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Optimal Induction of Tumor Necrosis Factor Production in Human Monocytes Requires Complete S-Form Lipopolysaccharide

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Optimal activation of human monocytes in vitro for the biosynthesis of tumor necrosis factor was achieved only with complete S-form lipopolysaccharide. Endotoxin preparations with shorter carbohydrate chains or the lipid A component of lipopolysaccharide were not able to induce release of comparable amounts of tumor necrosis factor by monocytes under the conditions described. The same differences in the level of tumor necrosis factor mRNA were observed. Moreover, addition of these agents to appropriate monocyte-activating substances inhibited the production of tumor necrosis factor. The regulatory implications of this phenomenon are discussed.

From numerous investigations, it seemed clear that the majority of the biological activities exhibited by lipopolysaccharide (LPS) reside in the lipid A portion of the molecule. Many of the endotoxic effects of LPS, however, are not direct effects of this lipid A moiety on the cellular system but are mediated via induction of biologically active endogenous factors such as tumor necrosis factor (TNF) or interleukin-1 (IL-1). Among other cells, macrophages and monocytes produce these factors after activation. TNF is able to induce all symptoms of endotoxic shock when administered in recombinant purified form (4, 20). For the production and secretion of TNF, a two-signal activation process has been elucidated in the murine macrophage model (18). In this murine system, it became obvious that LPS alone could provide all signals required for macrophage activation when given to cells from LPS-sensitive animals (25). Macrophage cytotoxicity or TNF release from cells obtained from LPS-low-responder mice or from high-responder mice, but using suboptimal amounts of LPS, was induced only after appropriate preactivation (first signal). Such preactivation was conferred in vivo by various bacterial or viral infections or by a tumor and in vitro by macrophage activation factors such as gamma interferon (IFN-γ). For such prepared macrophages, the lipid A 3-deoxy-D-mannooctulosonic acid (KDO) molecule of a Re mutant of *Salmonella minnesota* was able to function as a second or triggering signal for the induction of tumor cytotoxicity or TNF release (19). Free lipid A was also found to induce TNF release into the sera of presensitized mice in vivo (11).

Human peripheral blood mononuclear leukocytes (PBMNL) produced little TNF upon stimulation with LPS alone. But again, priming with IFN-γ before LPS stimulation greatly augmented TNF release (23). IFN-γ activated the monocytes for tumor cell cytotoxicity, but LPS was required for the release of TNF from those cells (14). Also, alveolar macrophages of sarcoidosis patients released TNF upon stimulation with LPS (1). Thus, LPS seems to act as a second signal for the induction of TNF release. In the murine system, the lipid A part of the LPS molecule was shown to be the structure required for this TNF release.

LPS is also a very good inducer of IL-1 activity from human peripheral mononuclear cells. Loppnow and coworkers demonstrated that for IL-1 release, the presence of 3-acyloxyacyl residues on the lipid A moiety of LPS were essential under the conditions used (17). Another group determined the heptosyl-(1, 5)-KDO disaccharide of the LPS molecule as the minimal structure required for IL-1 release from human monocytes (16).

In this study, we tested several LPS preparations of S- and R-form mutants and lipid A for the ability to induce TNF and IL-1 in human PBMNL, in the adherent fraction of human PBMNL, and in THP-1 cells. We found that optimal release of TNF and IL-1 from PBMNL from monocytes obtained from healthy blood donors and from THP-1 cells was induced only with the complete S-form LPS molecules. Shortening of the polysaccharide chain of LPS led to reduced IL-1 release. LPS R forms and lipid A induced only small amounts of IL-1α and TNF mRNAs compared with the amounts produced by optimally stimulated cells. When the same agents were added to cultures of monocytes stimulated with other activating agents, they were inhibitory.

