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Role of Interleukin-1 in Augmenting Serum Neutralization of Bacterial Lipopolysaccharide

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We have previously described an assay to quantify the serum neutralization of bacterial lipopolysaccharide which is based on a spectrophotometric *Limulus* amoebocyte lysate test (T. J. Novitsky, P. F. Roslansky, G. R. Siber, and H. S. Warren, J. Clin. Microbiol. 21:211–216, 1985). Studies since have shown that serum samples drawn from patients with leukemia and fever, gram-negative or gram-positive bacterial infections, or shock caused by gram-negative bacteria neutralize approximately 10-fold more lipopolysaccharide than do samples from normal controls. These findings suggested that the increased neutralization might reflect an acute-phase response and raised the question of whether it might be under the control of interleukin-1. To answer this question, we studied the neutralization of lipopolysaccharide in serum samples drawn from rabbits before and after the administration of crude interleukin-1, prepared from activated macrophage supernatants, and recombinant human interleukin-1. Crude interleukin-1 induced a 5.7-fold increase in serum neutralization 24 h after intravenous injection, and cloned interleukin-1 induced a 3.0-fold increase ($P \leq 0.01$ and 0.05, respectively). In individual rabbits given identical doses of crude interleukin-1 on a weight basis, the serum-neutralizing ability correlated significantly with three activities of interleukin-1: rise in temperature ($r^2 = 0.558$; $P \leq 0.01$), decrease in serum iron ($r^2 = 0.534$; $P \leq 0.01$), and increase in serum copper ($r^2 = 0.323$; $P \leq 0.05$). We conclude that the increase in neutralization of bacterial lipopolysaccharide by serum samples drawn from patients with inflammatory states is mediated, at least in part, by interleukin-1, presumably through the induction of acute-phase serum proteins.

For the last 50 years there has been interest in host mechanisms of protection against the toxic effects of bacterial lipopolysaccharide (LPS). Early studies used LPS-induced fever as a convenient biological assay, and it was reported that rabbits rapidly became tolerant to LPS. Careful studies revealed that there are two phases of pyrogenic tolerance to LPS, an early nonspecific phase that is present from 8 h to 6 days and a late phase that is specific to the LPS strain and coincides with the appearance of antibody directed to the polysaccharide moiety of LPS (5). Passive transfer of tolerance by large quantities of serum in the absence of anti-LPS antibodies has also been reported (4, 6, 15), suggesting that a factor exists in early-phase tolerant serum capable of augmenting the neutralization of heterologous LPS. The elucidation and control of the mechanisms involved in this phenomenon are of practical interest because they could perhaps be manipulated for prophylactic or therapeutic advantage.

We have recently described a sensitive and quantitative assay for measuring the serum neutralization of LPS (8). The assay is based on the ability of serum to inhibit the effects of LPS on *Limulus* amoebocyte lysate (LAL). Whereas earlier studies reported that serum contained inhibitors of the lysate itself, we have shown that the inhibition reflects the neutralization of LPS by serum, thus permitting its use as a method for quantitating LPS detoxification (14).

Using this assay, we have found that all rabbit sera neutralize LPS but that serum samples drawn from rabbits 24 h after the induction of tolerance neutralize increased amounts of homologous and heterologous LPS (13a). Another study demonstrated that human serum samples from patients with leukemia and fever or clinically diagnosed shock caused by gram-negative bacteria neutralize more LPS than do samples from normal control patients or patients with cardiogenic shock (14a). These findings suggested to us that the augmented detoxification of LPS by these sera may represent an acute-phase response by the host. Many acute-phase factors, including serum amyloid A, C-reactive protein, and fibrinogen are regulated, at least in part, by interleukin-1 (IL-1) (1). We therefore studied the effect of intravenous administration of IL-1 on the ability of serum to neutralize LPS, as measured by the *Limulus* assay. We report here that activated macrophage supernatants containing IL-1 and human recombinant IL-1 (rHuIL-1) administered to rabbits induced increased serum neutralization of LPS.

