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The cell wall components peptidoglycan and lipoteichoic acid from *Staphylococcus aureus* act in synergy to cause shock and multiple organ failure

(inducible nitric oxide synthase/tumor necrosis factor α/interferon γ)

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ABSTRACT **Although the incidence of Gram-positive sepsis has risen strongly, it is unclear how Gram-positive organisms (without endotoxin) initiate septic shock. We investigated whether two cell wall components from *Staphylococcus aureus*, peptidoglycan (PepG) and lipoteichoic acid (LTA), can induce the inflammatory response and multiple organ dysfunction syndrome (MODS) associated with septic shock caused by Gram-positive organisms. In cultured macrophages, LTA (10 μg/ml), but not PepG (100 μg/ml), induces the release of nitric oxide measured as nitrite. PepG, however, caused a 4-fold increase in the production of nitrite elicited by LTA. Furthermore, PepG antibodies inhibited the release of nitrite elicited by killed *S. aureus*. Administration of both PepG (10 mg/kg; i.v.) and LTA (3 mg/kg; i.v.) in anesthetized rats resulted in the release of tumor necrosis factor α and interferon γ and MODS, as indicated by a decrease in arterial oxygen pressure (lung) and an increase in plasma concentrations of bilirubin and alanine aminotransferase (liver), creatinine and urea (kidney), lipase (pancreas), and creatine kinase (heart or skeletal muscle). There was also the expression of inducible nitric oxide synthase in these organs, circulatory failure, and 50% mortality. These effects were not observed after administration of PepG or LTA alone. Even a high dose of LTA (10 mg/kg) causes only circulatory failure but no MODS. Thus, our results demonstrate that the two bacterial wall components, PepG and LTA, work together to cause systemic inflammation and multiple systems failure associated with Gram-positive organisms.**

Septic shock is now the most common cause of death in intensive care units (1). It is associated with a systemic inflammation, circulatory failure, and multiple organ dysfunction syndrome (MODS) (1, 2). Gram-negative, as well as Gram-positive, organisms can cause septic shock. The outer membrane of Gram-negative organisms contains endotoxin (lipopolysaccharide; LPS) and the release of LPS in patients elicits many of the features observed in septic shock (3, 4). In contrast, Gram-positive organisms cause septic shock without the involvement of LPS: indeed LPS is not always found in the serum of patients with sepsis (3, 5, 6). Furthermore, the incidence of sepsis resulting from Gram-positive organisms has risen in the last decade (7–9), but it is not yet clear how Gram-positive organisms cause septic shock. A minority of the Gram-positive organisms produce entero- or exotoxins—e.g., toxic shock syndrome toxin 1 or pyrogenic exotoxin A—which are able to elicit toxic shock-like syndrome in the absence of bacteremia (10). However, septic shock is also caused by Gram-positive organisms which do not produce these toxins (5, 11, 12). The cell walls of Gram-positive organisms contain various components, such as lipoteichoic acid (LTA) and peptidoglycan (PepG), which can produce an inflammatory response (6, 7, 13, 14). We have previously shown that LTA elicits tumor necrosis factor α (TNF-α) release, inducible nitric oxide synthase (iNOS) induction, and circulatory failure in anesthetized rats (15). However, in contrast to LPS, LTA or PepG does not cause lethality or organ toxicity at an immunostimulatory dose in mice or rats (16–19).

Here, we demonstrate that PepG and LTA (from *Staphylococcus aureus*) act in synergy to cause systemic release of TNF-α and interferon γ (INF-γ), induction of nitric oxide synthase (NOS), circulatory failure, MODS, and death.

MATERIALS AND METHODS

Induction of Nitric Oxide (NO) Release from Cultured Macrophages. Murine macrophages (J774.2 cell line) were cultured to confluency in 96-well plates containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal calf serum and 3.5 mM L-glutamine. Macrophages were activated with PepG (1–100 μg/ml), LTA (10 μg/ml), or PepG and LTA simultaneously. In another set of experiments, *S. aureus* (10^8 cells per ml; killed by boiling for 10 min boiling and sonicating for 1 min) or LTA (10 μg/ml) was used to activate macrophages in the absence or presence of a polyclonal antibody against PepG (0.25 mg/ml). After 24 h, nitrite accumulation in the supernatant was assayed by the Griess reaction (20).

