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Hemoglobin Increases Mortality from Bacterial Endotoxin

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Cell-free hemoglobin (Hb) is being developed as an erythrocyte substitute. We have previously demonstrated that cell-free Hb is an endotoxin-binding protein which disaggregates endotoxin and subsequently increases the biological activity of endotoxin in several in vitro assays. Because much of the morbidity and mortality associated with gram-negative bacterial infection is the result of pathophysiologic responses to bacterial lipopolysaccharide (LPS; endotoxin), we studied the effect of Hb on LPS-mediated mortality. Hb infused intravenously into mice before, coincident with, or after intraperitoneal LPS injection substantially increased LPS-related mortality from <5% to 50 to 70% 24 h after administration of LPS and from 50% to 60 to 90% at 48 h. Enhanced mortality was observed over a range of doses of injected LPS. At a given LPS dose, enhancement of mortality was shown to be dependent on the dose of Hb administered. Unmodified native human Hb, α-α-cross-linked human Hb, and β-β-cross-linked human or bovine Hb all were shown to enhance LPS-mediated mortality. Depressed reticuloendothelial cell function may have contributed to the enhanced mortality from LPS in the presence of Hb. Therefore, Hb-based blood substitutes, which are currently undergoing clinical trials, may intensify the potentially fatal effects of the sepsis syndrome in patients with trauma, infection, or hypotension who receive Hb for erythrocyte replacement.

Because of the known risks and limitations of transfused erythrocytes as oxygen carriers (infections, donor-recipient incompatibility, limited shelf life, requirement for refrigerated storage), there is great interest in the development of acellular hemoglobin (Hb)-based oxygen carriers (cell-free Hb). Both the Food and Drug Administration and the military have recognized the need for high-priority development of a stable, infectious-agent-free erythrocyte substitute. However, cell-free Hb exhibits specific biochemical properties that are either intrinsically toxic or capable of eliciting deleterious host responses. One worrisome characteristic of Hb is its potential interaction with infectious agents. In particular, it has been well documented that lethality associated with gram-negative infection can be enhanced by Hb (7, 8, 18, 19), and elucidation of the mechanism(s) that contributes to this deleterious effect of Hb is presently of major interest. In some studies, increased bacterial toxicity in the presence of Hb has been attributed to stimulation of bacterial growth by iron (7, 8), although this result has not been uniformly reported (16, 17). In other studies, inhibition by Hb of leukocyte chemotaxis, phagocytosis, and killing of bacteria has been demonstrated (9, 10). Depression of reticuloendothelial cell system function also has been implicated in the enhanced mortality from bacterial infections that occurs in the presence of hemoglobinemia due to erythrocyte hemolysis (14, 15). Finally, a decade ago, synergistic toxicity was observed following administration of Hb and bacterial endotoxin (lipopolysaccharide [LPS]) (30), the cell wall component of gram-negative bacteria responsible for much of the toxicity produced by bacterial sepsis. In that report, lethality in rabbits which simultaneously received both intravenous stroma-free Hb and *Salmonella enteritidis* LPS was significantly greater than lethality observed after LPS alone (30).

Recently, there has been renewed interest in the potentially important role of deleterious LPS-Hb interactions. Our laboratory has demonstrated that Hb forms stable complexes with a wide variety of LPSs and that LPS macromolecular particles become disaggregated after binding to Hb (11, 12). In a series of in vitro studies, Hb was shown to enhance the ability of LPS to activate coagulation (11, 25), stimulate procoagulant activity from peripheral blood mononuclear cells (25), and stimulate production of procoagulant activity by cultured human endothelial cells (23). LPS binding to endothelial cells was increased when LPS was complexed with Hb (24), suggesting a possible mechanism for the enhanced cellular response to LPS-Hb complexes compared to LPS alone.

