This study demonstrates that lipopolysaccharide (LPS), a major membrane component of Gram-negative bacteria, plays an important role in the pathogenesis of Gram-negative sepsis leading to septic shock (1). LPS is a potent stimulator of monocytes and macrophages, which release pro-inflammatory cytokines such as TNF (2) and IL-1 (3). LPS activates monocytes and macrophages via CD14, a glycosyl-phosphatidylinositol-anchored surface protein (4). However, LPS receptors other than CD14 may also contribute to LPS signaling (5-7).

A wide variety of other cell types are also affected by LPS, and some of these cells do not express membrane CD14. Thus, LPS has been reported to stimulate arachidonate metabolism and surface expression of adhesion molecules in endothelial cells, and it can induce aggregation of platelets, stimulate cytokine release from mast cells and fibroblasts, and lead to generation of chemotactic factors in epithelial cells (8). Although CD14 is not present on the plasma membrane of these cells, soluble (s)CD14 present in serum is essential for their stimulation by LPS (9, 10).

Transcription factors activated by LPS include NF-xB (10, 11) and NF-IL6 (12, 13). TNF is also known as a potent inducer of NF-xB as well as of other transcription factors including AP-1, NF-IL6, IRF-1, and NF-GMa (14). NF-xB belongs to the rel family of transcription factors which form a number of different hetero- and homodimers participating in the regulation of a large number of genes involved in the immune response (15). NF-xB proteins are constitutively expressed in the cytoplasm, bound to inhibitor I-xB, and are released and translocated to the nucleus upon phosphorylation and degradation of I-xB (16-18). Most likely, more than one I-xB kinase is involved, and different stimuli lead to phosphorylation and degradation of different I-xB species. Thus, while I-xB-alpha is degraded both by TNF and LPS, I-xB-beta is only degraded in response to LPS and IL-1, but not in response to TNF (19).

TNF can induce NF-xB via two different receptors, TNFR p55 and TNFR p75 (20). In most cells, TNF p55 is responsible for activation of NF-xB (21, 22), and recent evidence indicates that TNF p55 signaling involves ceramide (23, 24). In some cell types, NF-xB may also be activated by TNFR p75 (20), but TNF p75 signaling mechanisms are still poorly understood. Association of serine/threonine kinases with cytosolic domains of both TNFR p75 (25) and TNF p55 (26, 27) have been reported, and recently, two cytosolic proteins which specifically associate with TNFR p75 intracellular domains were identified and cloned (28). However, no intracellular pathways activated by any of these proteins have yet been identified, and it is not known whether they are involved in TNF-mediated activation of NF-xB.

LPS-mediated activation of NF-xB in monocytes is dependent on intracellular protein tyrosine kinase activity (29). Similarly, TNF-mediated activation of NF-xB in lysates of the monocyte-derived cell line U937 was inhibited by a tyrosine kinase inhibitor (20). Also, both LPS- and TNF-induced activation of NF-xB is inhibited by the antioxidant pyrrolidine dithiocarbamate (31, 32) indicating that reactive oxygen intermediates play a role in both LPS- and TNF-mediated NF-xB activation.

So far, LPS signal transduction has been most extensively studied in cells expressing a functional membrane CD14. However, LPS effects on CD14 negative cell types may also contribute...
ute to LPS-mediated pathology. In the present study, we show that LPS activates the transcription factor NF-kB and the cytoskeletal protein (CMV) promoter-enhancer in SW480 human adenocarcinoma cells which do not have a functional membrane CD14. Since both TNFR p55 and TNFR p75 independently activate SW480 cells (20), we considered it important to compare the LPS signaling mechanism to the signal transduction pathways activated by the TNF receptors. The results demonstrate that TNF induces LPS tolerance in SW480 cells and that TNFR p55 and LPS may activate overlapping pathways.

