

Antibodies against CD14 Protect Primates from Endotoxin-induced Shock

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Abstract

Lipopolysaccharide (LPS), residing in the outer membrane of all gram-negative bacteria, is considered a major initiating factor of the gram-negative septic shock syndrome in humans. LPS forms a complex with the LPS binding protein (LBP) in plasma, and LPS-LBP complexes engage a specific receptor, CD14, on the surface of myeloid cells, leading to the production of potent proinflammatory cytokines. The major goal of this study was to test the importance of the CD14 pathway *in vivo* in a primate model that is similar to human septic shock. Primates were pretreated with one of two different inhibitory anti-CD14 mAbs, then challenged with intravenous endotoxin (375 µg/kg/h) for 8 h. The anti-CD14 treatment regimens were successful in preventing profound hypotension, reducing plasma cytokine levels (TNF-α, IL-1β, IL-6, and IL-8), and inhibiting the alteration in lung epithelial permeability that occurred in animals treated with LPS and an isotype-matched control antibody. These results demonstrate for the first time the importance of the CD14 pathway in a primate model that is similar to human septic shock. Inhibition of the CD14 pathway represents a novel therapeutic approach to treating this life-threatening condition. (*J. Clin. Invest.* 1996. 98:1533–1538.) Key words: lipopolysaccharides • antigens, CD14 • primates • shock • acute lung injury

Introduction

Gram-negative sepsis is a syndrome characterized by fever, hypotension, disseminated intravascular coagulation, renal and hepatic failure, acute respiratory distress syndrome (ARDS),¹ and high mortality (1, 2). These findings have been duplicated in experimental animals after intravenous administration of

purified LPS (3). It is now well established that the mechanism by which the LPS activates cells involves an initial interaction with a specific LPS binding protein (LBP) (4, 5). The resultant LBP-LPS complexes are recognized by CD14, present either anchored to the cell membrane by a phosphatidylinositol linkage, or free as a soluble plasma protein (6, 7). Host cells bearing the CD14 receptor respond to LPS by releasing potent inflammatory cytokines, including TNF-α, IL-1β, and IL-6. Cells that do not express membrane-associated CD14 (e.g., endothelial and epithelial cells) are capable of releasing cytokines and upregulating adhesion molecules in response to LPS via a soluble CD14 (sCD14) pathway, in which LPS-sCD14 complexes are recognized by an unknown component on the cell surface (8, 9).

The goal of this study was to test the significance of blocking the CD14 pathway in the overall response to LPS in primates with endotoxin-induced shock, using two different murine mAbs directed at distinct functional domains of human CD14. This strategy was successful in reducing the major systemic consequences of endotoxin shock and in protecting the lungs from injury.

Methods

mAbs. Two murine mAbs were generated using a recombinant soluble form of human CD14. The mAbs prevented downstream signaling events in cells stimulated with LPS. These anti-CD14 mAbs, identified as 28C5 and 18E12 (both IgG₁), map to different sites on the CD14 molecule (10). Anti-CD14 mAb 28C5 prevents LPS-LBP complexes from binding to CD14, while 18E12 halts signaling events, without affecting LPS binding (11). Both mAbs were evaluated for their ability to detect rabbit, murine, cynomolgus, or baboon CD14. Only cynomolgus CD14 was recognized by both anti-CD14 mAbs. Both mAbs blocked LPS-induced cytokine release in a whole blood assay using either cynomolgus or human blood (data not shown).

Animal protocols. To evaluate the efficacy of these two mAbs *in vivo* in a model relevant for humans with gram-negative sepsis, we tested the ability of these mAbs to inhibit LPS-induced toxicity in cynomolgus monkeys (*Macaca fascicularis*). We developed an *in vivo* protocol that required low sensitizing doses of LPS, used a long infusion period (8 h), resulted in endotoxin shock, and allowed survival for a minimum of 24 h. Because IFN-γ potentiates the effects of LPS *in vitro* and *in vivo* (12, 13), we determined that recombinant human IFN-γ enhanced TNF-α release in cynomolgus blood *ex vivo* in response to LPS (data not shown). These data, as well as *in vivo* studies in other species showing that IFN-γ pretreatment sensitizes to the effects of LPS and gram-negative bacteria (13, 14), led us to pretreat the animals with IFN-γ to enhance the effects of LPS *in vivo*.