MATERIALS AND METHODS

Reagents. LPS preparations consisted of LPS from *Salmonella montevideo* SH94 prepared by the phenol-water extraction method, followed by the phenol-chloroform petrol ether procedure (9). LPS of *Salmonella abortus-equi* H1178, LPS of *S. minnesota* H907 (R7 preparation), LPS of *S. minnesota* H306 (R595 preparation), and lipid A from *Escherichia coli* (AcP [acetate buffer] preparation) were all generously supplied by H. Brade in solutions of 1 mg/ml in phosphate-buffered saline (PBS). All preparations had been converted to the uniform triethylamine and sodium salt forms, respectively, by electrodialysis and neutralized by the respective bases (8). Clear nonopalescent solutions were obtained. The protein content of the *S. abortus-equi* LPS was <0.2%, and that of R595 and lipid A was <0.5%, as determined by amino acid analysis. The DNA content of all preparations was <0.2%. Corresponding portions of the same batches as the preparations used for this study had biological activities (e.g., direct toxicity, galactosamine toxicity, Schwartzman and pyrogen activity, and B-cell mitogenicity) in other test systems. Glutaraldehyde-fixed *Staphylococcus aureus* cells (Pansorbin; Calbiochem-Behring, La

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Jolla, Calif.) were used as a 0.1% (wt/vol) suspension. Recombinant human IFN-γ (rhIFN-γ) (specific activity, 2×10^7 U/mg) was provided by G. R. Adolf, Ernst Boehringer Institut für Arzneimittelforschung. Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma Chemical Co., Deisenhofen, Federal Republic of Germany.

Culture medium. The culture medium used was RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) with 10% heat-inactivated fetal calf serum (GIBCO) and gentamicin (50 μg/ml; Sigma) unless stated otherwise.

Preparation of cells. Human PBMNL were prepared from buffy coats of healthy blood donors by Ficoll-Hypaque density gradient centrifugation (3). For preparation of the adherent cell fractions, the cells (3×10^6/ml) were seeded in 24-well plastic Falcon tissue culture plates (Becton Dickinson Labware, Oxnard, Calif.) and incubated for 2 h at 37°C in a humid atmosphere containing 5% CO_2. Nonadherent cells were removed by washing the cultures three times with culture medium. The remaining cell fraction consisted of >90% monocytes, as determined by morphology and phagocytosis. Cells of the human leukemic cell line THP-1 (26) were cultured in the manner described for human PBMNL.

TNF assay systems. The cytotoxicity assay for TNF was carried out on sensitive L929 mouse fibrosarcoma cells (2×10^4 cells per 0.2 ml of culture) in the presence of actinomycin D (2 μg/ml; Sigma). The cultures contained serial dilutions of TNF samples. After 20 h of culture, the surviving cells were fixed and stained with crystal violet (0.5% crystal violet, 3% [vol/vol] formaldehyde, 0.17% NaCl, 22.3% ethanol) for 15 min. Excess dye was washed off with water, and the remaining dye was solubilized in 33% acetic acid. A_{540} was measured. Concentrations of TNF are expressed as titers indicating the reciprocal dilution of the samples in triplicate at which 50% survival was measured. Sensitivity of this assay varied between 1 and 0.5 ng of rhTNF per ml. Titers below 8 were considered background.