MATERIALS AND METHODS

Cell Cultivation and Stimulation—Human colon adenocarcinoma cells, SW480/β-gal (generously provided by Dr. Gerald Ranges, Miles Inc., West Haven, CT), contain a β-galactosidase (β-gal) gene under the control of the CMV immediate early promoter-enhancer (33). SW480/β-gal were grown in RPMI 1640 (Life Technologies, Inc. Laboratories, Paisley, Scotland), supplemented with 2 mM l-glutamine, 10% heat-inactivated fetal calf serum (HyClone, Logan, UT), and 40 μg/ml garamycin (fetal calf serum medium). Stimulation with LPS was routinely carried out in RPMI 1640 medium supplemented with glutamine, 20% human A-β serum (The Blood Bank, University Hospital of Trondheim, Trondheim, Norway), and garamycin (A-β medium). Experiments conducted at serum-free conditions were performed in AIM medium (Life Technologies, Inc.).

Reagents—LPS from smooth Salmonella minnesota 6261 and from rough S. minnesota Re595 (Sigma) as well as S. minnesota lipid A di-and monophosphate (Sigma) were solubilized in 0.9% NaCl at stock solutions of 2 mg/ml (LPS) or 1 mg/ml (lipid A). Recombinant (r)CD14 and (r)LBP were generously provided by Dr. M. Lichenstein and Dr. M. Zulowski, AMGEN (Thousand Oaks, CA) and were prepared as described (34). Human recombinant TNF, with specific activity 7.6 × 10^4 units/mg protein, was generously supplied by Dr. Refaat Shalaby, Genentech Inc. (South San Francisco, CA). Anti-CD14 monoclonal antibody (mAb) 3C10 was obtained from a subclone of the hybridoma ATCC TIB 228 (American Type Culture Collection).

TNFR p75 antisemur (p75 AS) was generated by multiple injections of a rabbit with recombinant soluble TNFR p75 (20). The mAb htr-9 against TNFR p55 (35) was generously provided by Dr. M. Brockhaus, Hoffmann LaRoche Ltd. (Basel, Switzerland). Biotinylation of htr-9 and mAb utr-4 against TNFR p75 (35) was performed as described (36). The mAb 6H8 directed against a widely distributed 180-kDa membrane protein was used as a control antibody. All mAbs were purified on a Sepharose goat anti-mouse IgG column (Zymed Laboratories Inc., South San Francisco, CA). Benzamidine (Sigma) was dissolved in 50% alcohol at 0.1 M.

β-Galactosidase Assay—The β-galactosidase assay was performed essentially as described previously (20). Substrate conversion was measured as optical density (OD) at 570 nm. For pretreatment studies, cells were seeded in A-β medium containing pretreatment reagents. After 72 h, the plates were washed three times in Hanks’ buffered salt solution, and test reagents were added in A-β medium.

Quantitative Band Shift Assays—Preparation of nuclear extracts and band shift analysis was performed essentially as described (20). Briefly, equal amounts of nuclear protein from each sample were incubated with 1 μg of poly(dI-dC) (Pharmacia Fine Chemicals, Upssala, Sweden), in binding buffer (20 mM HEPES, pH 7.9, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, 2% Ficoll) (20 μg final volume) for 10 min at room temperature. Then, 17 fmol (1–5 × 10^6 counts/min) of end-labeled NF-kB-specific double-stranded oligonucleotide probe 5’-AGTTGAGGGGACTTTCCCAGG-3’ (Promega Corp., Madison, WI) was added, and the mixture was incubated for another 15–20 min. The samples were applied on native polyacrylamide gels (7% acrylamide, 0.25 × Tris borate-EDTA, 2.5% glycerol) and run at 100 V for 1 h and then at 130 V for 2–3 h, after which the gels were dried and exposed to x-ray film (X-Omat AR, Kodak, Rochester, NY) for 2–16 h. Specific band shifts were quantitated with a PhosphoImager (Molecular Dynamics, Sunnyvale, CA) by measuring the radioactivity within a rectangle enclosing the band shift in gel scans after exposures of 18–24 h. Radioactivity counts, which are a

