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Inhibition of Endotoxin-Induced Activation of Human Monocytes by Human Lipoproteins

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Toxicity of lipopolysaccharide (LPS) (endotoxin) is, to a large extent, mediated by the activation of monocytes/macrophages and subsequent release of monokines, such as interleukin-1 (IL-1) and tumor necrosis factor alpha (TNF-α). It is known that LPS binds readily to serum lipoproteins and that LPS-lipoprotein complexes are less toxic than unbound LPS. Here we present data analyzing the impact of the LPS-serum interaction at the cellular level. By measuring IL-1 TNF-α, and IL-6, the interaction of different LPSs or lipid A with human serum could be shown to prevent the activation of human monocytes. The amounts of LPS inactivated by normal human serum did not exceed 10 ng/ml. The LPS-inactivating capacity of serum was shown to be a function of the lipoproteins. Other serum components, such as naturally occurring anti-LPS immunoglobulin G, complement, or nutritive lipids, had no significant influence in our system. Our experiments suggest that serum lipoproteins control endotoxin-induced monocyte activation and monokine release.

Endotoxin (lipopolysaccharide [LPS]) is the main pathogenic factor of gram-negative bacteria. The most prominent toxic effects of LPS in vivo are fever, diarrhea, and hypotension, which may lead to shock and death. Minimal toxic concentrations of LPS vary widely in mammalian species, with humans being one of the most sensitive. Serum is known to interact with LPS and decrease its toxicity, a fact which has been under investigation since LPS was first isolated more than 30 years ago. In early experiments (31), inhibition of pyrogenicity was seen after incubation of bacterial polysaccharide with native serum as well as with serum treated at 58°C. One hypothesis was that detoxification of LPS by proteins was due to enzymatic degradation of LPS (34, 45). This was not, however, supported by other experiments indicating protein-endotoxin complexes, possibly involving immunoglobulins (32). Later (41), it was found that LPS detoxification occurred in many different mammalian species, that it was independent of the hemolytic complement system, and that it was optimal at 37°C. Although partial deacylation of LPS may occur inside neutrophils (23), it seems that LPS molecules, which remain in plasma, do not undergo major cleavage for many hours (20). It is firmly established that LPS binds to plasma lipoproteins (8, 39). The evidence for this was first reported by Skarnes in 1968 (33). LPS binding to lipoproteins, more specifically to the high-density lipoproteins (HDL), has been shown to diminish the LPS toxicity in vivo (20, 24, 38) with respect to pyrogenicity, neutropenia, and anticomplementary activity. Inactivation of LPS by HDL did not, however, proceed to completion, and HDL-complexed LPS retained much of its ability to induce hypotension, disseminated intravascular coagulation, and death.

The actual mechanisms of LPS toxicity are not fully understood. Nevertheless, there is accumulating evidence suggesting that LPS toxicity is mediated by cytokines, namely, tumor necrosis factor alpha-cachectin (TNF-α), interleukin-1 (IL-1), and IL-6. In vitro, these monokines are concomitantly produced by cells of the monocyte/macrophage lineage, fibroblasts, or endothelial cells upon stimulation by minute amounts of LPS. The central role of monocytes/macrophages in mediating LPS toxicity in vivo has been emphasized by several observations, including adoptive transfer of LPS sensitivity by C3H/HeN macrophages into LPS-nonsensitive C3H/HeJ mice (9). Further, TNF or IL-1 could mimic the whole spectrum of LPS toxicity in animals (18, 19, 30, 36), and passive immunization against TNF could prevent some of the LPS toxicity in vivo (4, 21, 37). IL-6 (previously hepatocyte-stimulating factor) is known to be the principal inducer of the acute-phase response of the liver (12, 13).

Inhibition of LPS effects by serum components has been demonstrated by several authors (15, 29, 42, 43), using the *Limulus* amebocyte lysate assay. Recently, we have shown that under certain conditions, interaction of serum with LPS could prevent LPS-induced mediator release (27, 28). Preliminary results in our laboratory indicated that those serum components which can inhibit LPS recognition by the *Limulus* amebocyte lysate assay may not be identical with the effective inhibitors of LPS-induced mediator release by human monocytes. It is the prime aim of this study to establish the extent to which serum interferes with LPS-induced monokine release, what the kinetics of this effect are, and which serum components are involved. In most experiments, IL-1 was measured and taken as representative, while in some experiments, TNFα and IL-6 release were also measured.

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MATERIALS AND METHODS

Materials. LPSs derived from *Escherichia coli* O128:B12, O127:B8, O111:B4, O55:B5, O26:B6, *Salmonella minnesota*