Monkeys were pretreated daily with recombinant, human IFN-γ (5 µg/kg, subcutaneously) (Genzyme Corp., Cambridge, MA) for 3 d be-

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1. Abbreviations used in this paper: ARDS, acute respiratory distress syndrome; BAL, bronchoalveolar lavage; CO, cardiac output; LBP, LPS binding protein; MAP, mean arterial pressure.

fore administration of LPS. Soluble CD14 levels in plasma did not increase during the 3-d treatment with IFN- γ , although plasma LBP levels rose approximately sevenfold (data not shown). *Salmonella typhimurium* Re595 LPS was prepared as described (15). Preliminary studies indicated that the dose of LPS that consistently induced sustained hypotension in the animals was 375 $\mu\text{g}/\text{kg}/\text{h}$ infused for up to 8 h. The animals were anesthetized with intravenous ketamine and treated with atropine 0.5 mg subcutaneously to prevent vagally mediated vomiting, then instrumented with arterial and venous lines and a urinary catheter. Anesthesia was maintained with periodic administration of ketamine, sufficient to keep the animals asleep but breathing spontaneously. After a period of 1–2 h of stable hemodynamic monitoring, the murine mAbs (18E12, 28C5, or an isotype-matched control antibody, murine IgG₁) were administered by bolus intravenous injection (5 mg/kg). 30 min later the LPS infusion was begun (375 $\mu\text{g}/\text{kg}/\text{h}$) and continued for 8 h. All antibody treatments were administered in a masked fashion so that the investigators were unaware of the experimental treatments. The mean arterial pressure (MAP) and cardiac output (CO) were determined hourly for 24 h via an indwelling arterial catheter. Lactated Ringer's solution was infused by bolus as needed to increase CO to $\geq 90\%$ of baseline after each hourly measurement. All of the animals were killed at the end of the 24-h study without recovering from the anesthetic. Survival beyond 24 h was not an endpoint for the study.

Table I. Effects of Anti-CD14 mAbs on Clinical Measurements in Septic Primates

	Group I (IgG ₁)	Group II (28C5)	Group III (18E12)
Number of animals	5	5	5
Weight (kg)	4.3 \pm 1.1	4.3 \pm 1.1	4.5 \pm 1.4
Peripheral circulating WBC (10 ³ cells/mm ³)			
t = 0	18.3 \pm 7.8	18.0 \pm 4.9	14.5 \pm 4.5
t = 0.5 h	5.4 \pm 2.7	16.1 \pm 6.2*	12.8 \pm 5.5*
t = 1 h	4.3 \pm 1.5	10.3 \pm 4.7*	7.5 \pm 4.4
t = 2 h	5.0 \pm 3.5	6.7 \pm 5.2	3.4 \pm 1.8
t = 12 h	20.2 \pm 16.6	12.9 \pm 1.0	23.4 \pm 11.1
t = 24 h	18.7 \pm 11.4	24.7 \pm 21.5	26.1 \pm 3.5
Temperature (°C)			
t = 0	37.4 \pm 0.4	37.1 \pm 0.2	37.1 \pm 0.4
t = 2 h	38.1 \pm 0.6	38.1 \pm 0.5	37.9 \pm 0.5
t = 12 h	37.9 \pm 0.5	37.6 \pm 0.5	37.4 \pm 0.8
t = 24 h	37.2 \pm 0.5	36.8 \pm 0.7	37.1 \pm 0.5
Heart rate (beats/min)			
t = 0	153 \pm 15	170 \pm 12	161 \pm 20
t = 2 h	211 \pm 20	220 \pm 17	207 \pm 11
t = 12 h	200 \pm 28	197 \pm 22	199 \pm 22
t = 24 h	188 \pm 22	183 \pm 11	194 \pm 16
PaO ₂ (mmHg)			
t = 0	95 \pm 25	93 \pm 10	95 \pm 6
t = 2 h	104 \pm 24	102 \pm 16	98 \pm 16
t = 12 h	91 \pm 8	100 \pm 9	86 \pm 17
t = 24 h	113 \pm 24	120 \pm 22	94 \pm 15
Arterial pH			
t = 0	7.4	7.4	7.4
t = 2 h	7.4	7.4	7.4
t = 12 h	7.5	7.5	7.5
t = 24 h	7.5	7.5	7.5

The data shown are mean \pm SD. * $P < 0.05$ for the comparison with group I.