2 B. Naume, A. Sundan, and T. Espevik, unpublished results.
Comparison between LPS and TNF Signal Transduction

RESULTS

LPS Activates the CMV Promoter-enhancer in the Colon Carcinoma Cell Line SW480—Transcriptional activation of the CMV promoter-enhancer was measured in a reporter gene assay in SW480 cells stably transfected with a plasmid containing β-gal under control of the CMV promoter-enhancer (33). Treatment of SW480/β-gal cells with LPS resulted in strong induction of β-gal activity (Fig. 1) which shows that LPS can activate intracellular signals leading to activation of the CMV promoter-enhancer in SW480 cells. S. minnesota LPS was found to be several orders of magnitude more potent than LPS from Escherichia coli or from Pseudomonas. S. minnesota mutant Re595 LPS gave rise to a higher maximal response than wild type 6261 LPS, while the lipid A part of S. minnesota LPS showed very little activity on its own (Fig. 1).

The LPS activity was strongly influenced by human serum which caused a dose-dependent enhancement of LPS mediated β-gal activity (Fig. 2A). The presence of neutralizing anti-CD14 mAb 3C10 completely inhibited LPS-mediated induction of β-gal activity (Fig. 2B), and we found that recombinant (r)CD14 could enhance LPS activity in serum-free medium (Fig. 2C). Recombinant LPS-binding protein (LBP) had little or no enhancing effect on the LPS response (Fig. 2C), and LBP did not further potentiate the rCD14-mediated enhancement of the LPS response (Fig. 2C). Taken together, the data indicate that SW480 cells do not express a functional membrane CD14 and that soluble (s)CD14 in human serum is necessary for the LPS response. The LPS effect was not mediated by TNF or LT-α as neutralizing antibodies against TNF or LT-α did not inhibit the LPS response (data not shown).

LPS Induces Activation of Transcription Factor NFκB at a Significantly Slower Rate than TNF—Nuclear extracts from SW480 cells stimulated with LPS or TNF were analyzed for induction of NFκB p50 and p65 and is strongly up-regulated. B, OCT band shift analysis of the same extracts as in A, C, quantitation of relative radioactivity in the slower migrating, strongly up-regulated p50/p65 NFκB complex from the band shift analysis shown in A, by PhosphorImager measurements (mean ± S.D. of triplicate measurements of the same band shifts). Similar results were obtained by analysis of two other series of nuclear extracts from cells stimulated as above.

Fig. 3. Activation of NFκB in SW480/β-gal cells by S. minnesota 6261 LPS, Re595 LPS, and TNF. A, NFκB band shift analysis of nuclear extracts from cells stimulated for 2 h with increasing amounts of TNF (lanes 1–6), Re595 LPS (lanes 7–12), or 6261 LPS (lanes 13–18), in A− medium. The two bands marked with arrowheads represent nuclear proteins binding specifically to the NFκB consensus sequence, as identified previously (20). The faster migrating complex (♦) is only weakly up-regulated and mainly contains NFκB p50 as judged by supershift analysis. The slower migrating complex (▪) contains both NFκB p50 and p65 and is strongly up-regulated.

B, OCT band shift analysis of the same extracts as in A. C, quantitation of relative radioactivity in the slower migrating, strongly up-regulated p50/p65 NFκB complex from the band shift analysis shown in A, by PhosphorImager measurements (mean ± S.D. of triplicate measurements of the same band shifts). Similar results were obtained by analysis of two other series of nuclear extracts from cells stimulated as above.

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significantly increase the amounts of activated NFκB and further increased during incubation of up to 120 min (Fig. 4, A and B). PhosphoImager quantitation of radioactivity in the slower migrating p50/p65 NFκB complex (mean ± S.D. of triplicate measurements of the same band shifts). Similar results were obtained by analysis of two other series of nuclear extracts from cells stimulated as above. C, induction of β-galactosidase activity in cells treated for the indicated time points with either Re595 LPS (0.1 µg/ml), TNF (10 ng/ml), or A+ medium. Results (mean values of duplicates) of a representative experiment are given.