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Re595 and *Salmonella abortus equi* were purchased from Sigma Chemical Co. (St. Louis, Mo.). LPS *Salmonella typhi* (strain 0901, no. 3124-25-6) was from Difco Laboratories (Detroit, Mich.). LPS *S. minnesota* was extracted by a phenol-chloroform-petroleum-ether extraction procedure; all other LPSs were phenol-extracted preparations and contained the complete molecule, including the O antigen. LPS was reconstituted in pyrogen-free distilled water at 1 mg/ml, sonicated for 10 min, passed through a 0.22-μm-pore-size filter (no. 8110; Costar, Fernwald, Federal Republic of Germany), and stored as stock solution at 4°C for a maximum of 4 months. Synthetic lipid A LA-15-PP(506) was obtained from Daiichi Co. (Tokyo, Japan), reconstituted as recommended by the producer, stored at 4°C as stock solution, and used within 2 weeks. Culture medium was RPMI 1640 (GIBCO, Karlsruhe, Federal Republic of Germany) containing 25 U of penicillin-streptomycin per ml, 2.5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, 0.02 mM 2-mercaptoethanol, and 6 mM L-glutamine and adjusted to pH 7.2 with 1 N HCl. Pyrogen-free water was prepared by bidistillation and frequently tested with a *Limulus* assay (Pyroquant, Walldorf, Federal Republic of Germany). All laboratory techniques were performed with disposable plastic materials to ensure pyrogen-free conditions throughout. C3H/HeJ-mice (4 to 7 weeks old) and Lewis rats (20 weeks old) were obtained from Bomholtgard (Ry, Denmark).

Preparation of sera. To obtain human serum, blood was drawn from healthy donors (blood group AB or A, checked for absence of irregular antibodies against erythrocytes), collected in bottles, and allowed to clot at 4°C overnight. The serum was withdrawn and frozen at −20°C until tested or immediately used for further preparation. Thawing was performed in a 37°C water bath. Serum fractions separated by ultracentrifugation were tested immediately after preparation. Samples of serum fractions stored at −20°C could also be used and produced equivalent results. Rat serum was obtained from Lewis rats by puncture of the abdominal artery under ether anesthesia and immediately used in experiments. Fetal calf sera (FCS) were purchased from different suppliers and pretested on more than 10 human monocyte preparations for lack of direct IL-1-inducing activity (28). Only those sera which were negative with all human monocytes were used in our experiments. One of them (lot no. 3R07; Seramed, West Berlin, Federal Republic of Germany) was used throughout the monocyte preparation. All sera were filtered through 0.45-μm-pore-size disposable filterware (Nalge Co., Rochester, N.Y.).

Preparation of serum fractions. (i) Density gradient ultracentrifugation. Human serum was adjusted with KBr to a density of 1.21 g/cm^3 and ultracentifuged in a SW50 Ti rotor (Beckman, Munich, Federal Republic of Germany) at 226,000 × *g* for 40 h at 4°C. The top one-quarter was separated from the lower three-quarters ("1.21 bottom") of the tubes (Quick-Seal; Beckman) by using needles for perforating the plastic. Representative samples were controlled for density. The lipoprotein-free serum ("1.21 bottom") was tested for apolipoproteins AI, AII, and B; cholesterol; and phospholipids by routine techniques and found to contain less than 3% of the physiological serum concentrations. Both serum fractions were dialyzed extensively under sterile conditions against RPMI 1640. Lipoprotein-free serum was diluted with RPMI 1640 to the original volume before use in monokine production. The upper fraction, which contained virtually all lipoproteins, was checked for apolipoproteins AI, AII, and B.

(ii) Kaolin absorption. Kaolin (E. Merck AG, Darmstadt, Federal Republic of Germany) was baked 4 h at 250°C and thoroughly washed. Human serum (10 ml) was treated with 5 g of kaolin gently held in suspension for 1.5 h at 4°C. Kaolin was removed from the suspension by centrifugation at 2,000 × *g* for 1 h and subsequent 0.2-μm-pore-size filtration.

(iii) Solvent extraction. Lipoprotein-free serum was also prepared by using a solvent system as described previously (7). Kaolin-absorbed or solvent-treated sera proved to be depleted of all lipoproteins, as controlled by apolipoprotein content.

Preparation of monocytes and monokine production. Mononuclear cells prepared by Ficoll density gradient centrifugation of buffy coat layers from normal blood units (ACD stabilizer, blood group O or A) were incubated for 2 h at 37°C in four (10 ml each) plastic flasks (no. 3024; Falcon, Heidelberg, Federal Republic of Germany) in RPMI 1640 medium containing 5% FCS. Nonadherent cells were removed by washing three times with RPMI 1640-FCS at 37°C. Adherent cells were recovered by gentle washing after 1 h of incubation on ice, adjusted to 10^6 cells per ml in RPMI 1640-FCS, and immediately used in monokine production. More than 90% of the cells were verified as monocytes by nonspecific esterase staining and immunofluorescence with monoclonal antibodies.

For testing the LPS-serum interaction, RPMI 1640 containing either 20% serum, lipoprotein-free serum, or lipoprotein-reconstituted serum was incubated with LPS for 24 h at 37°C (total volume of 100 μl; no. 3595; Costar) before the addition of monocytes. Alternatively, RPMI 1640 containing 20% serum was incubated without LPS and LPS was added immediately after the start of the culture. As a further control, LPS was incubated with RPMI 1640 alone and serum was reconstituted at start of culture. Cultures were initiated with 100 μl of monocyte suspension added to microdilution wells containing 100 μl of the serum-supplemented RPMI 1640. In another control, all media and agents (RPMI 1640, serum, LPS, and monocytes) were added at once without any incubation prior to culture. Serum or lipoprotein-free serum was always present at a final concentration of 10%. In reconstitution experiments, the lipoprotein-rich fraction was added to the lipoprotein-free serum to yield concentrations as indicated in the results. The culture supernatants were harvested after 24 h of monocyte culture and stored at −20°C until being tested for monokines.

