ASSOCIATION BETWEEN PROTECTIVE EFFICACY OF ANTI-LIPOPOLYSACCHARIDE (LPS) ANTIBODIES AND SUPPRESSION OF LPS-INDUCED TUMOR NECROSIS FACTOR α AND INTERLEUKIN 6

Comparison of O Side Chain-specific Antibodies with Core LPS Antibodies

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The core region of LPS has a relatively similar structure in various Gram-negative bacteria, whereas the outermost O side chains are responsible for the marked antigenic diversity among Gram-negative bacteria. O side chain-specific antibodies protect against homologous strains but not against heterologous strains in animal models. The core region of LPS is exposed at the surface of the O side chain-lacking rough mutants, among which *Escherichia coli* J5 and *Salmonella minnesota* R595 have been the most studied. After immunization with such mutants, antisera of rabbits or humans contain antibodies directed against core LPS. These antisera have been reported to protect against challenge with heterologous Gram-negative bacteria or smooth LPS (1-5). Recent publications of crossprotection afforded by core LPS mAbs seemed to support the concept that core LPS antibodies might have a role in the management of Gram-negative infections. The crossprotection reported against cutaneous Shwartzman reactions in rabbits or against Gram-negative bacterial peritonitis in mice with an anti-lipid A human IgM mAb HA-1A (6) was so impressive that a major multicenter clinical study has been undertaken in patients with severe Gram-negative bacteremia. The mechanisms of the postulated protection afforded by core LPS antibodies remain, however, largely unknown. Whereas O side chain-specific antibodies have been shown to increase the serum bactericidal activity against homologous Gram-negative bacteria and to increase the intravascular clearance of homologous bacteria or LPS (7, 8), core LPS antibodies did not increase significantly

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the serum bacterial activity against smooth Gram-negative bacteria and the intravascular clearance of smooth bacteria or LPS (8).

It is now recognized that the biological effects of LPS are mainly mediated by interactions with the immune system. TNF-α is released by LPS-stimulated macrophages, and, when secreted in high amounts, has been shown to be a pivotal mediator of LPS-induced toxicity (9). IL-6 is another pleiotropic cytokine that appears to be a key member of the cytokine network involved in the host responses to infection (10). In the present study, in order to investigate the mechanisms of protection afforded by O side chains or core LPS antibodies, we determined whether these antibodies would interfere with LPS-induced serum TNF or serum IL-6 production in mice, and whether serum TNF or IL-6 levels would correlate with the protective efficacy of these antibodies. We also reassessed the potential for crossprotection afforded by polyclonal anti-*E. coli* J5 rabbit antiserum or by the anti-lipid A HA-1A mAb against heterologous Gram-negative bacteria or LPS, using O side chain-specific rabbit antisera as controls.

Materials and Methods

Bacteria and LPS. The bacteria *E. coli* O111:B4, its J5 mutant, and *Pseudomonas aeruginosa* 3 have been kindly provided by E. J. Ziegler (University of California Medical Center, San Diego, CA). LPS from *P. aeruginosa* 3 was extracted in our laboratory with the hot phenol water method. O111 LPS and J5 LPS were commercially acquired (Sigma Chemical Co., St. Louis, MO; and Ribi ImmunoChem Research, Inc., Hamilton, MT).

Antisera. Female 2-3-kg New Zealand albino rabbits (Madörin, Füllinsdorf, Switzerland) were vaccinated with stationary phase bacterial cells (*E. coli*, J5, *E. coli* O111, or *P. aeruginosa* 3) heated to 100°C for 150 min and resuspended to a density of 5×10^9 bacteria/ml in normal saline. Groups of five rabbits were injected with 1 ml of bacterial vaccines intravenously three times weekly for 2 wk, and blood was collected 7 d after the last injection with aseptic techniques. Sera were pooled, heated at 56°C for 30 min, and kept frozen at −20°C until used.

mAb. HA-1A is a human IgM mAb resulting from a fusion of heteromyeloma cells with B lymphocytes from the spleen of a patient with Hodgkins disease immunized with *E. coli* J5. Purified HA-1A was provided by Merieux Institute (Lyon, France) and was prepared from the clone originally isolated at Stanford University (6). LPS was not detectable with a chromogenic Limulus assay (KabiVitrum, Stockholm, Sweden), which had a sensitivity of 12.5 pg LPS/ml.

Bioassay for TNF Serum Levels. A highly sensitive mouse fibrosarcoma cell line, WEHI 164 clone 13, a generous gift from J. Tschopp (Institute of Biochemistry, Lausanne, Switzerland), was used to measure serum TNF levels, as originally described by Espevik and Nissen-Meyer (11). Twofold serial dilutions of mouse serum from 1:100 to 1:12,800 were tested in duplicates, and the amount of TNF was extrapolated from a standard curve using mouse rTNF kindly provided by B. Allet (Glaxo IMB, Carouge, Switzerland). The LD_{50} of clone 13 cells was between 1 and 3 pg mouse rTNF/ml. The specificity of the test was confirmed by neutralization with a rabbit anti-mouse rTNF polyclonal antibody.