Fig. 4. Kinetics of Re595 LPS- and TNF-mediated activation of NFκB and CMV promoter-enhancer in SW480/β-gal cells. A, band shift analysis of nuclear extracts from cells treated for the indicated time points with either Re595 LPS (1 µg/ml) or with TNF (10 ng/ml) in A+ medium. The two specific NFκB complexes are indicated as in Fig. 3. B, PhosphoImager quantitation of radioactivity in the slower migrating p50/p65 NFκB complex (mean ± S.D. of triplicate measurements of the same band shifts). Similar results were obtained by analysis of two other series of nuclear extracts from cells stimulated as above. C, induction of β-galactosidase activity in cells treated for the indicated time points with either Re595 LPS (0.1 µg/ml), TNF (10 ng/ml), or A+ medium. Results (mean values of duplicates) of a representative experiment are given.

Transcription factor NFκB and activate the CMV promoter-enhancer in SW480 cells, raises the question whether these agents mediate their effects through similar intracellular mechanisms. In order to compare the LPS and TNF responses, we first analyzed the kinetics of NFκB activation for each of the stimuli. It was found that LPS activated NFκB at a significantly slower rate than TNF (Fig. 4, A and B). Activation of NFκB by TNF was clearly detectable at 10 min and reached a plateau level after approximately 45 min. However, LPS-induced NFκB activation was only detected after 60 min and further increased during incubations of up to 120 min (Fig. 4, A and B). Stimulation with LPS for more than 2 h did not significantly increase the amounts of activated NFκB (data not shown). In the reporter gene assay, LPS required at least 6 h to yield maximal response, while the TNF response reached plateau levels after 4 h of incubation (Fig. 4C), indicating that LPS also activates the CMV promoter-enhancer at a slower rate than TNF. These results suggest that LPS activates NFκB and CMV promoter-enhancer via mechanisms that are different from the TNF-activated mechanisms.

Pretreatment with TNF Inhibits LPS-induced Activation of NFκB but LPS Pretreatment Does Not Inhibit TNFR p55 or TNFR p75 Responses—LPS- and TNF-induced NFκB activation was further compared in a series of experiments where SW480 cells were pretreated for 72 h with either LPS, TNF, or agonistic antibodies against TNFR p55 or TNFR p75, followed by stimulation with LPS, TNF, or agonistic TNFR antibodies. Pretreatment of the cells for 72 h with TNF only marginally influenced the expression of TNFR p55 and TNFR p75 receptors (Fig. 5A). Furthermore, pretreatment of the cells with TNF or LPS did not affect the binding of FITC-LPS from S. minnesota (Fig. 5B).

Band shift analysis of nuclear extracts from pretreated cells stimulated with LPS, TNF, or with agonistic anti-TNFR antibodies are shown in Fig. 6. The data demonstrate that the LPS response is inhibited by pretreatment with either LPS or with TNF or agonistic TNFR antibodies (Fig. 6, A and B), while the TNF response is only inhibited by pretreatment with TNF or TNFR antibodies and not by LPS pretreatment (Fig. 6, C and D). Activation of NFκB by TNF p55 mAb htr-9 was inhibited by pretreatment with htr-9 or TNF (Fig. 6, E and F), while the TNF p75-mediated response was mainly inhibited by p75 AS and TNF pretreatment (Fig. 6, G and H). Taken together, the results suggest that pretreatment with a given stimulus leads to depletion or reduction of active intracellular components involved in the signal transduction pathway induced by that stimulant. In addition, pretreatment with agents like htr-9 or TNF may also reduce the level of active components involved in signal transduction by other agents like LPS.