TNF activity was also measured by an enzyme-linked immunosorbent assay (ELISA). For ELISA, 96-well flat-bottom plates (Titertek; Flow Laboratories, Meckenheim, Federal Republic of Germany) were coated with affinity-purified (protein A-Diasorb; Diagen, Düsseldorf, Federal Republic of Germany) polyclonal rabbit anti-recombinant human TNF antibodies (5 μg/ml in 0.05 M $NaHCO_3$ buffer [pH 9]) for 16 h at 4°C. Serial dilutions of the test samples in 0.1 M phosphate buffer (2% EDTA–1% bovine serum albumin [pH 7.5]) were applied to the plates for 2 h at room temperature after blocking with 1% bovine serum albumin in PBS for 2 h at room temperature. Plates were washed with PBS containing 0.05% Tween, and biotin (Sigma)-conjugated affinity-purified (protein A-Diasorb) polyspecific rabbit anti-rhTNF antibodies (2.7 μg/ml) were added to the wells for 1.5 h at room temperature. After extensive washing with PBS containing 0.05% Tween, a 1:2,000 dilution of streptavidin-peroxidase complex (Bethesda Research Laboratories, Inc., Karlsruhe, Federal Republic of Germany) was applied for 30 min at room temperature. The plates were washed again with PBS containing 0.05% Tween, and the substrate solution (3,3′,5,5′-tetramethylbenzidine [Miles Scientific, Munich, Federal Republic of Germany], 10 mg in 100 ml of 0.1 M sodium acetate-citric acid buffer [pH 4.9], and 14.7 μl of 30% H_2O_2) was added to the complex. The reaction was stopped with 2 M H_2SO_4, and A_{450} was measured. The absorption curves obtained with the test samples were compared against a standard curve obtained with rhTNF, and TNF content was expressed as nanograms per milliliter. The sensitivity of this TNF assay was 0.5 ng of rhTNF per ml.

IL-1 assay system. IL-1 activity was determined by the conventional costimulator assay. In brief, single-cell suspensions of C3H/HeJ thymocytes (5×10^5 per culture) were cultured in the presence of phytohemagglutinin (PHA-M; 50 μg/ml; Sigma) and serial dilutions of the test samples for 3 days. The cultures were pulsed with 1 μCi of tritiated thymidine ([6-^{3}H]thymidine; specific activity, 50 Ci/mmol [185 GBq/mmol]; Amersham Buchler, Braunschweig, Federal Republic of Germany) for 6 h, and incorporated counts were determined. IL-1 titers are given as the final dilutions of the supernatants in triplicate that gave counts per minute 2.5-fold higher than background.

RNA extraction and dot blot analyses. The procedure used has been described in detail recently (5). Cells (10^5 to 10^6 per culture) were solubilized with 1 ml of 7.6 M guanidine hydrochloride in 0.1 M potassium acetate buffer (pH 5), and DNA was sheared by aspiration five times through a 21-gauge needle. A 0.6-ml amount of 95% ethanol was admixed, and RNA was precipitated at −20°C for 12 h. RNA was pelleted by 20 min of centrifugation at 15,000 × *g*, the pellet was dissolved in 150 μl of 15% formaldehyde in water, and 150 μl of 20× SSC (SSC is 0.15 M sodium chloride plus 0.015 M sodium citrate) was added. The solution was heated for 15 min at 50°C and chilled on ice. Equal portions or serial dilutions were applied to nylon filters (Compas; Genofit, Heidelberg, Federal Republic of Germany) prewetted with water and 10× SSC. The RNA was fixed on the nylon filters by exposure to UV light for 2 min, and hybridization was performed as described in detail by Khandjian (13) at 42°C in the presence of dextran sulfate. The filters were washed twice under high-stringency conditions (65°C, 30 min, 2× SSC containing 1% sodium dodecyl sulfate). Probes were labeled with [^{32}P]GTP and [^{32}P]CTP (specific activity, 3,000 Ci/mmol; Amersham, Frankfurt, Federal Republic of Germany) by the random-primer method, using a hexamer (Pharmacia, Freiburg, Federal Republic of Germany).

The TNF cDNA probe, a 425-base-pair *Pst*I fragment of the nontranslated 3′ region of human TNF, was obtained from BASF (Ludwigshafen, Federal Republic of Germany). The IL-1α and IL-1β probes, gifts of U. Gubler (Hoffmann-La Roche Inc., Nutley, N.J.), consisted of a 460-base-pair human IL-1 *Eco*RI-*Bam*HI cDNA fragment [p3(IL1α)] and a 530-base-pair human IL-1β *Bam*HI-*Nde*I cDNA fragment [p11(IL1β)], respectively, of the coding region. The human β-actin cDNA probe, described by Moos and Gallwitz (21), consists of a 560-base-pair *Sal*I-*Eco*RI cDNA fragment.