IL-1 assay. IL-1 was determined in the costimulator assay as described previously (26). In brief, supernatants were titrated and C3H/HeJ mouse thymocytes were added at 7.5 × 10^6 cells per ml and incubated for 72 h at 37°C in the presence of suboptimal phytohemagglutinin concentrations (50 μg/ml; no. 27658; Serva, Heidelberg). Cultures were pulsed with [^{3}H]thymidine for the final 24 h. Background levels in the presence of LPS and suboptimal phytohemagglutinin were always less than 800 ± 200 cpm (mean ± standard deviation; done in triplicate). The minimal potentiation accepted as significant was a 2.5-fold increase in incorporated radioactivity over background. International units of IL-1 were calculated by comparison to the international standard for IL-1 (NBSB, Oxford, England) by using a computer-based program (Hochgeladen, Neu-Ulm, Federal Republic of Germany) for logit transformation.

TNF-α assay. TNF-α was determined by enzyme-linked immunosorbent assay (ELISA) by using a polyclonal rabbit antiserum as described previously (17).

IL-6 assay. IL-6 was determined by using the IL-6-de-

pendent cell line 7TD1 (kindly provided by J. van Snick, Brussels, Belgium). Supernatants of monocyte cultures were titrated, and 7TD1 cells were added at 2.5×10^4 cells per ml in 1% FCS and incubated for 72 h at 37°C. Cultures were pulsed with [^{3}H]thymidine for the final 24 h. Units were calculated by comparison to an arbitrary standard for IL-6.

Assay for naturally occurring anti-LPS antibodies. A total of 200 human sera were screened for immunoglobulin G (IgG) antibodies against LPS from *E. coli* O128:B12, O127:B8, O111:B4, O55:B5, and O26:B6; *S. abortus equi*; and *S. typhi* by the method of Gaffin et al. (10). The endotoxins were coupled to microdilution plates (Greiner, Nürtingen, Federal Republic of Germany) at an endotoxin concentration of 70 μg/ml (10 μg/ml of each LPS) in 100 μl per well. The wells were blocked with 10 mg of bovine serum albumin per ml and 1 mg of gelatin per ml in pyrogen-free phosphate-buffered saline. The sera to be tested were diluted 1:100 in phosphate-buffered saline with 20% goat serum. Diluted sera (100 μl per well) were incubated in triplicate at 37°C for 1 h. The bound IgG was detected by peroxidase-labeled goat anti-human IgG (Medac, Hamburg, Federal Republic of Germany) by using *o*-phenylenediamine (Sigma) as a substrate. Concentrations of anti-LPS antibodies were determined by comparison to a standard of 100 μg of anti-LPS IgG per ml (a kind gift of S. L. Gaffin). The 10 sera with the largest amounts of anti-LPS IgG showed cross-reactivity to all LPS species used in screening for antibodies, when tested separately.

Complement depletion. Freshly drawn human serum (2-ml aliquots) was either incubated at 56°C for 30 min (to destroy C1, C2, C5, C8, C9, and factor B), treated with 1 ml of packed baker's yeast at 37°C for 60 min (to deplete native C3), or passed over a controlled pore glass column (Boehringer GmbH, Mannheim, Federal Republic of Germany) coupled with anti-human-C3c antibody (no. A062; Dako, Hamburg, Federal Republic of Germany). The yeast was killed by 56°C for 60 min and washed six times before treatment of the serum. After treatment, the yeast was removed by centrifugation and the serum was incubated for a further 60 min at 37°C. Native control serum was stored on ice until use (approximately 3 h). All samples were passed through 0.22-μm-pore-size cellulose acetate filters before use in the monocyte assay, and aliquots were tested for total hemolytic complement activity. Complete depletion of complement was achieved by either method. Affinity column-treated serum was also controlled for lack of C3 by rocket immune electrophoresis.

RESULTS

Inactivation of LPS by human serum. Interaction of serum from normal donors with LPS was studied first. Previous experiments (27) had shown that freshly prepared monocytes which are cultured in the presence of 10% serum (human serum or FCS) will usually respond to minute amounts of LPS (0.1 to 100 pg of LPS *S. typhi* per ml) by a measurable release of IL-1. The threshold depended on the sensitivity of the individual monocyte population, providing that LPS was added at the start of the culture. Three parallel series of experiments were performed to test the effect of LPS-serum interaction upon monokine release. First, serial $\log_{10}$ dilutions of LPS were incubated with serum-supplemented RPMI 1640 for 24 h at 37°C. Following this incubation period, the cell culture was initiated by the addition of monocytes. Second, serum-supplemented culture medium was incubated for 24 h at 37°C and LPS was added along with monocytes at the start of the culture. Finally, all media and agents (RPMI 1640, sera, LPS, and monocytes) were added together at the start of the culture. Results of a representative experiment out of a series of three repetitive experiments are shown in Fig. 1. Incubation of LPS with serum clearly reduced or abrogated measurable IL-1 release by monocytes with concentrations of LPS *S. typhi* of ≤0.1 ng/ml. As a further control, a parallel dilution series of LPS was incubated with culture medium RPMI 1640 alone and serum was added with the monocytes. IL-1 release was comparable to that of both other controls, showing that adsorption of LPS to the plastic was not responsible for the observed effects (data not shown).