In all experiments performed, inhibition of the LPS response by pretreatment with TNFR p55 mAb htr-9 was of a similar magnitude as inhibition by LPS pretreatment (Fig. 6, A and B), indicating that activation of NFκB by LPS is dependent on cellular components which are reduced or inactivated by long term stimulation of TNFR p55. Pretreatment with TNFR p75 antiserum led to a weak but consistent inhibition of the LPS response (Fig. 6, A and B) and thus, components activated via TNFR p75 may also be involved in LPS signal transduction. However, neither TNFR p55- nor TNFR p75-mediated activation of NFκB was inhibited by pretreatment with LPS (Fig. 6, E-H), suggesting that long term stimulation with LPS does not lead to a reduction or inactivation of components involved in either of these pathways. In contrast to the lack of inhibition of the TNFR p75 response by LPS, pretreatment with TNFR p55 mAb htr-9 caused a low but reproducible inhibition of the TNFR p75 response. This inhibition, however, was markedly

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Fig. 5. Flowcytometric analysis of TNFR mAb and FITC-LPS binding to pretreated SW480/β-gal cells. Cells were pretreated for 72 h with TNF (1 ng/ml) or LPS (0.1 µg/ml) followed by labeling of the cells with p55/p75 mAb (A) or FITC-LPS (B) as described under “Materials and Methods.”
lower than the inhibition of the TNFR p75 response caused by p75 AS pretreatment (Fig. 6, G and H). The TNFR p55-mediated response was not inhibited by pretreatment with p75 AS in any of the experiments. Thus, it seems that TNFR p55-induced activation of NFκB does not depend on components activated via TNFR p75.

Pretreatment with TNFR p55 Agonistic Antibodies Can Also Suppress LPS-induced Transcriptional Activation of the CMV Promoter-enhancer—LPS- and TNFR-mediated activation of the CMV promoter-enhancer was compared by subjecting the cells to similar protocols of pretreatment and stimulation as described above. It was found that LPS-induced β-gal activity was markedly reduced after pretreatment with LPS or with the TNFR p55 mAb htr-9, while pretreatment with p75 AS had less inhibitory effect (Fig. 7A). The TNFR p55-mediated activity was almost completely abolished after pretreatment with TNFR p55 mAb htr-9 and very weakly inhibited by pretreatment with p75 AS, while pretreatment with LPS showed no inhibitory effect (Fig. 7B). Thus, pretreatment of the cells affected the subsequent activation of both the CMV promoter-enhancer and NFκB in a similar manner.

DISCUSSION

The present paper demonstrates that LPS can induce activation of transcription factor NFκB, as well as activation of the CMV promoter-enhancer in the human adenocarcinoma cells SW480. These cells do not express a functional membrane CD14 because addition of LBP did not enhance the LPS effect under serum free conditions. Such lack of enhancement is a typical phenomenon in cells which do not express a functional membrane CD14 (39, 40). Other CD14 negative cells where LPS has been found to mediate activation of NFκB include the murine pre-B-cell line 70Z/3 (10) and endothelial cells (11). The LPS response in SW480 cells was strongly dependent on hu-
man serum and could be completely inhibited by neutralizing antibodies against CD14. Recombinant soluble (s)CD14 could only partly compensate for the human serum enhancing effect since the activity of LPS in the presence of rCD14 was markedly lower than LPS activity in the presence of 20% human serum. Thus, sCD14 is necessary but not sufficient for LPS activity, and other serum factors in addition to sCD14 are necessary for maximal LPS response in SW480 cells.

LPS stimulates activation of NFκB at a markedly slower rate than TNF, indicating that the LPS and TNF signal transduction pathways are not identical. As shown earlier, the kinetics of NFκB activation by agonistic antibodies against the TNFR p55 is identical to the TNF kinetics with maximal levels reached after ~45 min, while stimulation of TNFR p75 results in maximum activation after ~60 min (20). Thus, in order to reach maximum NFκB activation, stimulation with LPS has to be continued for a significantly longer period of time than stimulation of any of the TNF receptors, indicating that the LPS signaling mechanism differs from the mechanisms employed by the two TNF receptors. Supershift analysis showed that stimulation of SW480 cells with LPS or with agonistic TNFR p55 or p75 antibodies resulted in activation of an identical pattern of NFκB hetero- and homodimers.3 Thus, SW480 is a cell system where the different pathways employed by LPS and the two TNF receptors can be compared, and where the question can be asked as to whether these pathways are independent or overlapping.