Hemodynamic Measurements. Male Wistar rats (200–300 g) were anesthetized with thiopentone sodium (Rhône Mérieux, Harlow, U.K.; 120 mg/kg, i.p.). The trachea was cannulated to facilitate respiration, and rectal temperature was maintained at 37°C. The right carotid artery was cannulated for the measurement of mean arterial blood pressure (MAP) and heart rate; the left jugular vein was cannulated for the administration of compounds. Cardiovascular parameters were allowed to stabilize for at least 20 min. At time 0, animals received (*i*) PepG (10 mg/kg, i.v.), (*ii*) LTA (3 mg/kg, i.v.), (*iii*) PepG and LTA, or (*iv*) vehicle (0.45 ml of 0.9% NaCl). PepG was administered over 60 min, and care was taken to maintain MAP above 80 mmHg (1 mmHg = 133 Pa) during injection. The pressor response to noradrenaline (1 μg/kg, i.v.) was determined 15 min before and every 60 min after treatment started. In some experiments, carotid artery blood was obtained at 45, 90, 135, 180, 240, 300, and 360 min for pH and blood gas analysis and measurement of plasma TNF-α and IFN-γ concentration. At the end of the experiment, a blood

Abbreviations: ALT, alanine aminotransferase; CK, creatine kinase; IFN-γ, interferon γ; iNOS, inducible nitric oxide synthase; LTA, lipoteichoic acid; MAP, mean arterial pressure; NO, nitric oxide; NS, not significant; Pa_{CO_2}, partial arterial carbon dioxide pressure; Pa_{O_2}, partial arterial oxygen pressure; PepG, peptidoglycan; TNF-α, tumor necrosis factor α; MODS, multiple organ dysfunction syndrome; LPS, lipopolysaccharide; iu, international unit(s); IL-*n*, interleukin *n*.

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sample from the carotid artery was obtained for the measurement of plasma markers for organ failure. Plasma concentrations of bilirubin (liver function), creatinine and urea (glomerular filtration and tubular excretion, respectively, for kidney function), and the intracellular marker enzymes (21), alanine aminotransferase (ALT; liver), lipase (pancreas), and creatine kinase (CK; cardiac and skeletal muscle and brain) were analyzed at a laboratory for veterinary clinical chemistry (Vetlab Services, Sussex, U.K.). Furthermore, part of the liver and lungs, kidneys, pancreas, heart, aorta, and mesentery were removed, quick frozen in liquid nitrogen, and stored at −80°C for the measurement of iNOS protein expression and activity (see below).

Serum TNF-α and IFN-γ Levels. TNF-α levels were measured in the serum by using a mouse TNF-α ELISA kit (Genzyme) which has also been used successfully to quantitate natural rat TNF-α (15). IFN-γ levels were measured in the serum by using a rat IFN-γ ELISA kit from Biosource International (Camarillo, CA).

iNOS-Activity Assay. Frozen tissues were homogenized on ice in Tris buffer (50 mM Tris·HCl, pH 7.4/0.1 mM EDTA/0.1 mM EGTA/12 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride). To determine calcium-independent iNOS activity in the homogenates, production of L-[^{3}H]citrulline from L-[^{3}H]arginine (7.4 kBq per tube) was measured in the presence of 10 μM L-arginine/1 mM NADPH/300 units of calmodulin per ml/5 μM tetrahydrobiopterin/50 mM L-valine/1 mM EGTA for 30 min at room temperature. Reactions were stopped by adding 1 ml of ice-cold 20 mM Hepes, pH 5.5/2 mM EDTA/2 mM EGTA. After separation by using Dowex 50W (sodium form), L-[^{3}H]citrulline activity was measured by counting scintillation. Experiments performed in the absence of NADPH determined the extent of L-[^{3}H]citrulline formation independent of iNOS activity.