Bioassay for IL-6 Serum Levels. IL-6 was measured using IL-6-dependent mouse-mouse hybridoma cells 7TD1, kindly provided by J. Van Snick (Ludwig Institute, Brussels, Belgium), according to his method (12). The limit of detection was <1 pg mouse rIL-6/ml.

Endotoxemic Lethality in Mice. Female 20-25-g C57BL/6 mice (Madörin) were acclimatized for 3-5 d before each experiment. 0.5 ml rabbit antisera, 100 μg HA-1A in 0.2 ml 1% human albumin, or 0.2 ml of the vehicle albumin was injected in the tail vein 1 h before subsequent intraperitoneal challenge with various doses of O111 LPS, J5 LPS, or *P. aeruginosa* 3 LPS in 0.5 ml PBS containing 15 mg galactosamine. Groups of 8-10 mice were used to determine the LD_{50}. For each experiment, groups of four mice without LPS challenge were

included. Lethality was monitored until 96 h. Blood was collected in the retro-orbital plexus 1 and 3 h after the intraperitoneal challenge to measure serum TNF and IL-6 levels, respectively.

Mucin-hemoglobin Peritonitis Model in Mice. The procedure was similar to that of the endotoxemic lethality model, except that female 20–25-g OF1 mice (Madörin) were used and that a suspension of bacteria (*E. coli* O111 or *P. aeruginosa* 3) with mucin (Sigma Chemical Co.) and hemoglobin (BBL Microbiology Systems, Cockeysville, MD) was injected intraperitoneally instead of LPS. The suspension of 15% mucin and 4% hemoglobin in PBS was prepared in advance, autoclaved, homogenized by sonication, and kept at −20°C in aliquots.

Cutaneous Shwartzman Reactions in Rabbits. Groups of 10 female 1.5–1.8-kg New Zealand rabbits (Madörin) were shaved along the side and submitted to a preparative intradermal injection of 50 μg O111 LPS. 22 h later, 10 ml rabbit antisera or 1 mg HA-1A diluted in 1 ml of 1% human albumin, or 1 ml vehicle albumin alone was injected intravenously. 2 h later, rabbits were submitted to a provocative intravenous injection of 12.5 μg O111 LPS. The presence of hemorragic necrosis at the site of intradermal injection was assessed after 24 h. The preparative and provocative doses of LPS were determined as the lowest doses still inducing a cutaneous reaction in ~80% of untreated rabbits.

Statistical Methods. The numbers of deaths or cutaneous Schwartzman reactions in experimental groups were compared with two-tailed χ^2 tests or Fisher exact tests when appropriate. Intergroups differences in TNF or IL-6 serum levels were compared with the nonparametric Kruskall-Wallis test.

Results

Mortality, Serum TNF, and Serum IL-6 Levels in Mice after Challenge with LPS. In C57BL/6J galactosamine-sensitized mice, the LD_{50} is very low for J5 LPS or O111 LPS, thus providing the possibility to administer antibody in large molar excess over LPS. Anti-J5 rabbit antiserum (J5RS)[1] protected against J5 LPS challenge. However, neither J5RS nor HA-1A increased the LD_{50} of O111 LPS, whereas anti-O111 rabbit antiserum (O111RS) afforded a very significant protection (Table I). When mice were challenged with 1.5 LD_{50} of *P. aeruginosa* 3 LPS (100 ng/mouse), the numbers of survivors were 1 of 10 in the normal rabbit serum (NRS) group, 0 of 10 in the J5RS group, and 0 of 10 in the HA-1A group. In contrast, 10 of 10 mice in the *P. aeruginosa* 3 rabbit antiserum group survived ($p < 10^{-5}$).

In another experiment (Fig. 1), serum TNF levels were measured 1 h after challenge with 50 ng O111 LPS (~10 LD_{50}). Levels were undetectable in the 10 mice pretreated with O111RS, including the two mice that died in this group. In the NRS-, J5RS-, HA-1A-, or albumin-recipient mice, TNF levels ranging from 0.2 to 8.3 ng/ml were detectable in all animals, except in one NRS-recipient mouse, which was the only survivor from these four groups. No significant difference in TNF levels between these four nonprotected groups was observed, whereas the difference between these groups and the O111RS group was highly significant ($p < 10^{-5}$).