Because these in vitro data suggested potentially substantial pathological consequences of the LPS-Hb interaction, we wished to determine whether coexistent hemoglobinemia and endotoxemia were deleterious in vivo. For these studies, we chose a murine model system frequently used for investigations of sepsis and bacteremia. To mimic a common clinical scenario produced by sepsis resulting from trauma to the gastrointestinal tract or gut ischemia, LPS was injected intraperitoneally into mice, creating prolonged endotoxemia as is characteristic of clinical peritonitis. We used *Escherichia coli* LPS because of the frequent occurrence of *E. coli* sepsis and because of our extensive characterization of LPS-Hb complex formation with use of this LPS (11, 12). Experiments were conducted both with purified native human Hb (HbA_0) and with cross-linked (stabilized) human or bovine Hb.

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MATERIALS AND METHODS

Animals. Female Swiss Webster and C3H/HeJ mice (28 to 32 g) were purchased from Simonsen (Gilroy, Calif.). In conducting research using animals, we adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (5a).

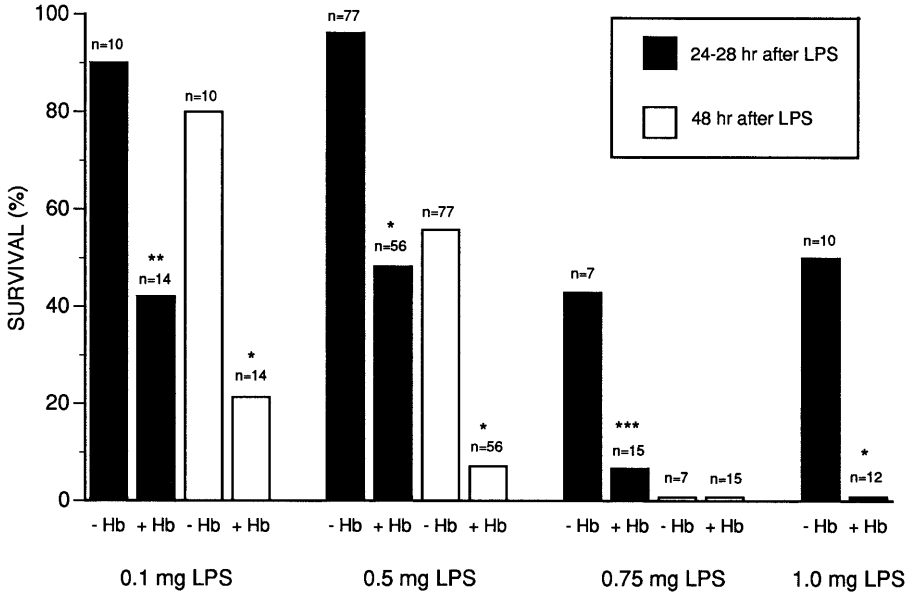

FIG. 1. LPS lethality in mice, at various LPS doses, in the absence (−Hb) and presence (+Hb) of ααHb. Swiss Webster female mice (28 to 32 g) were injected intraperitoneally with 0.1, 0.5, 0.75, or 1.0 mg of LPS (*E. coli* O55:B5 LPS, in sterile, pyrogen-free saline). Eight to 10 h after LPS injection, mice were infused by tail vein with either 0.6 to 0.8 ml of saline or 0.6 to 0.8 ml ααHb in Ringer's acetate (pH 7.4) (60 mg/mouse), prepared as described in the text. Survival was monitored at 24 to 28 and 48 h after LPS injection. *n*, number of mice in each group. *, $P < 0.01$, +Hb versus −Hb; **, $P = 0.03$, +Hb versus −Hb; ***, $P = 0.07$, +Hb versus −Hb (Fisher's exact *P* value).

Hb. Human Hb, α-α-cross-linked (>98% cross-linked between the α99 lysine residues) with bis(3,5-dibromosalicyl) fumarate (31) (ααHb), was provided by collaborators at the Blood Research Detachment, Walter Reed Army Institute of Research, Washington, D.C. Chromatographically purified, unmodified human HbA_0 (5) also was provided by collaborators at Walter Reed Army Institute of Research. Hb solutions were in Ringer's acetate (pH 7.4), contained <5% methemoglobin, and were sterile and essentially endotoxin free (<100 pg/ml). They were stroma free as determined by phosphate analysis for stromal lipid. Three β-β-cross-linked Hb (ββHb) preparations were generously provided by Enrico Bucci (University of Maryland School of Medicine, Baltimore): bovine fumaryl ββHb, cross-linked between the β81 lysine residues in the β cleft as described previously (29); decasebacyl-cross-linked human β-β/α-α-cross-linked Hb (ββ/ααHb), 50% cross-linked between β82 lysine residues and 50% simultaneously cross-linked between β82 lysine residues and between α99 lysine residues as described previously (4); and another bovine ββHb currently being characterized.