Western Blot Analysis. Lung homogenates were centrifuged (5000 × *g* for 10 min), and the supernatant was boiled for 5 min with an equal volume of gel-loading buffer [20 mM Tris·HCl, pH 6.8/2 mM EDTA/2% (wt/vol) SDS/20% (vol/vol) glycerol/10% (vol/vol) 2-mercaptoethanol/2 mg of bromophenol blue per ml]. Total protein equivalents for each sample were resolved on SDS/7.5% polyacrylamide gels and transferred to nitrocellulose. The membranes were incubated for 20 h at 4°C with an iNOS antibody (1:2000 dilution), a generous gift from our colleague C. E. Bryant (The William Harvey Research Institute). Bands were detected by using a horseradish peroxidase-conjugated antibody and 3,3′-diaminobenzidine.

Materials. Unless stated otherwise, all compounds were purchased from Sigma. *S. aureus* was kindly provided by L. Bryan (Department of Medical Microbiology of St. Bartholomew's Hospital, London). The *S. aureus* PepG polyclonal antibody raised in sheep was a generous gift of D. Smith (Therapeutic Antibodies Ltd., Medical College of St. Bartholomew's Hospital, London). The suspension containing PepG was sonicated for 2 min before use.

Statistical Evaluation. All data are presented as the mean ± SEM of *n* observations. For cultured macrophages, at least three independent experiments were performed in duplicate or triplicate. For *in vivo* experiments, all rats surviving more than 300 min were included in the calculations. The vasopressor response to norepinephrine was determined as the area under the curve and expressed in mmHg·min. Statistical analysis was performed using analysis of variance (ANOVA) followed by Bonferroni's test (one-way ANOVA) or Fisher's test (two-way ANOVA) for multiple comparisons of single means.

RESULTS

PepG and LTA Act in Synergy in the Induction of iNOS. Incubation of macrophages with LTA (10 μg/ml for 24 h) resulted in an increase in the nitrite concentration in the culture medium. Incubation of these cells with PepG (1–100 μg/ml) alone did not influence the basal release of nitrite. However, PepG potentiated the release of nitrite elicited by LTA in a concentration-dependent manner (Fig. 1). Importantly, the induction of nitrite release from cultured macrophages activated with killed *S. aureus* (10^8 cells per ml for 24 h) was decreased by a polyclonal antibody against PepG (0.25 mg/ml) from 44 ± 6 μM (n = 16) to 16 ± 2 μM (n = 20) nitrite in the supernatant ($P < 0.01$). In contrast, the response to LTA was not influenced in the presence of anti-PepG (94% ± 2% of control LTA response; n = 6).

In anesthetized rats, injection of PepG (10 mg/kg, i.v.) or LTA (3 mg/kg, i.v.) resulted in a statistically significant induction of iNOS protein expression and activity ($P < 0.05$) in lungs 6 h after administration (Fig. 2). Only a small increase in iNOS activity was found in the liver [$P < 0.05$ for PepG; not significant (NS) for LTA], while no alterations were observed in other investigated organs (Table 1). Injection of both PepG and LTA resulted in a substantial increase in iNOS protein expression and activity in lungs (Fig. 2). This iNOS activity was significantly greater than (*i*) the calculated additive effect of the bacterial components alone and (*ii*) the iNOS activity induced by a 3-fold higher dose of LTA (10 mg/kg, 139 ± 8 vs. 825 ± 179 pmol of L-citrulline per 30 min per mg of protein; $P < 0.01$). The combination of PepG and LTA caused an increase in iNOS activity in all organs investigated with the following rank order: lung > liver > mesentery ≈ aorta > heart ≈ kidney > pancreas (Table 1).

PepG and LTA Act in Synergy to Release Cytokines *in Vivo*. In anesthetized rats, injection of either PepG (10 mg/kg) or LTA (3 mg/kg) alone resulted in an increase in the plasma concentration of TNF-α (Fig. 3). A similar increase was observed with a higher dose of LTA (10 mg/kg, 10.1 ± 0.8 ng/ml at 90 min). Significantly higher plasma levels of TNF-α were observed after coadministration of PepG and LTA (3 mg/kg) (Fig. 3). Furthermore, the combination of PepG and LTA caused a pronounced elevation in the plasma levels of IFN-γ, while the individual cell wall components had only a marginal effect on the plasma levels of IFN-γ (Fig. 3).