MATERIALS AND METHODS

Preparation of cells. Rabbit peritoneal exudates were induced by an intraperitoneal injection of 50 ml of sterile mineral oil. The rabbits were sacrificed 72 h later by administration of an intravenous overdose of pentobarbital, and peritoneal exudate cells (PEC) were harvested from the peritoneal cavity by using 300 ml of heparinized (5 U/ml) pyrogen-free saline. The PEC were washed twice by centrifugation and suspended at a density of 10^7 PEC per ml in

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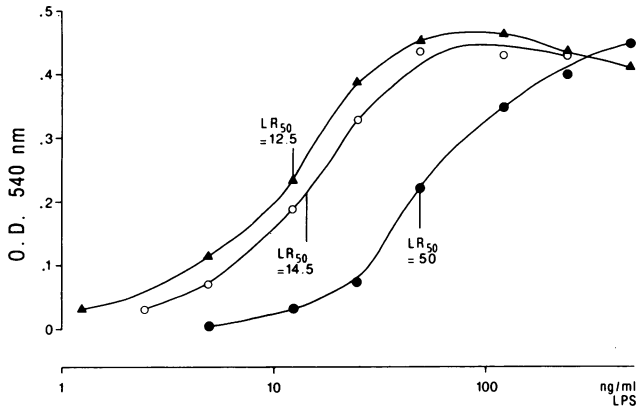

FIG. 1. Sample curve illustrating method of calculation of LR_{50} (also see reference 8). Serum samples drawn at different times after injection of crude IL-1 were incubated with various concentrations of LPS for 3 h at 37°C. This solution was then combined with reconstituted LAL and incubated for another 60 min. The LR_{50} was determined by calculating the concentration of LPS (in nanograms per milliliter) that elicited 50% maximal optical density (OD) at 540 nm. Serum samples from the same rabbit were always studied in parallel on the same microtiter plate. Three serum samples from a representative rabbit were drawn: preinjection serum (▲), serum drawn 8 h after injection of crude IL-1 (○), and serum drawn 24 h after injection of crude IL-1 (●).

RPMI 1640 medium (Seromed, Munich, Federal Republic of Germany) supplemented with antibiotics (penicillin-streptomycin solution) and 0.01 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (pH 7.1 to 7.3). The cell population contained at least 90% macrophages, as estimated by nonspecific esterase staining.

Preparation of supernatant fluids. PEC were incubated (15 ml) in 75-cm^2 tissue culture flasks (Falcon 3024; Becton Dickinson Labware, Grenoble, France) for 18 h at 39°C in an incubator containing 7% CO_2. Cultures were incubated with or without muramyl dipeptide (MDP) at a concentration of 100 μg/ml, added to stimulate the macrophages to produce IL-1 (9). The cells and medium were then centrifuged at 1,000 × *g* for 25 min, and the cell-free supernatants were dialyzed overnight against 200 volumes of pyrogen-free saline to remove the MDP. The supernatants were stored at −20°C until use. All supernatants were shown to have <1.0 ng of LPS per ml by the *Limulus* lysate clot test (Associates of Cape Cod, Inc., Falmouth, Mass.).

rHuIL-1. The preparation of rHuIL-1 has been previously described (2). It is 223 amino acids long and has a molecular weight of 24,556. The rHuIL-1-producing bacteria were grown at 37°C and lysed, and the rHuIL-1 was extracted from the insoluble cellular fraction with 8 M urea. It was then purified by sequential ion-exchange chromatography and either gel-filtration chromatography or high-pressure liquid chromatography and stored at −70°C in 0.1 M phosphate buffer (pH 6.8) containing 10 mM 2-mercaptoethanol and 10 mM dithiothreitrol. The identity of the purified rHuIL-1 was confirmed by amino acid composition and the sequence of the terminal heptapeptide. Protein concentration was determined by the Bradford method with a bovine serum albumin standard. The preparation was estimated to be greater than 99% pure, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie blue or silver staining. The endotoxin concentration of the homogeneous rHuIL-1 was determined to be less than 20 pg/μg of IL-1 protein by the *Limulus* amoebocyte clot lysate test (Associates of Cape Cod).

Rabbit pyrogen assay. Male New Zealand White rabbits (2.5 to 3.5 kg) were used throughout the study. Glassware, needles, syringes, and phosphate-buffered saline (Seromed) were pyrogen free. Rectal temperature was recorded every 3 min for 5 h after intravenous injection with thermistor probes connected to a telethermometer interfaced to an HP85 computer (Hewlett-Packard Co., Palo Alto, Calif.). Changes in temperature are expressed as maximum deviation from the base line recorded at the time of injection.

Determination of iron and copper concentrations in sera. Blood samples were collected in iron- and copper-free tubes from the median ear artery of the same groups of rabbits used for fever determinations. Serum samples were prepared from blood obtained 24 h before and 8, 24, and 48 h after injection and frozen at −80°C until testing. The serum (2 ml) was mixed with 1 ml of HCl solution (1 N) and left standing for 20 min at 20°C. Trichloroacetic acid (1 ml; 1.23 M) was then added for deproteinization. Total iron and copper concentrations were then determined by colorimetric micromethods adapted to a test combination (Boehringer GmbH, Mannheim, Federal Republic of Germany). The results are expressed as the percent change in metal concentration.