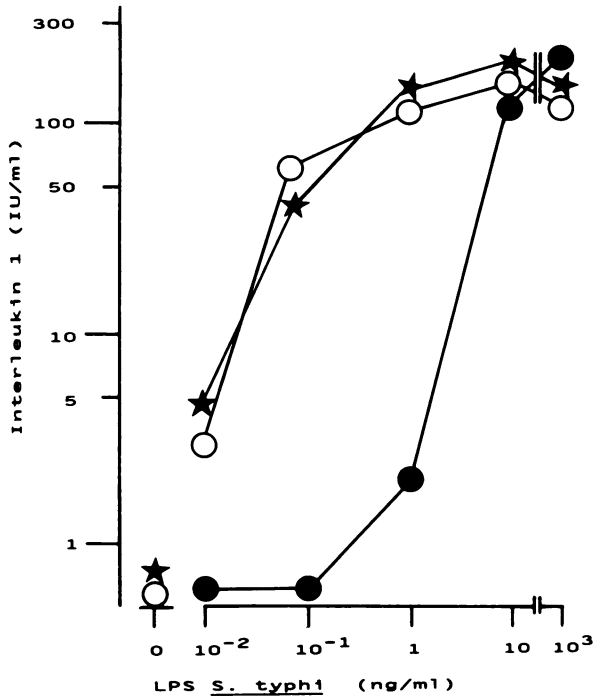
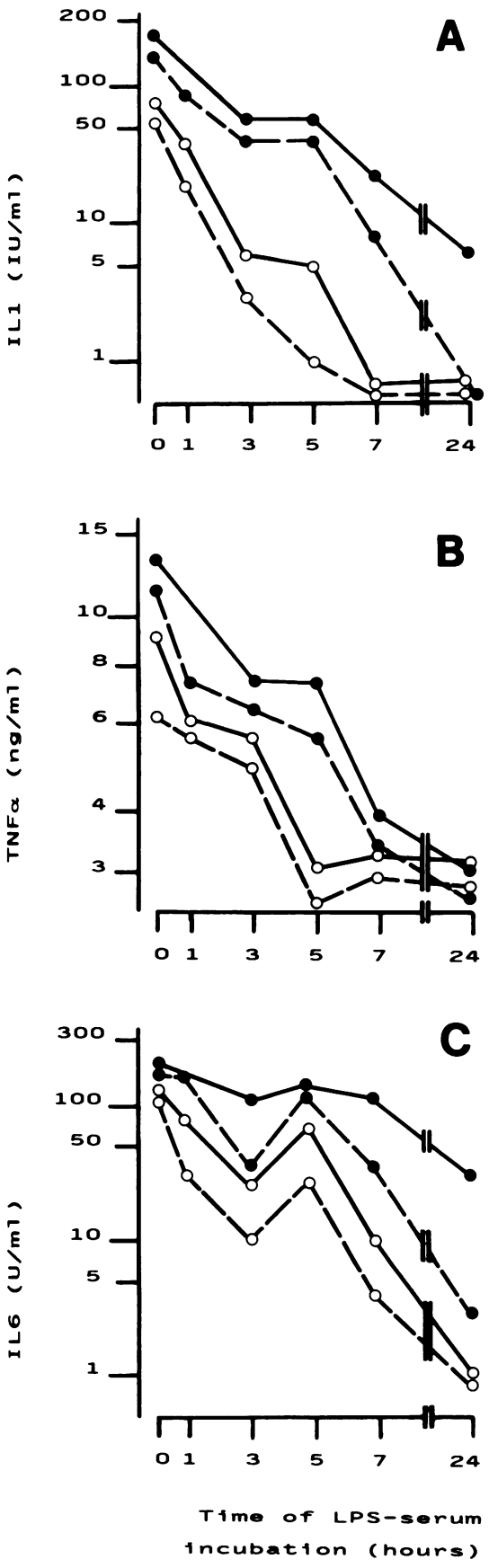


FIG. 1. Effect of LPS-serum interaction on IL-1 release by human monocytes. Monocytes were stimulated for 24 h with LPS *S. typhi* in the doses indicated on the abscissa. Induced IL-1, as measured by thymocyte assay and expressed in international units, is shown on the ordinate. ★, RPMI 1640, serum, LPS, and monocytes added at start of the culture; ○, Serum-supplemented RPMI 1640 incubated for 24 h at 37°C, with LPS added at start of culture; ●, LPS incubated with RPMI 1640 and human serum for 24 h at 37°C.

Testing more than 50 monocyte preparations and sera from 20 different donors revealed that the threshold dose of LPS required for minimal IL-1 induction was always raised by a factor of 10 to 100 following incubation with serum. Inactivation of LPS by incubation with serum could not be demonstrated under the given conditions when LPS *S. typhi* was used in doses ≥10 ng/ml. The fact that inactivation of LPS could always be overcome by high LPS doses excluded toxicity of the employed serum preparations as a possible mechanism of inactivation. All species of LPS tested (five strains of *E. coli* and two of *Salmonella* species) and a synthetic lipid A component could be inactivated. Inactivation could also be achieved with five human cord sera and five batches of FCS. Finally, we tested pooled rat sera and found the inactivation capacity to be at least 10 times greater than that of human sera (data not shown).