Delayed Circulatory Failure Elicited by PepG and LTA. Injection of PepG (10 mg/kg) and LTA (3 mg/kg) caused a transient decrease in MAP from 122 ± 3 mmHg (time 0) to 82 ± 3 mmHg at 30 min. MAP partially recovered, and a second delayed hypotension commenced at ≈180 min, when MAP fell from 101 ± 4 mmHg to 77 ± 5 mmHg at 360 min. Although either PepG or LTA alone decreased MAP, the hypotension caused by PepG or LTA was less pronounced than the hypotension caused by the combination of the two (Fig. 4). Neither PepG nor LTA caused a significant alteration in heart rate, while the combination of PepG and LTA caused a

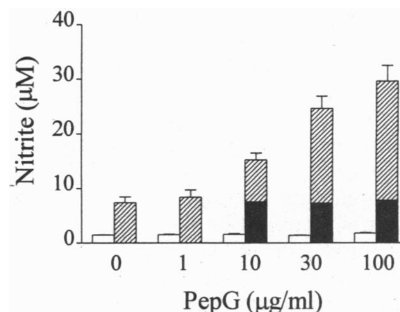

FIG. 1. Effect of PepG and LTA on the accumulation of nitrite in the supernatant of J774.2 macrophages. Macrophages were incubated for 24 h with PepG alone (0–100 μg/ml; n = 9; open bars) or with both PepG and LTA (10 μg/ml; n = 12; hatched bars). The additive individual effects of PepG and LTA are indicated by solid black bars. $P < 0.01$ for 10, 30, and 100 μg of PepG per ml and LTA vs. LTA alone.

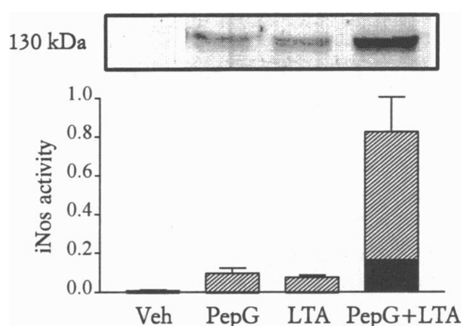

FIG. 2. Effect of PepG and LTA on the induction of iNOS protein (*Upper*) and enzyme activity (*Lower*) in lungs from anesthetized rats. Rats were treated with vehicle (Veh), PepG, LTA, or PepG and LTA (PepG+LTA) for 360 min before lungs were removed. After tissue homogenization, iNOS activity was determined ($n = 6-8$) and iNOS protein was measured by Western blot analysis using a selective iNOS antibody (the data are representative of six experiments). iNOS activity is defined as nmol of L-citrulline formed per mg of protein in 30 min. The additive individual effect of PepG and LTA is shown by a solid black bar. $P < 0.01$ for the increase in iNOS activity by PepG+LTA compared with vehicle and the individual cell wall components.

pronounced tachycardia which coincided with the delayed hypotension (Fig. 4). Furthermore, coadministration of PepG and LTA decreased the pressor response to norepinephrine from 38 ± 6 mmHg·min (time 0) to 11 ± 1 mmHg·min at 60 min and to 5.3 ± 0.5 mmHg·min at 360 min. Administration of either PepG or LTA alone caused only a transient decrease in the pressor response to norepinephrine at 60 min (results not shown), but the pressor response was restored at 360 min (Fig. 4).

