS are necessary for the induction of LPS desensitization because of the rapid elimination of the pyrogen from the blood (9, 10, 30), in our system preculture of Mono-Mac-6 cells with small amounts of LPS over a defined period of time is sufficient. Similar observations on in vitro desensitization with respect to LPS have been made with the human THP-1 cell line looking at the interleukin 1 response (14), with the murine RAW 264.7 cell line looking at TNF (32), and with murine peritoneal macrophages looking at c-fos expression (13). However, the molecular mechanisms involved remained unclear thus far.

Desensitization phenomena seem to occur in most cases by uncoupling, down-regulation, or modification of cellular receptors (11). Our studies on LPS-induced desensitization, however, provide several lines of evidence indicating that the refractory state of LPS-tolerant cells is not or is not alone due to occupancy or modification of the receptor molecule. First of all, we could not detect a modulation of CD18, the putative LPS receptor (33). In addition, when Mono-Mac-6 cells were cultured in the presence of LPS for 2 days, followed by extensive washing and LPS-free culture for 24 hr, the Mono-Mac-6 cells still remained refractory to stimulation by LPS (data not shown). This indicates that after a period that may allow for ligand release and receptor recovery, LPS is still...
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