Time kinetics and temperature dependence. To determine the kinetics of LPS inactivation, LPS was incubated for different periods of time at 37°C with RPMI 1640 containing human serum. With LPS doses of up to 300 pg/ml, significant inactivation was achieved within 3 h. Complete inactivation took 7 h. The results of one of two repetitive experiments are shown in Fig. 2A. In this experiment, 1 ng of LPS *S. typhi* per ml could not be inactivated completely within 24 h. Similar results were obtained for induction of TNF-α (Fig. 2B) and IL-6 (Fig. 2C). In separate experiments, larger LPS doses (10 to 1,000 ng/ml) were incubated with serum-containing medium for up to 1 week. There was no measurable effect upon IL-1 release as compared to the controls. The inactivation of LPS was largely dependent on the temperature. Incubation for 24 h at 4°C had no or only marginal effects for LPS doses that were completely inactivated at 37°C. On the other hand, the degree of inactivation achieved at 37°C after 24 h could be neither reversed nor increased by an additional 24 h of incubation at 4°C (data not shown).

Inactivation of LPS by the lipoprotein-rich serum fraction. Since binding of LPS to lipoproteins is well documented, we suspected that lipoproteins might also be involved in LPS inactivation in our system. Using ultracentrifugation, we prepared a serum devoid of lipoproteins. This lipoprotein-free serum (density of >1.21 g/cm^3), which contained the remaining serum proteins, was tested for its ability to prevent LPS from inducing IL-1 release by monocytes. As seen in Fig. 3A, incubation of LPS with lipoprotein-free serum had no significant influence on the induced IL-1 release compared with the control. When, however, lipoprotein-free serum was reconstituted with the lipoprotein-rich fraction (density of <1.21 g/cm^3) to the original serum levels (by apolipoprotein), this mixture had regained the capacity to inactivate LPS upon incubation (Fig. 3B). The results shown in Fig. 3 are from one representative experiment of a series of five comparable tests. The effect of lipoproteins on LPS was dose dependent. When graded amounts of lipoproteins were titrated into lipoprotein-free serum, the mixture showed increasing capacity to inactivate LPS proportional to the amount of lipoproteins present (Fig. 4, results from one of two experiments). To corroborate our assumption that we were dealing with the effects of lipoproteins and not with any effect of proteins contaminating the lipoprotein-rich fraction, we also tested other ways of removing lipoproteins from serum, namely, adsorption to kaolin and extraction by a solvent system (diisopropylether-butanol). Both manipulations resulted in a lipoprotein-free serum, which was unable to inactivate LPS upon incubation (data not shown).

Nutritive lipids and LPS inactivation. Since the lipoprotein-rich fraction also contained free lipids, we tried to determine the influence of diet on inactivation of LPS by serum. A meal rich in fatty acids and cholesterol was given to four persons prior to blood donation. As expected, their sera proved to be

FIG. 2. Time kinetics of LPS inactivation by human serum. (A) Different doses of LPS *S. typhi* were incubated at 37°C with serum-supplemented RPMI 1640 for the periods of time indicated on the abscissa. Then, monocytes were added and cultured for another 24 h, and supernatants were tested for IL-1 by thymocyte assay. Activity of IL-1 in these supernatants is depicted on the ordinate. (B) The same supernatants as in panel A were tested for TNF-α by ELISA, and concentrations are given on the ordinate. (C) The same supernatants as in panel A were tested for IL-6 by induction of hybridoma (7TD1 cell) proliferation. IL-6 activity is shown on the ordinate. LPS amount: ●——●, 1 ng/ml; ●----●, 0.6 ng/ml; ○——○, 0.3 ng/ml; ○----○, 0.1 ng/ml.

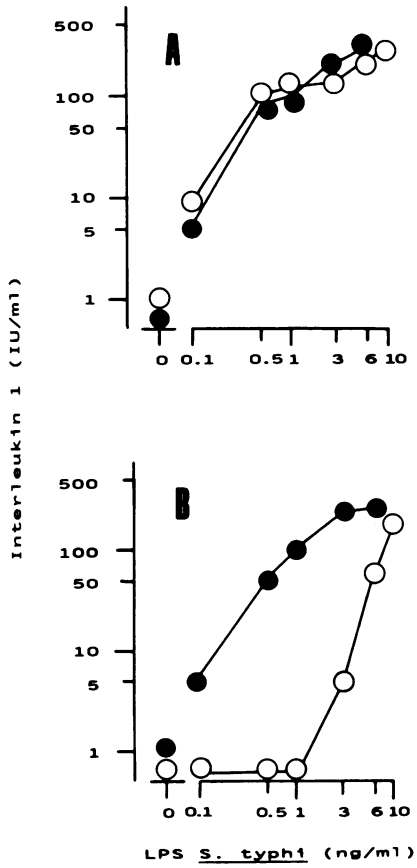


FIG. 3. Effect of removal of lipoproteins from human serum. (A) LPS *S. typhi* was titrated into RPMI 1640 supplemented with lipoprotein-free serum prepared by ultracentrifugation. LPS was either added at start of culture together with the monocytes (●) or incubated with supplemented RPMI 1640 for 24 h at 37°C previous to the start of the monocyte culture (○). All other conditions are as described in the legend to Fig. 1. (B) Same conditions as in panel A except that lipoprotein-free serum was reconstituted with lipoprotein-rich fraction.