MODS Elicited by PepG and LTA. From 16 rats receiving PepG and LTA, 8 rats survived, 4 rats died between 240 and 300 min, and another 4 rats died between 300 and 360 min. At 120 min, only the concentration of urea was slightly increased (9.4 ± 0.5 mM at 120 min vs. 6.2 ± 0.1 mM for control; $P < 0.05$), but none of the other markers of organ injury was significantly influenced—e.g., ALT, 0.10 ± 0.04 international unit (iu)/ml at 120 min vs. 0.06 ± 0.01 iu/ml for control; NS. Rats surviving more than 300 min exhibited an increase in plasma concentrations of bilirubin and ALT (liver failure and hepatocellular damage), urea and creatinine (renal failure and tubular damage), lipase (pancreatic injury), and CK (skeletal, muscle, brain, or cardiac injury) (Fig. 5). Neither PepG nor LTA alone caused mortality or significant changes in the concentration of these plasma markers of MODS at 360 min after injection (Fig. 5). A high dose of LTA (10 mg/kg) caused only a moderate increase in plasma concentration of urea (12 ± 1 mM; $P < 0.01$ vs. control; $P < 0.01$ vs. 18 ± 1 mM for PepG and LTA), but not of any of the other plasma markers of

Table 1. Induction of iNOS activity by PepG and LTA

Organ	iNOS activity, pmol of L-citrulline per 30 min per mg of protein			
	Vehicle	PepG	LTA	PepG + LTA
Liver	5 ± 3	27 ± 6	17 ± 6	101 ± 24
Mesentery	8 ± 5	8 ± 5	11 ± 6	38 ± 5
Aorta	5 ± 2	4 ± 6	5 ± 1	37 ± 2
Heart	4 ± 2	1 ± 2	4 ± 4	28 ± 6
Kidney	0 ± 2	0 ± 1	0 ± 1	25 ± 4
Pancreas	2 ± 1	3 ± 1	3 ± 2	14 ± 1

Data are presented as the mean ± SEM ($n = 3-8$). For all organs: PepG + LTA vs. vehicle ($P < 0.01$) and vs. the individual cell wall components ($P < 0.05$).

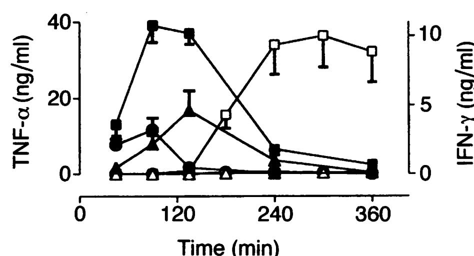

FIG. 3. Effect of PepG and LTA on the systemic release of TNF-α (closed symbols) and IFN-γ (open symbols). Rats were treated with PepG (triangles; $n = 4-6$), LTA (circles; $n = 4-6$), or PepG and LTA (squares; $n = 5$ or 6). Immunoreactive TNF-α and IFN-γ were measured by ELISA. $P < 0.01$ for PepG and LTA vs. the individual cell wall components.

MODS—e.g., creatinine, 39 ± 5 μM; and ALT, 0.08 ± 0.01 iu/ml.

Injection of both PepG and LTA resulted in a significant decrease in partial arterial oxygen pressure (Pa_{O_2}) at 360 min indicating a respiratory failure (Table 2). Blood pH decreased, while Pa_{CO_2} was not significantly affected (Table 2). Therefore, the calculated HCO_3^- concentration and base excess decreased. These results show a metabolic acidosis elicited by coadministration of the bacterial components. PepG or LTA alone did not cause any significant alterations in these parameters (Table 2) and neither did a higher a dose of LTA (10 mg/kg; Pa_{O_2}, 79 ± 2 mmHg; HCO_3^-, 28.0 ± 0.7 mM).

DISCUSSION

This study demonstrates that, in anesthetized rats, coadministration of two major cell wall components from *S. aureus*, PepG and LTA, results in septic shock. This is seen from the delayed hyperdynamic circulatory failure (hypotension, vascular hyporeactivity, and tachycardia) and MODS, as indicated by respiratory failure, liver failure, acute renal failure, pancreatic injury, and skeletal or cardiac muscle injury. There is also a metabolic acidosis and 50% mortality. Most of these features were not apparent either at 120 min or at 360 min after injection of PepG or LTA alone. This indicates that organ