HSA. Human serum albumin (HSA) for infusion, in 0.9% NaCl with sodium bicarbonate (pH 7.4), was obtained from Baxter Corp. (Miami, Fla.).

LPS. *Escherichia coli* LPS O55:B5 and *Salmonella typhosa* LPS O901 were obtained from Difco (Detroit, Mich.). Solutions of 1 mg of LPS per ml in sterile, endotoxin-free 0.9% NaCl (Lymphomed, Deerfield, Ill.) were vortexed and briefly sonicated (1 min) prior to use. *E. coli* K-12 LPS, biosynthetically radiolabeled with ^3H, was obtained from List Biological Laboratories, Inc. (Campbell, Calif.).

Mortality experiments. Mice were injected intraperitoneally with LPS (0.6 to 0.8 ml) and infused intravenously by tail vein with Hb (<1 ml in most experiments). In most experiments, Hb was infused intravenously 8 to 10 h after injection of LPS. In selected experiments, Hb was infused either 12 h prior to or coincident with LPS. In control animals which received intraperitoneal LPS only, sterile, endotoxin-free 0.9% NaCl was infused intravenously so that all animals in an experiment received equal volumes of parenteral fluid. Similarly, in control animals which received intravenous Hb only, NaCl was injected intraperitoneally. In a separate set of control experiments, hemin chloride (Sigma, St. Louis, Mo.) in 0.9% NaCl was infused intravenously 10 h following intraperitoneal administration of LPS or NaCl. Mortality was monitored at 24 to 28 h and at 48 h after injection of LPS. Animals were given standard mouse chow and water ad lib throughout the experiments. Care and manipulation of mice were in accordance with institutional guidelines. Experimental protocols were approved by the Sub-Committee for Animal Studies, Veterans Affairs Medical Center, San Francisco, Calif.

Plasma LPS concentrations. Blood for LPS levels was serially obtained from the retro-orbital plexus in EDTA-containing capillary tubes at various time points after LPS injection, and plasma was prepared by centrifugation of the capillary tubes in a serofuge (International Equipment Co., Needham Heights, Mass.). Variable but low levels of LPS contamination (<0.05 to 2.2 ng/ml) in samples obtained by this technique were deemed acceptable, since 10^2- to 10^4-fold-higher peak plasma LPS levels were achieved in the LPS-injected mice. Plasma LPS concentrations were determined with an Endospecy microplate assay kit generously provided by Seikagaku Kogyo Co. (Tokyo, Japan). Plasma samples were diluted in water (typically 1:1,000 to 1:100,000) until LPS concentrations were within the evaluable range and then assayed with the Endospecy *Limulus* amebocyte assay (LAL) reagent, containing amebocyte lysate prepared from *Tachypleus tridentatus*, buffer, and chromogenic substrate. This range of dilutions was sufficient to obviate use of the alkalinization step recommended by the manufacturer to remove inhibitors. Incubations were carried out with an SK601 well reader (Seikagaku), and LPS concentrations were determined by kinetic analyses of absorbances at 405 nm, with standard *E. coli* O111:B4 LPS provided by Seikagaku as the reference.

Plasma LPS concentrations were also determined by using recovery of radiolabeled LPS. In these experiments, mice received an intraperitoneal injection of ^3H-LPS (5×10^5 cpm; 0.3 μg of LPS) with or without 500 μg of unlabeled LPS. Plasma samples were obtained at various time points after LPS injection, and plasma LPS concentrations were determined from ^3H measurements by using a liquid scintillation counting system (Tracor Analytic, Elk Grove Village, Ill.).