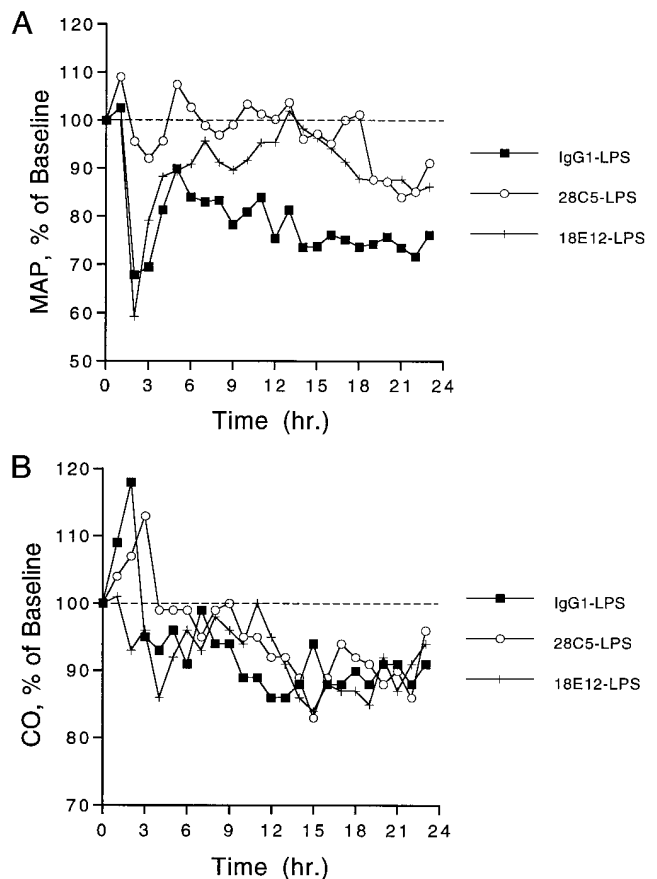


Figure 1. The MAP and CO are plotted in A and B, respectively. The means of each of the five animals per group are included in each graph (1 \times 24 h). A Wilcoxon signed rank test was used to analyze the trends over time in each of the groups. The differences in MAP for groups II (28C5-LPS) and III (18E12-LPS), versus the control group I (IgG1-LPS), were statistically significant ($P < 0.0001$). No significant differences in the CO measurements were evident when group I was compared with either group II or III. Values were plotted as percentage of baseline.

Effect of anti-CD14 treatment on lung permeability. To determine the effect of LPS and the mAb treatments on the permeability of the lung endothelial and epithelial barriers to different sized proteins, the animals were treated with an intravenous bolus of sterile pyrogen-free BSA (Calbiochem-Novabiochem Corp., La Jolla, CA) (0.5 grams/kg) 1 h before the end of the experiment. At the end of each experiment, the animals were killed with an overdose of pentobarbital. An endotracheal tube was inserted rapidly into the trachea and the lungs were lavaged (BAL) with three separate 50-ml aliquots of sterile pyrogen-free 0.89% NaCl containing 0.6 mM EDTA. The BAL fluid was spun at 200 g and the cells were resuspended in RPMI 1640 media and counted in a hemacytometer. The supernatant BAL fluid was aliquoted and stored frozen at -70°C until analyzed. Total protein was measured by the bicinchoninic acid method. The concentrations of BSA (67,000 mol wt) and murine IgG (150,000 mol wt) in serum and BAL were measured using specific immunoassays. The immunoassays for BSA and murine IgG did not cross-react with primate serum albumin or primate IgG, respectively.

Statistical analysis. Hemodynamic data, collected as repeated measures over time, were analyzed using the sign rank test (16). Differences at individual times were assessed using one-way ANOVA. Physiologic and BAL measurements at specific times were analyzed using one-way ANOVA or Student's t test on log₁₀ transformed data.