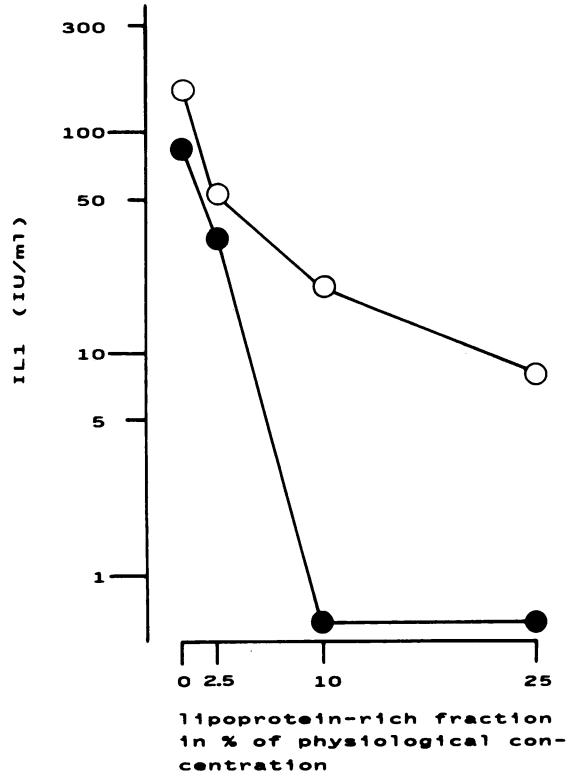


FIG. 4. Titration of lipoproteins into lipoprotein-free serum. LPS *S. typhi* was incubated for 24 h at 37°C with RPMI 1640 containing a fixed amount of lipoprotein-free serum but different amounts of lipoprotein-rich fraction. Following incubation, monocytes were added and cultured for another 24 h. Final concentrations of LPS were 0.1 ng/ml (●) and 1 ng/ml (○). Final concentrations of lipoproteins are indicated on the abscissa, expressed as a percentage of the original concentration of lipoprotein in serum.

visibly lipemic. These sera were compared in regard to their ability to inactivate LPS with sera from three blood donors who had fasted overnight previous to blood donation. Both groups of sera inactivated LPS to comparable extents (Table 1). It may be noted that lipemic sera caused somewhat enhanced absolute counts per minute in low-power dilutions of the thymocyte assay, possibly because of scavenging of prostaglandins. The titers and units of IL-1 were, however, unaffected. To avoid any unexpected influence, we did not use lipemic sera in other experiments.

Influence of naturally occurring antibodies against LPS. Almost all human sera contain measurable amounts of anti-LPS antibodies. We investigated whether the naturally occurring antibodies against LPS would interfere with the capacity of LPS to induce IL-1 release. Sera from normal donors with high (>60 μg/ml) and low (≤5 μg/ml) concentrations of anti-LPS IgG determined by ELISA were spiked with the LPS species used to screen for antibodies. The sera were incubated with LPS for 1 h at 4°C to avoid LPS inactivation by lipoproteins and used in monokine production. Comparison of sera with high and low anti-LPS concentrations showed no significant differences in IL-1 induction (Table 2). Binding of naturally occurring antibodies against LPS did not, therefore, influence IL-1 release in our system. LPS inactivation by serum was also not due to naturally occurring anti-LPS antibodies.

Influence of complement. LPS is known to activate the bypass mechanism of complement activation. Monocytes possess receptors for and are also possibly activated by complement. Therefore, it was important to evaluate whether complement depletion or activation might have any influence on LPS inactivation observed in our system. We approached this question by using C3-depleted serum and serum treated at 56°C or yeast. Comparison of complement-active serum with C3-depleted or yeast-treated serum re-

TABLE 1. Comparison of visibly lipemic human sera with sera from fasted blood donors

LPS *S. typhi* (ng/ml)[a]	IL-1 (IU/ml) (mean ± SD)			
	LPS incubated with serum		LPS added at start of culture	
	Lipemic (n = 4)	Nonlipemic (n = 3)	Lipemic (n = 4)	Nonlipemic (n = 3)
10	101.0 ± 31.1	124.0 ± 49.2	110.0 ± 24.5	99.3 ± 29.1
1	36.3 ± 7.7	36.3 ± 10.1	110.5 ± 25.4	98.0 ± 31.2
0.1	0	0	75.5 ± 8.1	70.3 ± 34.8

[a] With no LPS, result was 0 for all columns.

TABLE 2. Comparison of IL-1 release induced by different LPSs in presence of normal human sera with low or high concentrations of anti-LPS IgG

Source of LPS	Concn of anti-LPS IgG[a]	n	IL-1 (IU/ml) (mean ± SD)[b] induced by LPS (ng/ml):		
			10	1	0.1
E. coli O55:B5	Low	6	317.0 ± 107.9	249.0 ± 69.3	136.5 ± 44.3
	High	6	273.0 ± 77.8	204.7 ± 26.2	144.0 ± 56.5
S. abortus equi	Low	6	138.4 ± 25.7	158.4 ± 57.9	57.4 ± 39.7
	High	6	138.2 ± 60.6	163.6 ± 61.0	54.6 ± 51.1
S. typhi	Low	10	97.2 ± 47.7	121.4 ± 46.1	42.7 ± 12.9
	High	10	87.5 ± 28.0	113.0 ± 27.5	30.9 ± 16.8
E. coli O127:B8	Low	10	239.6 ± 62.1	145.9 ± 33.2	36.5 ± 34.3
	High	10	287.6 ± 67.0	203.3 ± 68.9	35.6 ± 25.4
E. coli O128:B12	Low	10	130.0 ± 46.9	56.3 ± 39.3	28.4 ± 5.3
	High	10	153.0 ± 55.5	71.5 ± 38.6	27.1 ± 4.8
E. coli O111:B4	Low	10	292.3 ± 157.6	169.0 ± 117.8	0
	High	6	313.0 ± 108.4	186.5 ± 49.1	0
E. coli O26:B6	Low	10	116.7 ± 39.1	10.5 ± 11.5	0
	High	10	97.0 ± 30.9	8.0 ± 10.0	0