Plasma glucose concentrations. Blood for determination of glucose levels was serially obtained from the retro-orbital plexus in EDTA-containing capillary tubes at various time points up to 48 h after LPS injection, with and without intravenous Hb infusion coincident with the LPS injection. In these experiments, all animals were fasted (water ad lib was provided) following LPS injection. Plasma was prepared by centrifugation of the capillary tubes in a serofuge (International Equipment Co.), and glucose concentrations were determined with a Glucose (HK) assay kit (Sigma).

Hb clearance. Blood for measurement of Hb levels was serially obtained from the retro-orbital plexus in similar capillary tubes at various time points after Hb infusion, and plasma was obtained by centrifugation as described above. Plasma samples were diluted 1:15 with 0.9% endotoxin-free saline, and Hb clearance was monitored at 405 nm in a BioWhittaker (Walkersville, Md.) Kinetic-QCL plate reader.

Particulate carbon clearance. Carbon (India ink) was purchased from Baxter Corp. and diluted in sterile, endotoxin-free 0.9% saline such that a subsequent 1:50 dilution had an absorbance of approximately 0.6 at 620 nm. Mice were injected with intraperitoneal NaCl or LPS followed by intravenous NaCl or Hb 10 h later, and after an additional 10 h, carbon solutions were infused intravenously by tail vein. Serial blood samples were obtained from the retro-orbital plexus with EDTA-containing capillary tubes at various time points (0.5 to 30 min) after injection. The samples of whole blood were diluted 1:10 in water to lyse the erythrocytes, and absorbances due to carbon were determined at 620 nm.

Blood cultures. Mice were injected intraperitoneally with LPS and 10 h later received Hb intravenously. Blood samples (~1 ml) were obtained for culture (Bacto Columbia Broth; Difco) by cardiac puncture 26 and 36 h after LPS injection. Cultures were incubated aerobically at 37°C for 30 days and periodically plated on G-C agar medium (Difco).