LAL assay. The assay for LPS neutralization has been described (8). Serum specimens were prepared from blood samples collected in pyrogen-free tubes from the median ear artery and were frozen at −80°C until use. Serum (50 μl) diuted 1:1 in normal saline (0.9% sodium chloride for injection, USP; Abbott Laboratories, North Chicago, Ill.) was added to 50 μl of serial twofold diutions of *Escherichia coli* O113 LPS in normal saline in 96-well microtiter plates (Flow Laboratories, Inc., McLean, Va.). After incubation of the plates for 3 h at 37°C, 100 μl of reconstituted LAL (spectrophotometric lot 99-01-347; Associates of Cape Cod) was added and mixed. After further incubation at 37°C for 1 h, the plates were read on a microplate reader (Flow) at 380 nm. These results were plotted, and the concentration of LPS needed to produce a 50% *Limulus* gelation response (LR_{50}) was calculated. In each assay, preimmune serum from the same rabbit was included for comparison. All assays were done at least in duplicate, and means were used for calculations. A sample curve illustrating the method of plotting is shown in Fig. 1. The increase (fold) in the LR_{50} at 8 and 24 h after injection of crude IL-1 or rHuIL-1 was determined by the formula: mean LR_{50} in serum after injection/mean LR_{50} in serum before injection.

Statistical analysis. The statistical test used for the results presented in Fig. 2 was the Mann-Whitney U test. Student's *t* test was used for the results shown in Table 1 (two tailed). The maximal change in temperature, decrease in iron at 8 h, and increase in copper at 24 h were correlated with the increase (fold) in the LR_{50} at 24 h after injection (LAL assay). The positive correlation between each physiological event and the LR_{50} was evaluated, and the strength of correlation (r^2) was calculated. The significance (*t*) was determined, and the probability (*P*) was estimated with (*n*-2) df.

RESULTS

Effect of crude IL-1 and rHuIL-1 on fever and metal concentrations in serum. MDP-stimulated macrophage supernatants (referred to as crude IL-1) and rHuIL-1 were first assessed for their pyrogenic activity and the ability to alter

iron and copper concentrations in serum (10). As expected, both crude IL-1 and rHuIL-1 were pyrogenic and induced a significant decrease in serum iron and increase in serum copper (Table 1).

Effect of crude IL-1 and rHuIL-1 on serum neutralizing ability. Both crude IL-1 and rHuIL-1 induced a significant increase in serum LR_{50} 8 and 24 h after intravenous injection ($P < 0.01$ for crude IL-1 and $P < 0.05$ for rHuIL-1 at each time [Mann-Whitney two-tailed test]) (Fig. 2). For each preparation the increase in the LR_{50} was significantly greater at 24 h than at 8 h ($P < 0.01$ [Mann-Whitney two-tailed test]). Crude IL-1 administered at 1.0 ml/kg appeared to be more active in augmenting the LR_{50} than was cloned IL-1 administered at 10 μg/kg. This difference in potency cannot be interpreted because there were probably numerous monokines in our crude macrophage supernatants and because the biological activity of the recombinant preparation was possibly diminished during expression, extraction, and purification.

Correlation of LPS-neutralizing activity with pyrogenicity and change in metal concentrations in serum after injection of activated macrophage supernatants. Since the rise in temperature and change in metal levels in serum varied considerably in different rabbits despite an identical dose of crude macrophage supernatant (administered on a weight basis), it was of interest to determine whether these parameters correlated with the increase in serum LR_{50}. The correlation analysis was performed as described in Materials and Methods at the time of maximal change for each parameter. The increase in LR_{50} at 24 h correlated significantly with maximal rise in temperature ($r^2 = 0.558$; $P < 0.01$; 12 df), decrease in serum iron at 8 h ($r^2 = 0.534$; $P < 0.01$; 12 df), and increase in serum copper at 24 h ($r^2 = 0.323$; $P < 0.05$; 10 df) in the 14 rabbits studied.

DISCUSSION

Our data provide direct evidence that the neutralization of LPS by serum is augmented by administration of activated macrophage supernatants and rHuIL-1, at least as measured by inhibition of LAL. Notably, the neutralization correlated with three different activities of IL-1 in rabbits given identical doses of activated macrophage supernatants. Further work will be needed to determine whether the neutralization is a direct or indirect (e.g., fever mediated) effect of IL-1. Whichever is the case, IL-1 would seem to be the perfect mediator for the serum neutralization of LPS from a teleological and homeostatic point of view. We have previously shown that LPS incubated in fresh frozen human plasma screened for high LR_{50} induces less fever in the rabbit pyrogen test than does LPS incubated in plasma screened for low LR_{50} (14). We hypothesize that LPS stimulates macrophages to produce IL-1 (and perhaps other factors such as tumor necrosis factor or hepatocyte-stimulating factor [16]), which then induces a change in serum such that it is capable of neutralizing greater amounts of LPS. Thus, there appears to be a macrophage-based mechanism for decreasing the induction of IL-1 by subsequent LPS challenge or continued LPS circulation.