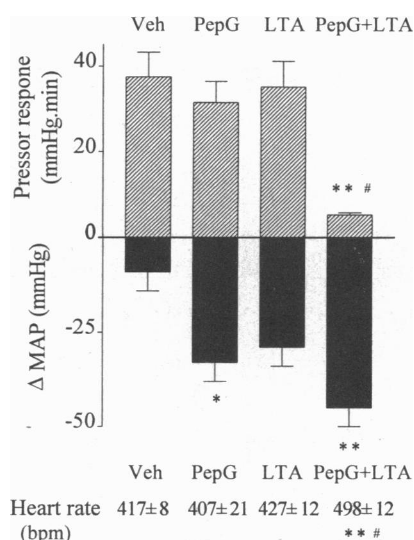

FIG. 4. Effect of PepG and LTA on the pressor response to norepinephrine (hatched bars), MAP (solid bars), and heart rate at 360 min. Rats were treated with vehicle (Veh; $n = 6$), PepG ($n = 7$), LTA ($n = 6$), or PepG and LTA (PepG+LTA; $n = 9$). *, $P < 0.05$ vs. vehicle; **, $P < 0.01$ vs. vehicle; #, $P < 0.01$ for PepG and LTA vs. the individual cell wall components. bpm, Beats per min.

Table 2. Blood gas and pH analysis of carotid artery blood at 360 min after injection of Gram-positive cell wall components

	Vehicle	PepG	LTA	PepG + LTA
Pa_{O_2}, mmHg	80 ± 3	74 ± 5	72 ± 3	59 ± 3*
Pa_{CO_2}, mmHg	38 ± 1	32 ± 2	40 ± 1	39 ± 3
pH	7.43 ± 0.01	7.49 ± 0.02	7.44 ± 0.02	7.29 ± 0.05*
HCO_3^-, mM	25 ± 1	24 ± 1	27 ± 1	19 ± 1*
Base excess, eq	2 ± 1	3 ± 1	3 ± 1	−7 ± 2*

Data are presented as the mean ± SEM. Rats were treated with vehicle (n = 6), PepG (n = 7), LTA (n = 6), or PepG and LTA (n = 7). Pa_{O_2}, partial arterial oxygen pressure; Pa_{CO_2}, partial arterial carbon dioxide pressure.

*$P < 0.05$ for PepG + LTA vs. vehicle and the individual cell wall components.

injury develops as a consequence of the host response to PepG and LTA together.

Little is known about the mechanism by which Gram-positive organisms induce shock. Plasma levels of TNF-α, interleukin 1 (IL-1) and IL-6 are increased in human septic shock (22). In animal models of septic shock, neutralization of proinflammatory cytokines, such as TNF-α, IL-1, or IFN-γ, reduces circulatory failure and mortality (12, 23–25). Indeed, administration of both PepG and LTA resulted in the release of TNF-α and IFN-γ. These cytokines cause the expression of iNOS in a wide variety of cells, including macrophages and vascular smooth muscle cells (26). Interestingly, plasma levels of nitrite and nitrate are increased in patients with septic shock (27). The present results show a pronounced increase in iNOS protein and activity in lungs obtained from rats treated with the combination of PepG and LTA. NO is a powerful vasodilator which contributes to the circulatory collapse in various animal models of septic shock (15, 28, 29). Furthermore, following the induction of iNOS, NO can bind to mitochondrial enzymes, inhibiting cellular respiration, and react with superoxide anion to form peroxynitrite, resulting in nitrosylation of proteins or formation of highly reactive hydroxyl radicals (30–32). Thus, NO in large amounts can be cytotoxic and contribute to the development of MODS. Indeed, after giving PepG and LTA, iNOS activity is abundant in lung, liver, aorta, mesentery, heart, kidney, and pancreas. The lack of MODS after injection of PepG or LTA alone correlates with (*i*) the minor induction of iNOS activity, (*ii*) the moderate release of TNF-α, and (*iii*) the marginal release of IFN-γ elicited by these individual cell wall components. Thus, PepG and LTA together cause a pronounced release of cytokines and induction of iNOS in the anesthetized rats. However, the exact mechanism by which this widespread inflammation results in MODS remains to be elucidated.