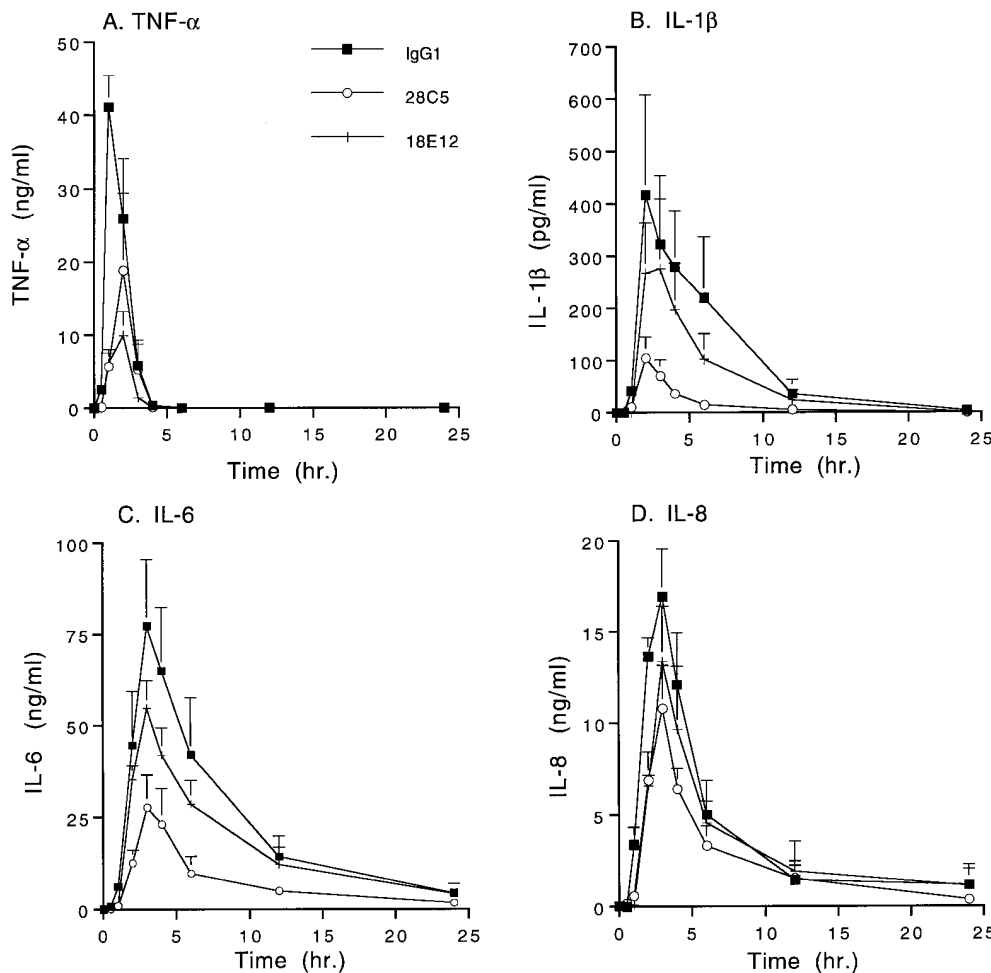


Figure 2. Plasma cytokine responses for all animals by time (mean \pm SE). An ANOVA for repeated measures and an unpaired *t* test were used to evaluate the differences between the groups ($P < 0.05$ was considered significant). The TNF- α response was significantly different at 1 h for both anti-CD14-treated groups versus the control group ($P < 0.001$). The IL-1 β response was significant for both 28C5 ($P = 0.02$) and 18E12 treatment groups ($P < 0.0001$) versus the control group at 1 h. The IL-6 response was significantly different for the 28C5 group versus control animals by ANOVA ($P < 0.05$) and *t* test at 1 and 3 h. The IL-6 levels at 1 h were significantly different in the 18E12 animals versus control animals ($P = 0.003$). The IL-8 levels in both anti-CD14 groups were significantly lower at 1 h ($P < 0.03$) and 2 h ($P < 0.007$) versus the control animals.

For the BAL data, the two treatment groups did not differ, so they were combined and compared with the control group of animals using Student's *t* test. $P < 0.05$ was accepted as significant.

Results

Three groups ($n = 5$) of cynomolgus monkeys, IgG₁ isotype control (group I), anti-CD14 28C5 (group II), and anti-CD14 18E12 (group III), were pretreated with mAb, and then treated with LPS infusion for 8 h. All of the animals survived the 24-h duration of the experiment; survival was not a predetermined endpoint in the study. All animals responded to the intravenously administered endotoxin with elevated core temperature and tachycardia (Table I). There were no significant differences among the groups over time in temperature, heart rate, arterial oxygen tension, or pH (Table I). A significant leukopenia occurred in group I animals beginning 0.5 h after the start of the LPS infusion. In groups II and III, the drop in white blood cell counts was delayed ($P < 0.05$) and did not occur until 1 h (group III) and 2 h (group II) after the start of the endotoxin infusion (Table I). By 12 h the white blood cell counts in all groups had increased, and there were no significant differences between groups at 12 or 24 h (Table I).