Similar findings were observed with serum IL-6 levels measured 3 h after challenge in the same experiment. Levels were undectable (<0.1 ng/ml) in the 10 mice that received O111RS. In all the other mice, levels were detectable: the median (range) was 2.8 ng/ml (0.4–6.4) in the NRS group, 2.3 (0.8–5.6) in the J5RS group, 1.7 (0.8–4.8) in the albumin group, and 1.7 (0.8–3.2) in the HA-1A group. The lowest value (0.4 ng/ml) was found in the only surviving mouse from the NRS group. There was no

[1] *Abbreviations used in this paper:* J5RS, anti-J5 rabbit antiserum; NRS, normal rabbit serum; O111RS, anti-O111 rabbit antiserum.

TABLE I

Protection against LPS Afforded in Galactosamine-sensitized C57BL/6J Mice by Rabbit Antisera or by the Human IgM mAb HA-1A

Sera or mAb	LD_{50} after LPS challenge	
	J5 LPS	O111 LPS
	ng LPS/mouse	
Nonimmune rabbit	2	4
Anti-J5 rabbit	214*	20
Anti-O111 rabbit	-	500*
Human albumin	-	4
HA-1A‡	-	2

* $p < 0.01$; the other comparisons between groups were not significant.
‡ Anti-lipid A human IgM mAb (6).

significant difference between the four nonprotected groups, whereas the difference between these groups and the O111RS group was highly significant ($p < 10^{-4}$).

Experiments of Protection In Mucin-hemoglobin Peritonitis in Mice. Since we found that J5RS and HA-1A did not improve the survival of mice challenged with purified LPS, we investigated whether these preparations would be more effective against challenge with living bacteria. In the peritonitis model, neither J5RS nor HA-1A increased the LD_{50} of *E. coli* O111 or *P. aeruginosa* 3 (a strain with a very low LD_{50}), whereas homologous rabbit antisera afforded a very significant protection (Table II).

Cutaneous Shwartzman Reactions in Rabbits after Challenge with O111 LPS. We extended our investigations to the Shwartzman reaction in rabbits because some of the original experiments of protection with J5RS and HA-1A have been performed using this model. We found that the number of rabbits with a cutaneous hemorragic necrosis was 8 of 10 after NRS administration, 8 of 10 after J5RS, 8 of 10 after albumin, and 6 of 8 after HA-1A (2 of 10 rabbits in this group died before 24 h). The hemorragic necrosis were slightly weaker in the J5RS group compared with the NRS, HA-1A, or albumin groups. In contrast, only 4 of 10 rabbits after O111RS showed a cutaneous reaction ($p = 0.08$).

Discussion

Although LPS can activate plasmatic mediators such as complement and contact (Hageman) factors, there is increasing evidence that many of the toxic effects of LPS

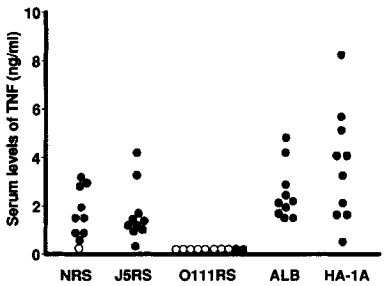

FIGURE 1. Serum TNF levels 1 h after intraperitoneal challenge of galactosamine-sensitized C57BL/6J mice with 50 ng O111 LPS/mouse after prophylaxis with rabbit antisera or HA-1A mAb. Groups of 10 mice were pretreated 1 h before LPS challenge with 0.5 ml NRS, 0.5 ml J5RS, 0.5 ml O111RS, 0.2 ml of 1% human albumin (ALB), or 100 μg human IgM mAb HA-1A diluted in 0.2 ml of 1% ALB per mouse. The open circles represents survivors, and the close circles represent nonsurvivors. The limit of detection of TNF in serum was 0.1 ng TNF/ml.

TABLE II

Protection against Gram-negative Bacteria Afforded in OF1 mice by Rabbit Antisera or by the Human IgM mAb HA-1A

Sera or mAb	LD_{50} after bacterial challenge	
	E. coli O111	*P. aeruginosa* 3
	CFU/mouse	
Nonimmune rabbit	1.7×10^5	5
Anti-J5 rabbit	1.3×10^5	7
Anti-O111 rabbit	$>4.5 \times 10^6$*	-
Anti-*P. aeruginosa* 3	-	>500*
Human albumin	4×10^4	<5
HA-1A‡	9×10^4	<5

* $p < 0.01$; the other comparisons between groups were not significant.
‡ Anti-lipid A human IgM mAb (6)

are mediated through activation of target cells in the immune system, especially of the macrophage (9). With regard to the pathophysiology of septic shock, several lines of evidence suggest that TNF is among the most important LPS-induced mediators. First, the injection of high doses of TNF to rats (13), mice (14), or rabbits (15) reproduced the picture of septic shock. Second, the lethality of experimental Gram-negative bacteremia or endotoxemia was prevented by the administration of anti-TNF antibodies (15–17). Last, high serum TNF levels correlated with the mortality of patients with meningococcemia (18) or septic shock (19). Therefore, an important mechanism of protection of anti-LPS antibodies in vivo could be to prevent the increased TNF serum levels due to the triggering of target cells by LPS. In the present study, we found that O side chain-specific rabbit antiserum markedly suppressed the serum TNF induced by homologous LPS challenge and protected mice from death. Thus, the suppression of LPS-induced serum TNF after administration of type-specific antibodies might be an important mechanism leading to increased survival. In contrast, the two preparations of core LPS antibodies, rabbit J5 antiserum and the anti-lipid A HA-1A mAb, had no detectable impact on serum TNF levels and showed no protection compared with controls.