RESULTS

PBMNL from normal blood donors were stimulated with complete S-form LPS preparations from *S. montevideo* and *S. abortus-equi*, with LPS from a rough mutant of *S. minnesota* (R7), with LPS from a Re mutant of *S. minnesota* (R595), consisting of the lipid A part with one KDO residue attached, and with purified lipid A from *E. coli*. After 16 h, cell-free supernatants from these cultures were tested in the biological assay for TNF activity (Table 1). Cultures were stimulated with *Staphylococcus aureus* as a positive control, which led to strong cytotoxic TNF activity in the supernatant. Only complete S-form LPS induced release of high levels of TNF activity. LPS from *S. montevideo* was a better inducer than *S. abortus-equi* LPS. The R7, R595, and lipid A preparations did not induce measurable TNF release even at the highest concentrations tested. Addition of IFN-γ to the culture in combination with *S. abortus-equi* LPS induced higher TNF titers than did LPS stimulation alone but again

TABLE 1. Release of cytotoxic activity by PBMNL after stimulation in vitro[a]

Stimulating agent	Concn (μg/ml)	TNF titer	
		− IFN-γ	+ IFN-γ
None		4	
Salmonella montevideo LPS	10	25	
	1	8	
	0.1	6	
	0.01	6	
	0.001	4	
Staphylococcus aureus	10	64	
	1	12	
Salmonella abortus-equi LPS	10	8	25
	1	6	12
	0.1	6	6
R7	10	2	2
	1	2	3
	0.1	2	4
R595	10	4	3
	1	2	4
	0.1	4	2
Lipid A	10	3	2
	1	3	3
	0.1	3	4

[a] PBMNL (3×10^6/ml) were stimulated for 16 h with the indicated preparations in the presence or absence of IFN-γ (100 U/ml). The cytotoxic activity in the supernatants was determined in the biological TNF assay.

did not lead to release of measurable TNF activity when given together with R7, R595, or lipid A. No inhibitory substance was released after stimulation with R7, R595, or lipid A, as determined in the biological test system in which a defined amount of TNF was mixed with these supernatants and tested for activity (data not shown).

The same pattern of TNF activity was obtained when the adherent cell fraction (>90% monocytes) was stimulated with different substances (Table 2). Again, only LPS from *S. montevideo* and *S. abortus-equi* released significant cytotoxic activity into the supernatant. Taken together, these data indicate that only S-form LPS was able to induce significant TNF production in monocytes.

Cells used for the experiment shown in Table 2 were probed for the presence of TNF mRNA to test whether only the release of TNF protein or also the expression of TNF mRNA was dependent on the complete S-form LPS as inducer. RNA from the cells was extracted and blotted on

TABLE 2. Release of cytotoxic activity by the adherent cell fraction of PBMNL after stimulation in vitro[a]

Stimulating agent	Concn (μg/ml)	TNF titer
Salmonella montevideo LPS	1	32
	0.001	32
Salmonella abortus-equi LPS	1	16
	0.001	4
R7	1	4
	0.001	8
R595	1	4
	0.001	4
Lipid A	1	4
	0.001	4
None		4
Staphylococcus aureus	1	32

[a] The adherent cell fraction of 3×10^6 PBMNL per ml was stimulated for 16 h with the indicated preparations. The cytotoxic activity in the supernatants was determined in the biological TNF assay.