While it was clear that all animals responded to the LPS infusion, the anti-CD14 28C5 pretreatment (group II) had a dramatic effect on MAP ($P < 0.0001$), blocking the severe and sustained hypotension that occurred in the animals pretreated

with the control IgG₁ (Fig. 1 A). Primates pretreated with anti-CD14 mAb 18E12 had an initial hypotensive response (0–3 h) similar to animals treated with the control IgG₁; however, the hypotension resolved by 5 h in this group, while it was sustained in the control group ($P < 0.0001$).

When infused slowly, endotoxin produces an early period of hyperdynamic cardiac function which later progresses to a hypodynamic state characterized by low arterial pressure and low CO (17). Animals pretreated with the isotype control IgG₁ antibody (group I) or anti-CD14 28C5 (group II) had an increase in CO over the first 2–3 h, which declined with time (Fig. 1 B). The animals pretreated with 18E12 did not show this early rise in CO, although a response similar to the other two groups was observed throughout the remainder of the experiment. CO did not fall significantly, because the animals were treated with fluids to maintain CO $> 90\%$ of baseline.

The cytokine responses in the groups of animals are shown in Fig. 2. The LPS infusion caused significant increases in TNF- α , IL-1 β , IL-6, and IL-8 in the isotype-control animals. Both antibody treatments significantly reduced circulating cytokine levels during the LPS infusions as compared with the control group. The TNF- α response was delayed in both anti-CD14 groups and was reduced in magnitude as compared with the control group at 1 h ($P < 0.0001$). The IL-1 β levels in anti-CD14-treated animals were lower than those observed in the control animals; the 28C5-treated animals showed the lowest overall response. IL-1 β levels were significantly lower at 1 h in

Table II. BAL Total Protein, BSA Levels, Lavage/Plasma BSA Ratio, and Cells

Animal	BAL total protein (µg/ml)	BAL BSA (µg/ml)	Plasma BSA (mg/ml)	BAL/plasma BSA ($\times 10^{-3}$)	BAL total WBC ($\times 10^6$)	PMN (%)
Group I (LPS + IgG₁)						
88102	630	87.04	8.19	10.63	10.4	15
85412	40	0.36	14.84	0.02	10.5	5
86136	200	3.28	10.16	0.32	1.3	5
88025	1470	36.05	13.93	2.59	12.8	32
91051	130	2.18	12.29	0.18	15.0	0
Geometric mean:	249.31*	6.05 [‡]	11.62	0.50 [§]	7.71	4.13
Group II (LPS + 28C5)						
88103	9	0.21	9.01	0.02	5.8	3
92023	70	0.12	13.31	0.01	6.8	2
91043	170	1.38	13.72	0.10	13.7	5
81033	250	9.38	9.83	1.00	6.6	2
92174	80	7.27	13.93	0.52	8.5	0
Geometric mean:	73.48	1.20	11.76	0.10	7.88	1.43
Group III (LPS + 18E12)						
92020	70	0.17	15.97	0.01	7.6	34
87096	20	0.17	10.90	0.02	3.7	4
86332	50	0.24	12.70	0.02	9.5	4
87348	40	0.55	7.05	0.08	5.6	3
Geometric mean:	40.91	0.25	11.17	0.02	6.22	6.36
Antibody only (no LPS)						
84198 (IgG ₁)	30	ND	ND	ND	7.8	3
82256 (28C5)	50	ND	ND	ND	15.0	4
86026 (18E12)	20	ND	ND	ND	8.0	0
Geometric mean:	31.07				9.78	1.06

Antibody only denotes animals pretreated for 3 d with IFN- γ , then treated with mAb but no LPS, and followed for 24 h. One animal treated with 18E12 and LPS was not included in the analysis, because it vomited and aspirated 10 h into the experiment. * $P = 0.028$ (group I vs. groups II and III). [‡] $P = 0.023$ (group I vs. groups II and III). [§] $P = 0.032$ (group I vs. groups II and III). *ND*, not determined.

the 28C5 ($P = 0.02$) and 18E12 ($P < 0.0001$) groups versus the control group. Similarly, the IL-6 levels were lower in both of the anti-CD14 groups as compared with the control group. The 28C5 animals had the lowest IL-6 response ($P < 0.05$) over the 24-h time course. The IL-6 levels in the 18E12 group were significantly lower at 1 h than in control animals ($P < 0.003$). IL-8

levels in both anti-CD14-treated groups were significantly lower at both 1 and 2 h versus control animals ($P < 0.03$ and $P < 0.007$, respectively).