TABLE 1. Effect of PEC supernatant fluid (crude IL-1) and cloned IL-1 on metal levels in serum and pyrogenic activity

Prepn (no. of rabbits)	Fever rise (°C) (mean ± SD)[a]	Decrease (%) in iron at 8 h (mean ± SD)[a]	Increase (%) in copper at 24 h (mean ± SD)[a]
Control (saline) (7)	0.09 ± 0.06	7 ± 4	10 ± 11
Crude IL-1 (1 ml/kg) (14)	0.81 ± 0.13	61 ± 11	83 ± 20
Cloned IL-1 (10 μg/kg) (5)	0.90 ± 0.19	47 ± 8	76 ± 15

[a] Data for crude and cloned IL-1 significantly different from data for control at $P \leq 0.01$ (Student's t test).

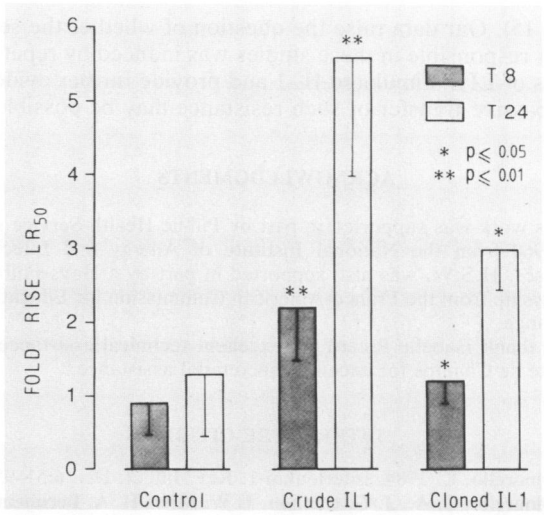

FIG. 2. Effect of crude IL-1 and rHuIL-1 on serum LR_{50}. Controls (7 rabbits) received 1.0 ml of unstimulated macrophage supernatants per kg. The crude IL-1 group (14 rabbits) received 1.0 ml of MDP-stimulated macrophage supernatants and the cloned IL-1 group (5 rabbits) received 10 μg per kg, of rHuIL-1 per kg. The effects of these preparations on fever and metal concentrations in the same rabbits are shown in Table 1. Since data reflect the fold rise, each rabbit was its own control. The calculation of fold rise and statistics are described in Materials and Methods.

The neutralization mechanism is under study. Several pieces of evidence suggest that the binding of LPS to serum lipoproteins may be important. Procedures that remove lipoproteins from serum, such as chloroform extraction (3) or ultracentrifugation (H. S. Warren, unpublished data), decrease the serum neutralization of LPS in the LAL assay. Delipidation by ether extraction removes the ability of serum to neutralize LPS in the rabbit pyrogen assay, whereas reconstitution with lipoproteins restores it (13). Radiolabeled LPS binds to serum lipoproteins in a time- and temperature-dependent manner (12), and LPS bound to lipoprotein is much less active than unbound LPS in numerous assay systems, including the induction of fever (7, 12) and LAL activation (7). In addition, the rate of binding of *E. coli* O113 LPS to lipoproteins and the neutralization of LPS in the LAL assay are both increased in serum samples drawn from tolerant rabbits (13a). A different possibility is that an acute-phase protein stimulated by IL-1 is responsible for the neutralization. A 60-kilodalton glycoprotein has recently been found in acute-phase sera that binds to LPS extracted from the rough mutant *Salmonella minnesota* Re595 and delays its binding to serum lipoproteins (11). It is possible that our results reflect stimulation of this or other acute-phase reactants.

The work reported here was done with the ultimate hope that protection from LPS might be provided either by the passive transfer or active stimulation of a serum factor. Previous studies have suggested that tolerance to LPS-induced fever can be passively transferred by large amounts of serum in the absence of demonstrable anti-LPS antibody

(4, 6, 15). Our data raise the question of whether the serum factor responsible in these studies was induced by repetitive bursts of LPS-stimulated IL-1 and provide further evidence that passive transfer of such resistance may be possible.

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