[a] Determined by ELISA.
[b] With no LPS, IL-1 induced was 0 IU/ml for all LPSs and all concentrations of IgG.

vealed that all three sera were equally supportive for monocyte activation by low doses of LPS when LPS was added at the start of the culture (Table 3). They were also comparable in their capacity to inactivate LPS upon incubation. Heat treatment (56°C, 30 min) of serum showed some influence on the dose of LPS required for IL-1 induction. When 0.1 ng of LPS *S. typhi* per ml was added at the start of the culture, this dose was no longer sufficient to activate monocytes with this serum. However, heat-treated serum still inactivated LPS upon incubation (Table 3). Maximum stimulation by a high dose of LPS (≥1 μg/ml; data not shown) was not affected at all, excluding toxicity of heat-treated serum. All sera mentioned in Table 3 were prepared and tested twice. Representative results are shown. Time kinetics experiments were also performed with heat-treated serum. These experiments revealed a slope parallel to that for native serum (data not shown). The results indicate that the complement system, at least starting from C3, is not involved in the LPS inactivation described in this report. This is in agreement with our finding that the capacity to inactivate LPS resides exclusively in the lipoprotein-rich fraction.

DISCUSSION

Inflammatory cytokines such as TNF-α and IL-1 appear to mediate much of the LPS toxicity in vivo, as already pointed out in the introduction. These cytokines are synthesized and released by susceptible cells upon contact with LPS. It is also known that serum components can interact with LPS and reduce its toxicity in vivo. Earlier, we reported that LPS *S. typhi* could be inactivated by incubation with serum from healthy donors, such that the LPS could no longer activate human monocytes to secrete IL-1 (27). The aim of this study was to give a detailed analysis of the impact of the interaction between LPS and serum on mediator secretion by monocytes in vitro.

TABLE 3. Effect of complement depletion of sera upon LPS inactivation

Human serum	IL-1 (IU/ml) induced by LPS					
	Added at start (ng/ml)			Incubated with serum (ng/ml)		
	0.1	1	10	0.1	1	10
Native[a]	78	130	103	0	0	103
Yeast treated[a]	73	ND[b]	98	0	ND	116
56°C treated[a]	0	ND	96	0	ND	33
C3 depleted[c]	95	190	210	0	0	200

[a] Results obtained in the same experiment by using identical monocyte preparations.
[b] ND, Not determined.
[c] Results obtained by using a different monocyte preparation.

Our findings comprise three points. (i) We found that under the conditions used, human serum could prevent the activation of human monocytes by all different species of LPS tested, including a synthetic lipid A, and that all three monokines tested (TNF-α, IL-1, and IL-6) were affected. We therefore favor the view that inactivation described in this system is not specific for the type of LPS and impairs recognition of LPS by monocytes rather than merely modifying the effect of LPS.

(ii) We found that the complement system and naturally occurring antibodies against LPS are not involved in the inactivation process described in this report. C3-depleted serum and yeast-treated serum both behaved in a way indistinguishable from that of native serum in our assay. This demonstrates that under the conditions used, LPS activation of monocytes, as well as serum inactivation of LPS, were both independent of the complement system, at least starting from C3, although complement may participate in separate pathogenic mechanisms of LPS in vivo. Our results are in agreement with those in a recent publication questioning whether complement could activate monocytes at all (3). Naturally occurring antibodies which are present in most human sera are also not involved in the inactivation process described, since high- and low-titer antisera, as well as FCS (which contains no antibodies), had LPS-inactivating capacities comparable to that of adult human serum. These results do not exclude the possibility that antibodies may have beneficial effects in vivo by affecting the clearance of LPS or whole microorganisms (22). Nevertheless, the inability of naturally occurring anti-LPS antibodies to prevent LPS activation of monocytes may contribute to our understanding of why the efficiency of therapy with anti-LPS plasma remains controversial.

Finally, we clearly show that the capacity of normal human serum to inactivate LPS in regard to monokine induction in vitro resides in the lipoproteins. Lipoproteins are the sole fraction in normal human serum to have this capacity, since lipoprotein-free serum did not show any LPS inactivation at all in our system. This does not exclude the possibility that the presence of nonlipoprotein serum components may be necessary or beneficial for the LPS-inactivating function of lipoproteins. Furthermore, components of lipoprotein-free serum have been shown to inactivate LPS in in vitro assays other than monocyte activation and to detoxify LPS in vivo (1, 2, 15, 35). The results obtained by different investigators vary considerably, probably depending on the type of LPS, assay system, or in vivo model used (see below). We therefore feel that data from other systems may not be directly transferable to the human model. With the data presented in this paper, we could link LPS-lipoprotein interaction to the inhibition of monocyte activation in a human in vitro model. On the basis of our findings, we speculate that LPS-lipoprotein interaction may well participate in reducing LPS toxicity in humans by suppressing monokine release.