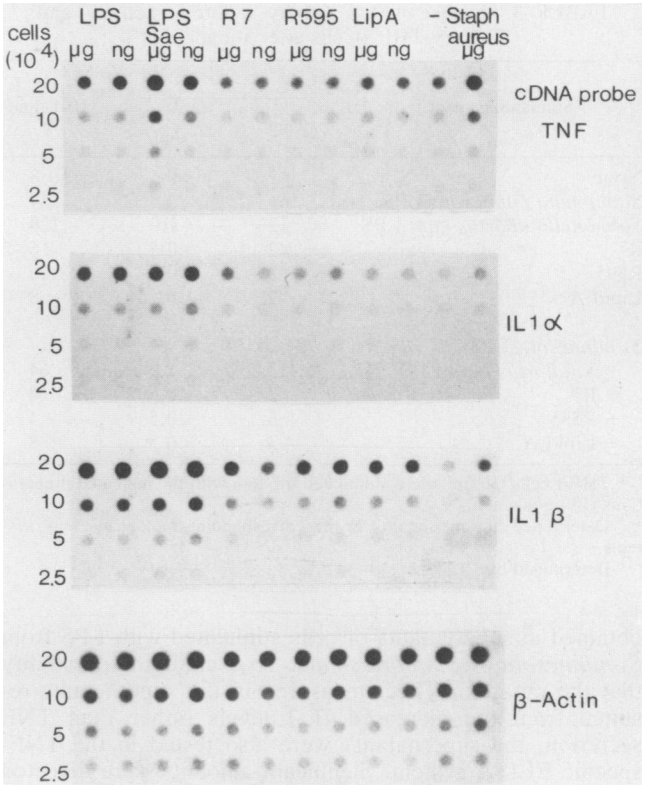

FIG. 1. TNF, IL-1α, IL-1β, and β-actin mRNA expression in adherent PBMNL after stimulation with different agents. Dilutions of mRNA from 2×10^5 adherent PBMNL stimulated with LPS from *S. montevideo*, *S. abortus-equi* (Sae), R7, R595, or *Staphylococcus aureus* (1 μg/ml or 1 ng/ml, as indicated) or unstimulated were extracted after 2 h and blotted onto a nylon filter. The filter was hybridized consecutively with cDNA probes for TNF, IL-1α, IL-1β, and β-actin and autoradiographed.

nylon filters in different concentrations. After hybridization with a TNF cDNA probe, it was obvious that LPS from *S. abortus-equi* induced a much higher signal than did the R7, R595, or lipid A preparation (Fig. 1). Thus, also at the level of mRNA expression, complete S-form LPS was required for optimal induction.

After hybridization of the same filter with an IL-1α cDNA probe, optimal IL-1α mRNA signals were again induced by S-form LPS (Fig. 1). On the other hand, when the same filter was probed with an IL-1β cDNA probe, the strongest signal was again obtained after stimulation with complete S-form LPS, but RNA of cells stimulated with R7, R595, and lipid A also showed positive signals. The amounts of RNA of all samples were comparable, as can be seen by the intensities of the dots after hybridization with β-actin cDNA. TNF mRNA expression was maximal 2 h after stimulation with *Staphylococcus aureus* or S-form LPS. To investigate whether the kinetics of TNF mRNA expression were shifted by using R7, R595, or lipid A, TNF mRNA was monitored up to 20 h after stimulation. No significant induction of TNF mRNA was detected (data not shown).

When the monocytelike tumor cell line THP-1 was stimulated, a similar pattern of results was obtained. Cytotoxic activity was detected only in supernatants of cells stimulated with complete S-form LPS (Table 3). Also, IL-1 activity was found in supernatants of THP-1 cells (Table 3). Unstimulated cells produced some IL-1 activity, but highest values were

TABLE 3. Release of IL-1 activity, cytotoxic activity, and TNF by THP-1 cells after stimulation[a]

Stimulating agent	TNF		IL-1 titer[b]
	Titer[b]	Concn (ng/ml)[c]	
None	<2	<0.5	8
Salmonella minnesota LPS	64	2.5	64
Salmonella abortus-equi LPS	12	1.0	128
R7	<2	<0.5	16
R595	<2	<0.5	16
Lipid A	<2	<0.5	8
S. minnesota LPS			
+ *S. abortus-equi* LPS	12	1.0	64
+ R7	3	<0.5	16
+ R595	3	<0.5	4
+ Lipid A	3	<0.5	8

[a] THP-1 cells (10^6/ml) were stimulated for 16 h with the indicated preparations (10 μg/ml each).
[b] Determined in supernatants in the corresponding biological assay systems.
[c] Determined by ELISA.