Thus, the anti-CD14 pretreatment resulted in significant overall reductions in the circulating levels of four different important proinflammatory cytokines that are produced in re-

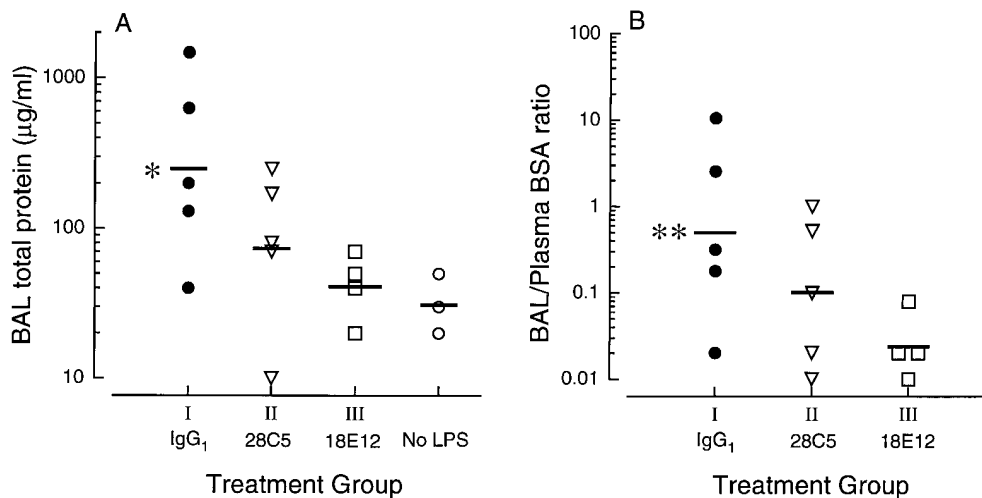


Figure 3. BAL total protein (A) and BAL/plasma ratios (B) in animals pretreated with mAb followed by LPS infusion (group I = isotype control; group II = 28C5; group III = 18E12). Animals were treated with 0.5 grams/kg sterile, pyrogen-free BSA by intravenous injection 1 h before the end of the experiment. Shown are the data points for individual animals and the geometric mean for each group. Statistical comparisons were made on log₁₀ transformed data using Student's unpaired *t* test. * $P < 0.021$ for group I versus groups II and III. ** $P < 0.032$ for group I versus groups II and III.

sponse to LPS. The anti-CD14 18E12-treated animals had the lowest TNF- α levels, whereas the 28C5-treated animals had the lowest levels of IL-1 β , IL-6, and IL-8.

The ability of anti-CD14 mAbs to prevent tissue damage induced by endotoxin was assessed by measuring changes in BAL total protein, and pulmonary permeability to two different exogenous proteins, BSA administered 1 h before the end of the experiment, and the murine IgG antibodies administered before the LPS treatment. BAL total protein, BSA, and lavage/plasma BSA ratios are shown in Table II and Fig. 3. The LPS treatment increased BAL total protein in four of five animals, although there was variability among the individual animals. The two treated groups of animals had significantly lower total protein concentrations and BSA levels than the control group ($P = 0.028$ and $P = 0.023$, respectively). In addition, the BAL:plasma BSA ratio was significantly lower in the anti-CD14-treated animals than in the control animals (Fig. 3 B). Similar differences for BAL murine IgG were found, with lower concentrations of murine IgG in BAL of the treated versus the control animals. No consistent changes in BAL leukocytes were noted.