Another cytokine that may play an important role in the defense against septic shock is IL-6. IL-6 expression is induced by LPS, by TNF, or by IL-1 in many cell types such as fibroblasts, endothelial cells, and macrophages. Among many biologic activities, IL-6 expands the production of hematopoietic cells, enhances the production of hepatic acute phase proteins, and induces an elevation in body temperature (10). In man, injection of LPS (20) or TNF (21) elicits increased circulating IL-6. High serum levels of IL-6 have been associated with death in patients with meningococcemia (22) or Gram-negative septic shock (23). However, this correlation may not represent a causal relationship between IL-6 and mortality, particularly in view of the fact that, in contrast to TNF, the administration of high doses of IL-6 to animals is apparently not deleterious (W. Fiers, personal communication). In the present study, we found that LPS-induced IL-6 production in mice was suppressed by O side chain-specific antibodies concomitantly with TNF production, while core LPS antibodies had no effect. The observed reduction of LPS-induced IL-6 production

may have been either a consequence of the TNF reduction, since IL-6 can be induced by TNF, or, alternatively, a direct effect of antibodies on the stimulation of the IL-6 secretion by LPS.

There are at least two possible mechanisms for the in vivo reduction of LPS-induced TNF or IL-6 production by anti-LPS antibodies: opsonization or neutralization. By opsonizing LPS or whole bacteria, antibodies might prevent LPS from reaching sensitive target cells. Alternatively, by binding to LPS, antibodies may directly neutralize the biologically active part of the molecule. Since the protection reported by others with antisera to rough mutants could not be attributed to opsonization (8), it was postulated that core LPS antibodies might neutralize LPS. However, recent experiments in vitro using O side chain-specific and core LPS mAbs failed to confirm this hypothesis (24), thus raising doubts about the existence of true LPS-neutralizing antibodies. In view of the absence of neutralization of LPS demonstrable in vitro (24), one might hypothesize that the reduction of TNF and IL-6 production after challenge of mice with LPS in the present experiments might have been due to opsonization of LPS by O side chain-specific antibodies. Such an hypothesis would need confirmation.

Our data do not support a role for core LPS antibodies in the crossprotection against Gram negative bacteria or LPS. Indeed, neither the polyclonal J5 rabbit antiserum nor the human monoclonal IgM HA-1A protected against challenge with the LPS from *E. coli* O111 or *P. aeruginosa* 3 in galactosamine-sensitized mice, against lethal peritonitis induced by these strains in mice, or against O111 LPS challenge in the cutaneous Shwartzman reaction in rabbits. Moreover, neither J5 rabbit antiserum nor the HA-1A mAb prevented the production of TNF or IL-6 in the serum of mice challenged with LPS, suggesting that these preparations do not have the ability to prevent the interactions between LPS and target cells in vivo. While these findings contrast with data previously reported using very similar models (6), they are in accordance with the results found by others with core LPS antisera (25). Therefore, there are several reasons to fear that the protection sometimes reported with core LPS antisera or mAbs might not be real: the precise epitopes of the LPS molecule responsible for the postulated crossreactions remain unknown, the mechanisms of the postulated crossprotection remain enigmatic, and the experiments of protection give discrepant results. The existence or not of a crossprotection afforded by core LPS antibodies is a crucial issue because major multicenter clinical trials with core LPS mAbs are presently being performed. Should these trials fail to demonstrate a clear-cut protection, in the light of the present study, the whole concept should seriously be questioned.

Summary

Two-core LPS antibodies, the rabbit J5 polyclonal antiserum and the human anti-lipid A IgM mAb HA-1A, did not improve the survival of mice challenged with *E. coli* O111 or *P. aeruginosa* 3, or with the LPS extracted from them, and did not decrease the incidence of Shwartzman reactions in rabbits challenged with O111 LPS. In contrast, O side chain-specific rabbit antisera were protective in these models. The protection afforded by O side chain-specific antisera against endotoxin lethality was associated with decreased LPS-induced serum TNF and IL-6 levels, whereas core LPS antibodies had no effect on TNF or IL-6 levels. The absence of reduction

of LPS-induced cytokines levels by core LPS antibodies suggests that these antibodies are not able to prevent the interactions between LPS and target cells.

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