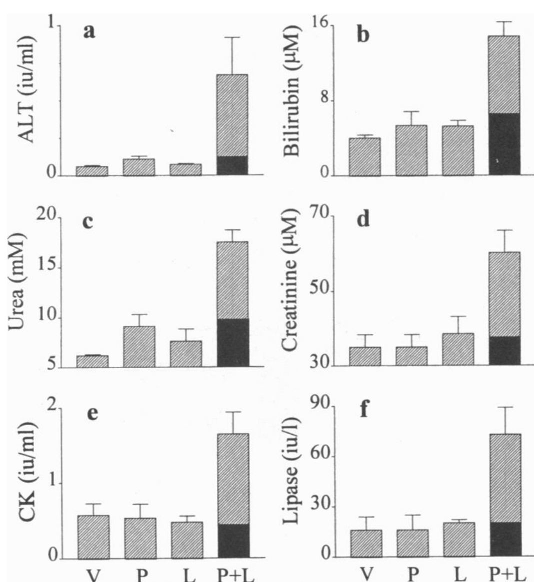

FIG. 5. Effect of PepG and LTA on the plasma concentration of markers of MODS in plasma. Rats were treated with vehicle (V; n = 6), PepG (P; n = 7), LTA (L; n = 6), or PepG and LTA (P+L; n = 11). ALT (*a*), bilirubin (*b*), urea (*c*), creatinine (*d*), CK (*e*), and lipase (*f*) were measured in plasma obtained at the end of the experiment. The additive individual effect of PepG and LTA is shown by a solid bar. $P < 0.01$ for the increase in plasma markers by P+L compared with vehicle and the individual cell wall components.

How Gram-positive organisms initiate an inflammatory response is not yet clear. It is difficult to pinpoint one component (such as LPS for Gram-negative organisms) that is responsible for initiating Gram-positive sepsis. Here, we demonstrate that the combination of two major cell wall components, PepG and LTA, mimics the pathophysiology and outcome of septic shock. Interestingly, antibodies to PepG and LTA have been detected in patients with sepsis due to *S. aureus* (33, 34), indicating that PepG and LTA are released and elicit a host response during infection in humans. The effect of combined administration of PepG and LTA on the release of cytokines, induction of iNOS, development of MODS, and mortality is significantly greater than the expected additive effect of the two components alone. Moreover, at a 3-fold higher dose, LTA alone still has only very limited effects on these parameters. These results show that PepG and LTA act synergistically. This conclusion is supported by our findings *in vitro* that (*i*) PepG alone does not cause the release of nitrite from macrophages but potentiates the release elicited by LTA and that (*ii*) antibodies against PepG selectively inhibit the release of nitrite elicited by killed *S. aureus*, despite the fact that PepG alone does not induce the release of nitrite. These results suggest that PepG and LTA act, at least partially, via different pathways to elicit an inflammatory response. LTA is amphiphilic and generally consists of a hydrophilic poly(glycerophosphate) chain attached to a glycolipid. PepG consists of polymers of alternating *N*-acetylneuraminic acid and *N*-acetylglucosamine molecules, which are linked by small peptide bridges. Like LPS, LTA induces the release of prostaglandin E_2 from murine macrophages (13). Preincubation of these macrophage with LTA rendered the cells insensitive to LPS, but they remained responsive to phorbol esters (13). This cross-tolerance suggests similarities in the mechanism of action of LTA and LPS. In contrast, repeat incubation of Kupffer cells with PepG induced no tolerance to TNF-α or IL-1 release (35). Furthermore, PepG-rich cell wall preparations stimulate TNF-α synthesis in macrophages derived from LPS-insensitive C3H/HeJ mice (36). These comparisons with LPS support our notion that LTA and PepG exhibit proinflammatory properties but that there are differences in their molecular action.

Thus, LTA and PepG from *S. aureus* act together to elicit widespread systemic inflammation and pathological features observed in septic shock. As these two cell wall components are common to other Gram-positive organisms, their joint activity may explain the initiation of Gram-positive septic shock in general.

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