TABLE 4. Release of cytotoxic activity by THP-1 cells after stimulation[a]

Stimulating agent	TNF titer
Salmonella minnesota LPS	48
+ *Salmonella abortus-equi* LPS	16
+ R7	<2
+ R595	<2
+ Lipid A	<2
PMA	48
+ *S. abortus-equi* LPS	48
+ R7	12
+ R595	<2
+ Lipid A	<2

[a] THP-1 cells (10^6/ml) were stimulated for 16 h with the indicated agents. The endotoxin preparations were used in concentrations of 10 μg/ml; PMA was used at 10 ng/ml. Cytotoxic activity in the supernatants was determined in the biological TNF assay.

obtained in supernatants of cells stimulated with LPS from *S. minnesota* or *S. abortus-equi*. To exclude the possibility that the cytotoxic effect measured in the supernatants resulted from the increased IL-1 levels rather than TNF secretion, the supernatants were also tested in the TNF-specific ELISA system. Significant amounts were detected only in supernatants of THP-1 cells stimulated with *S. minnesota* or *S. abortus-equi* LPS and thus correlated with the cytotoxic activity.

Furthermore, stimulation of monocytes with R7, R595, and lipid A together with LPS from *S. minnesota* abrogated the appearance of measurable TNF in the supernatants of those cells and reduced the levels of secreted IL-1 activity (Table 3). Thus, R7, R595, and lipid A were not inactive preparations but were capable of inhibiting the activation of LPS-induced TNF release.

The same inhibition of release of TNF activity by R7, R595, and lipid A into the supernatant of THP-1 cells was observed when the cells were stimulated with PMA instead of LPS (Table 4). Other functions of these cells were not inhibited. Trypan blue exclusion, conversion of the tetrazolium salt MTT (22), and uptake of *Staphylococcus aureus* were the same as in untreated cells or cells treated with complete S-form LPS (data not shown).

The same results were obtained at the TNF mRNA level in THP-1 cells. Only LPS from *S. minnesota*, LPS from *S. abortus-equi*, PMA, and *Staphylococcus aureus* induced significant signals (Fig. 2). R7-, R595-, and lipid A-stimulated THP-1 cells showed only background signals, but these agents reduced the intensity of the TNF mRNA specific signal when added to either *S. minnesota* LPS, *Staphylococcus aureus*, or PMA during stimulation. The same amounts of RNA had been blotted onto the filters, as can be seen from the results of hybridization with a β-actin cDNA probe.

DISCUSSION

This study shows that as in the murine system, in human PBMNL cultures complete S-form LPS is the favored agent to induce both the production and release of TNF. Although the sensitivities of cells from different donors varied considerably, in all experiments performed only the complete S-form LPS molecule induced high levels of TNF release and TNF mRNA expression over the background level of unstimulated cells when used in microgram amounts per milliliter. Incomplete R-form LPS and the lipid A part of the molecule were not effective inducers of TNF production in this system. As shown in Table 1 and in accordance with