Comparison of LPS and TNF signal transduction pathways was performed by analyzing activation of NFκB and the CMV promoter-enhancer in cells pretreated with LPS, TNF, or with agonistic antibodies against TNFRs. We found that pretreatment of the cells did not result in down-regulation of TNF receptors or reduction in the binding of LPS. Thus, it is likely that the observed inhibition of activation of NFκB and CMV promoter-enhancer is due to intracellular effects of the pretreatment analogous to depletion of protein kinase C by long term treatment with the phorbol ester PMA. Such treatment renders cells unresponsive to subsequent activation of NFκB by PMA, while the TNF response remains unaffected, indicating

3 A. Lægreid and L. Thommesen, unpublished observations.
that TNF does not depend on PMA-responsive protein kinase C for activation of NFκB (41, 42).

The results from the pretreatment experiments are in agreement with our previous results which indicated that TNFR p75 mediates NFκB activation through a different signal transduction mechanism than TNFR p55 (20). Thus, the TNFR p75-mediated response is maximally inhibited by pretreatment with p75 AS while pretreatment with TNFR p55 mAb htr-9 led to a markedly lower reduction of the TNFR p75 response. Furthermore, lack of inhibition of the TNFR p75 response by LPS pretreatment suggests that the TNFR p75-activated signaling mechanism is independent of components activated by LPS. Thus, the TNFR p75 pathway leading to NFκB activation is different from the LPS, as well as from the TNFR p55 pathway, although it appears to include intracellular components which are depleted or inactivated by long term stimulation of TNFR p55.

TNFR p55, which mediates rapid NFκB activation, apparently employs a signaling mechanism which is independent of intracellular components activated by LPS or TNFR p75, since pretreatment with LPS or TNFR p75 AS did not inhibit the TNFR p55 response. On the other hand, the LPS response seems to be mediated by a pathway which is partly overlapping with the TNFR p55 pathway, since pretreatment with TNFR p55 mAb htr-9 inhibited the LPS response to a similar extent as the LPS pretreatment. The observation that TNFR p55 and LPS signaling pathways may be partly overlapping suggests that TNFR p55 employs more than one pathway which mediates rapid activation of NFκB and is independent of intracellular components activated by LPS or p75 AS, and another pathway which overlaps with the LPS signal transduction pathway.

The stage at which the LPS and TNFR p55 signaling pathways overlap may involve ceramide, a lipid messenger which participates in the activation of NFκB in several cell lines including Jurkat (23), HL-60 (43), and SW480 (42). TNFR p55-mediated activation of NFκB in Jurkat cells as well as 702/3 cells has been found to proceed by ceramide generated by an acidic sphingomyelinase (23, 44), while TNFR p55-mediated activation of NFκB in HL-60 cells is reported to involve a 97-kDa ceramide-activated protein kinase which is activated via ceramide generated by a neutral sphingomyelinase (43, 45, 46). Recently, LPS was found to stimulate ceramide-activated protein kinase in HL-60 cells directly, in the absence of detectable sphingomyelinase activity (47). A possible reason for this LPS activity may be structural similarities between LPS and ceramide (47). Thus, ceramide-activated protein kinase may be an intracellular component putatively involved in both LPS- and TNFR p55-mediated NFκB activation in SW480 cells.

Release of LPS during Gram-negative infections may induce high levels of circulating TNF which can lead to shock and death (48). Our finding that TNF pretreatment inhibits LPS-induced NFκB activation may have important clinical implications since release of low TNF levels during Gram-negative infections could render cells resistant to subsequent LPS stimulation. This is supported by in vivo data showing that pretreatment of mice with TNF or IL-1 induces partial tolerance to LPS (49). Thus, release of low TNF levels during Gram-negative infections may have an important function in limiting harmful effects of LPS in vivo.

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