Discussion

The major goal of these studies was to determine whether inhibition of the CD14 pathway would be protective in an animal model that simulates many of the effects of gram-negative sepsis in humans. The results indicate that inhibition of the CD14 pathway by two functionally different mAbs to CD14 protects primates from most of the physiologic and proinflammatory consequences of endotoxin shock. Pretreatment with the anti-CD14 mAb 28C5, which inhibits interactions between LPS-LBP complexes and membrane CD14, protected against severe, sustained hypotension and reduced cytokine release during prolonged infusion of LPS. Animals pretreated with anti-CD14 mAb 18E12, which blocks signaling events without affecting the binding of LPS to CD14, experienced an early drop in blood pressure but were protected from the sustained hypotension that occurred in the control animals (Fig. 1 A).

Each of the mAbs also reduced plasma levels of four different major proinflammatory cytokines, TNF- α , IL-1 β , IL-6, and IL-8, confirming that a strategy directed at the most proximal interactions of LPS with cell membranes has a broad inhibitory effect on the resulting inflammatory response in vivo. The 18E12-treated animals had the lowest TNF- α responses and the least change in protein permeability in the lungs. The sample size in this study was sufficient to show significant beneficial effects on hemodynamics, cytokine release, and lung protein permeability, but a larger number of animals would be needed to fully evaluate other clinical consequences after LPS infusion.

This model of sustained LPS infusion simulates many aspects of septic shock in humans, including fever, severe leukopenia, hypotension, and organ damage that included changes in lung epithelial and endothelial permeability (2, 18). The animals were pretreated with IFN- γ to test the ability of the anti-CD14 antibodies to prevent the most severe effects of LPS. IFN- γ has been found to enhance the sensitivity to LPS in vitro (12) and in vivo (13), and also to gram-negative bacteria (14). Mice deficient in IFN- γ receptors are insensitive to the effects of LPS (19). Our observation that IFN- γ enhanced

LPS-dependent TNF- α production in cynomolgus blood ex vivo provided relevance for IFN- γ in this experimental primate model. The sevenfold rise in circulating LBP levels during pretreatment indicated that the IFN- γ had a systemic effect in the animals, including the induction of acute-phase response proteins produced by the liver. Although the mechanism by which IFN- γ enhances LPS effects is not clear from existing studies, the present data suggest that CD14-dependent pathways are involved in an important way.

Sustained LPS infusion also was associated with increased lung endothelial and epithelial permeability, indicated by an increase in BAL total protein and the movement of the BSA tracer (67,000 mol wt) and murine IgG (150,000 mol wt) into the BAL fluid of the control animals. Changes in lung epithelial permeability are characteristic of ARDS (20) and reflect loss of the normal sieving properties of the alveolar epithelium after damage to type I and type II pneumocytes (21). The anti-CD14 treatment significantly reduced the lung permeability changes, as reflected by lower BAL total protein, BSA, murine IgG, and BAL:plasma ratios of BSA and murine IgG. In contrast to the changes in BAL proteins, the LPS infusions did not cause consistent increases in BAL neutrophils. This is similar to the effects of LPS infusion in humans (22), but differs from findings in the lungs of humans with ARDS, in which intraalveolar PMN are a consistent finding (23, 24).

Endotoxemia occurs in many patients with septic shock, and patients with circulating LPS have more severe physiologic changes and a higher mortality (25). Endotoxemia is associated with organ damage, including ARDS (1, 26). Infusion of LPS in humans induces hemodynamic changes and cytokine responses that are similar to the responses that we found in the primates (17, 27–29), providing further support for the similarities between this animal model and endotoxemia in humans. Endotoxin is not present in all patients, however, and as many as 50% of patients with clinically defined sepsis have gram-positive or other infections (2, 30). Recent evidence suggesting that CD14-dependent pathways are involved in some responses to gram-positive organisms raises the possibility that these results may be relevant to host responses to other bacterial products that circulate in humans with sepsis, in addition to LPS (2, 31, 32).

The results described here demonstrate for the first time the significance of blocking the LPS/LBP/CD14 pathway in an in vivo model of endotoxin shock that is similar in many ways to the events that occur in humans with septic shock. The results confirm that blocking very proximal interactions between LPS and membrane receptors on the surface of myeloid cells has profound effects on physiologic responses and the production of at least four different important proinflammatory cytokines. This strategy has the advantage of minimizing the complex proinflammatory cytokine cascades that are triggered by LPS, in contrast to strategies aimed at single points in complex inflammatory networks. Overall, the findings suggest a new approach to developing therapeutics that may ameliorate the deleterious effects of gram-negative sepsis.

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