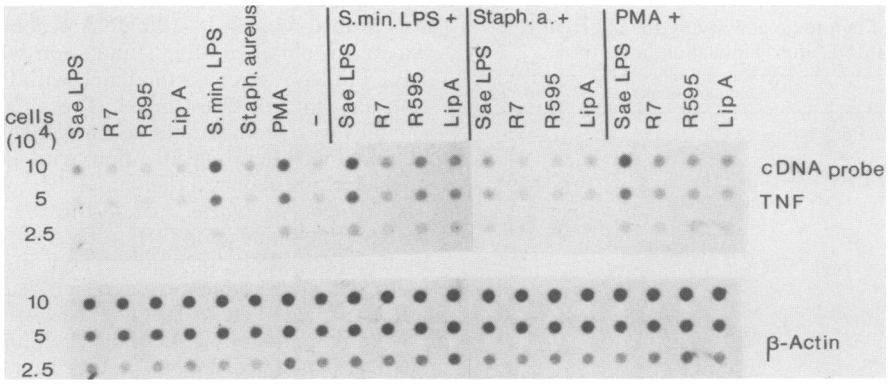

FIG. 2. TNF and β-actin mRNA expression in THP-1 cells after stimulation with different agents and combinations thereof. Dilutions of mRNA from 10^5 THP-1 cells stimulated with *S. abortus-equi* (Sae) LPS, R7, R595, lipid A, LPS from *S. minnesota*, or *Staphylococcus aureus* (10 μg/ml each), PMA (10 ng/ml), or combinations of these agents as indicated were extracted after 2 h and blotted onto a nylon filter. The filter was hybridized consecutively with a TNF cDNA probe and a β-actin cDNA probe and autoradiographed.

previously published findings (23), the simultaneous activation of monocytes by LPS and IFN-γ led to higher TNF titers but was not able to restore the activity of the incomplete LPS preparations. A similar difference in effectiveness of complete LPS versus lipid A has recently been reported for studies in which TNF production induced in vitro was compared in a murine system (15). One could argue that the polysaccharide part of endotoxin carries distinct immunomodulatory activities (7) or that physicochemical properties of the endotoxin preparations influence the biological activity. Therefore, it is possible that the incomplete forms of the endotoxins did not convey all of the necessary signals. Addition of IFN-γ did not provide the missing signal.

It is conceivable that culture conditions or cell preparation procedures used for the experiments described here contributed to the differences in activities of the endotoxin preparations. Most important, the presence of 10% fetal calf serum in all media could interfere with the potency of the activating agents. Therefore, all experiments were repeated in serum-free culture medium. The results obtained in these experiments were qualitatively identical to those described above. The general difference was a higher amount of TNF protein as well as stronger TNF mRNA signals.

In several cases, high levels of IL-1 and TNF production without prior in vitro stimulation were observed. It is possible that this was due to preactivation of the blood cells of the individual donors. These cells were not used for further experiments. Except for the experiments shown in Fig. 1 and Table 2, which were performed with the same cells, all experiments were carried out with cells from different donors. All experiments were repeated at least three times with cells from different blood donors. The monocytic leukemia cell line THP-1 showed the same reactivities toward the different endotoxin preparations. This finding is in full accordance with the recent observation by Russmann et al., who found that complete S-form LPS molecules were necessary for TNF release from THP-1 cells (E. Russmann, personal communication).

On the molecular level, Beutler and colleagues found that in cells of LPS-low-responder animals or dexamethasone-treated cells, a posttranscriptional blockade inhibited the release of mature TNF molecules; however, a partial transcriptional blockade was also found (2). In the murine system, it was found that LPS was responsible for the release of TNF from activated macrophages (19). The data presented here show, in addition, that mRNA levels of TNF are also regulated by LPS.

With respect to IL-1 production, the results for IL-1α and IL-1β and for TNF appear to be very similar. The requirements seem not to be as stringent for the production of IL-1β. Since soluble IL-1 measured in the supernatant consists mainly of IL-1β (12), reports determining parts of the lipid A structure essential for IL-1 release (15, 16) are not contradictory to our results.

It was surprising to find that substances such as the R7, R595, and lipid A preparations and, in some cases, the relatively less potent *S. abortus-equi* LPS (Tables 3 and 4) actively inhibited TNF production and TNF mRNA expression of monocytes that were stimulated with potent TNF inducers. Similar observations were reported by others. Ding and Nathan reported that after exposure to trace levels of LPS, mouse peritoneal macrophages were refractory for a respiratory burst response (6). Possible mechanisms for such antagonizing effects, e.g., receptor blockage, receptor down-regulation, or cyclooxygenase product formation, have been considered. Furthermore, Proctor et al. reported that mice were protected against lethal endotoxemia by a monosaccharide precursor of lipid A, lipid X (24). Several lines of evidence point to inhibition by lipid X of toxic mediator release from effector cells (10). It remains open to speculation whether such a phenomenon could be part of a regulatory mechanism.

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