In the present report, we confined ourselves to the investigation of serum from healthy individuals to exclude possible interference from acute-phase proteins, including an LPS-binding protein (35) and a macrophage factor controlling LPS-lipoprotein binding (44). Such acute-phase proteins, elicited as a response to previous LPS challenge, may interfere with the lipoprotein-dependent LPS inactivation in vitro and may constitute different LPS-inactivating principles in vivo. It is known that the basal level of individual acute-phase proteins present in normal serum varies widely between different species. These differences may well contribute to the massive variation of results in models from different species.

The LPS-inactivating capacity of serum was resistant to 56°C and was optimal at 37°C; this is in agreement with results of in vivo studies (41). The kinetics of the inactivation were relatively slow. Together with the fact that the presence of serum is often viewed as a prerequisite for optimal culture conditions (6), this may be the reason why LPS inactivation has long gone undetected in systems using monocytes/macrophages. At first glance, the amount of LPS which can be inactivated by normal human serum (about 10 ng/ml) may also not seem very impressive. It may, however, be significant, since monocytes can respond to picogram concentrations of LPS (27). In relation to the extraordinary sensitivity of monocytes to LPS, the inactivating capacity of serum amounts to between 100- and 1,000-fold.

The amounts of LPS inactivated by serum as described here are in contrast to the vast amounts of LPS which can be bound by lipoproteins (39, 40). The mere binding of LPS to lipoproteins can therefore not be equated with inactivation of LPS. Hence, LPS inactivation as described in this report may represent a distinct biological function of the lipoproteins. Our findings are in agreement with observations from the literature which show that lipoprotein-complexed LPS retained much of its biological activity (21, 25) or its toxicity in animals (24, 38). The amounts of LPS used in these experiments clearly exceeded the amounts which can be inactivated as judged from our findings.

Using lipoprotein subfractions, we observed that HDL possess a rather small LPS-inactivating capacity compared with that of the low-density lipoproteins (LDL) (W. A. Flegel, manuscript in preparation). This observation is in contrast to results of binding studies (39) showing that HDL

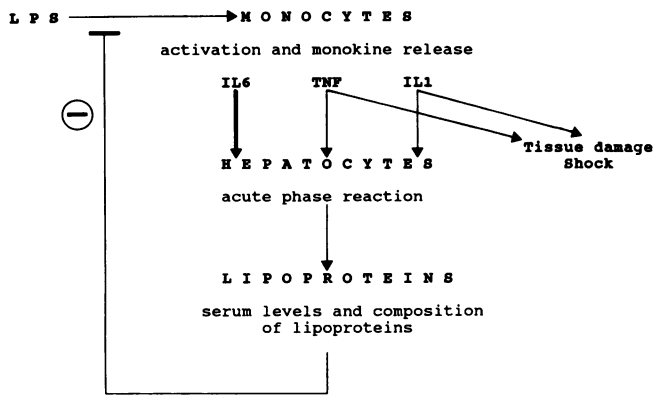


FIG. 5. Proposed feedback mechanism for LPS detoxification by lipoproteins in acute-phase reaction. Massive release of monokines causes tissue damage and shock. Monokines also activate hepatocytes, leading to the acute-phase reaction, which includes changes in serum levels of lipoproteins and changes in the ratio of lipoprotein classes. These changes lead to an enhanced inactivation of LPSs. Other acute-phase proteins which may constitute different ways of endotoxin detoxification are not included in the figure (see text).

can bind more LPS than LDL can. Extrapolating our observations, we conclude that mere changes in the ratio of lipoprotein classes may considerably alter the LPS-inactivating capacity of total lipoproteins. A relative increase in LDL and decrease in HDL should increase the LPS-inactivating capacity of serum. Changes of composition and levels of serum lipoproteins during the acute-phase response showing the predicted pattern have recently been described (5, 11, 14, 16).

We believe that lipoproteins are likely to play a role in handling and controlling the everyday influx of LPS from the gut or minor infections. We speculate that lipoproteins constitute a kind of buffer system for LPS, sheltering the organism from being massively activated by minor amounts of LPS, but low enough in their inactivating capacity to allow for sensible activation of the immune system in case of increased influx of LPS. In response to endotoxemia, the inactivation capacity of serum could then be up-regulated by a change of composition or concentration of serum lipoproteins in addition to the generation of other detoxifying principles as mentioned above. Our view of a possible regulation circuit contributing in part to the inactivation of LPS is depicted schematically in Fig. 5.

In conclusion, we showed that lipoproteins contained in normal human sera can interact with LPS to prevent LPS from activating human monocytes in vitro. Lipoproteins are the sole serum fraction of healthy individuals which has this capacity. The LPS-inactivating capacity of lipoproteins is clearly lower than their capacity to bind LPS. However, in view of the exquisite sensitivity of monocytes to LPS, we favor the concept that lipoproteins constitute a system to control the effects of low doses of LPS on monocyte activation in vivo. A shift of serum lipoprotein levels and composition during the acute phase of an infection might then contribute to an increased detoxification rate of LPS.

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