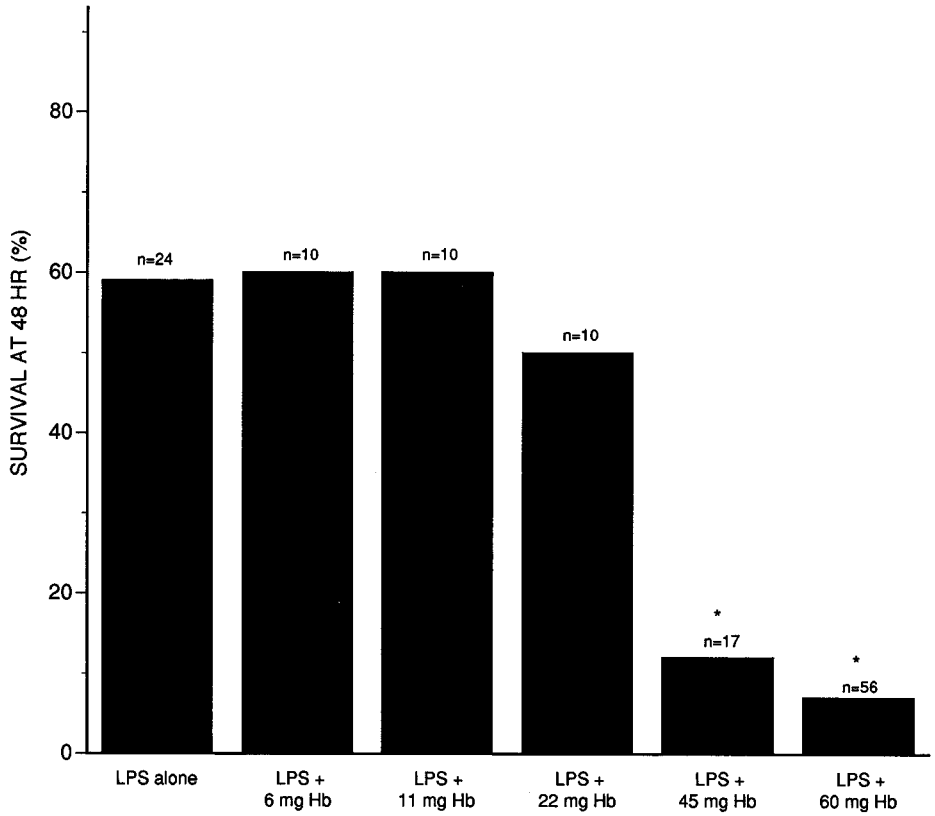

FIG. 2. LPS lethality in mice after the administration of various doses of ααHb. Swiss Webster female mice (28 to 32 g) were injected intraperitoneally with 0.5 mg of LPS (*E. coli* O55:B5 LPS, in sterile, pyrogen-free saline). Eight to 10 h following LPS injection, the mice were infused by tail vein with either 0.6 to 0.8 ml of saline or ααHb in Ringer's acetate (pH 7.4) (doses of Hb ranged from 6 to 60 mg/mouse). Survival was monitored at 48 h. *, $P < 0.01$ versus LPS alone (Fisher's exact *P* value).

Tissue histology. Tissues were obtained from untreated animals, from animals which had received intraperitoneal LPS 18 h prior to sacrifice, and from animals which had received intraperitoneal LPS 18 h prior to sacrifice and intravenous Hb 8 to 10 h after LPS. Mice were sacrificed by cervical dislocation, and portions of liver, spleen, kidney, lung, heart, adrenal, brain, ovary, uterus, and skeletal muscle were fixed in 10% formalin, sectioned, and stained with hematoxylin and eosin. Tissue sections were examined for evidence of inflammation, hemorrhage, thrombosis, or necrosis.

RESULTS

Effect of Hb on LPS-induced mortality. When 60 mg of ααHb/mouse was infused intravenously 8 to 10 h after intraperitoneal injection of LPS, animals died at a much faster rate than with LPS alone, and overall survival was greatly diminished (Fig. 1). Peak plasma Hb levels achieved by this infusion were approximately 4.0 to 5.5 g/dl. The deleterious effect of Hb was observed at all LPS doses tested (0.1 to 1 mg of LPS/mouse, doses of LPS which alone produced a 20% lethal dose to a 100% lethal dose at 48 h). Seventy-two-hour mortality data were also obtained for the mice which received 0.5 mg of LPS. This dose of LPS produced 72% mortality in the absence of Hb and 96% mortality in the presence of Hb at 72 h. Control experiments demonstrated that infusion of Hb alone (in the absence of LPS) was well tolerated, with no evidence of toxicity (e.g., there was no ruffling of the fur or lethargy and no mortality). Hb enhancement of LPS-induced mortality was dependent on the dose of subsequently infused Hb, as follows: 48-h survival rates with 6 and 11, 22, 45, and 60 mg of Hb/mouse were 60, 50, 12, and 7%, respectively (Fig. 2). Hb infused coincident with LPS also resulted in increased mortality, as did non-cross-linked HbA_0 infused coincident with LPS (Fig. 3). Intraperitoneal injection of preincubated ααHb-LPS mixtures likewise resulted in increased mortality, approximating the mortality observed when Hb was administered intravenously 10 h after LPS. Hb infused intravenously 12 h prior to LPS produced 24-h mortality which was statistically greater than that observed with LPS alone; 48-h survival in the animals which received Hb prior to LPS (36%) was also lower but not statistically different from survival in the animals which received LPS alone (56%).

As for ααHb, infusion of human ββ/ααHb or bovine fumaryl ββHb preparations subsequent to LPS produced greater mortality at both 24 and 48 h than LPS alone (Fig. 3). An additional bovine ββHb produced similar mortality (data not shown). In additional control experiments, infusion of HSA coincident with or subsequent to LPS injection had no statistically significant effect on baseline LPS-induced mortality, in contrast to the synergistic mortality observed with Hb. Finally, to demonstrate that the ability of Hb to enhance LPS mortality was not restricted to the *E. coli* LPS used in the above-described experiments, we also performed experiments with *S. typhosa* LPS. Infusion of Hb 10 h after intraperitoneal injection of *S. typhosa* LPS produced statistically significant enhancement of LPS mortality from 36 to 72 h, similar to the effects of Hb with *E. coli* LPS (data not shown).

We performed a series of experiments to determine whether the enhancement of LPS-induced lethality could be attributed to the heme component of Hb. Preliminary studies indicated that a 0.48-mg dose of hemin chloride (equivalent to the heme

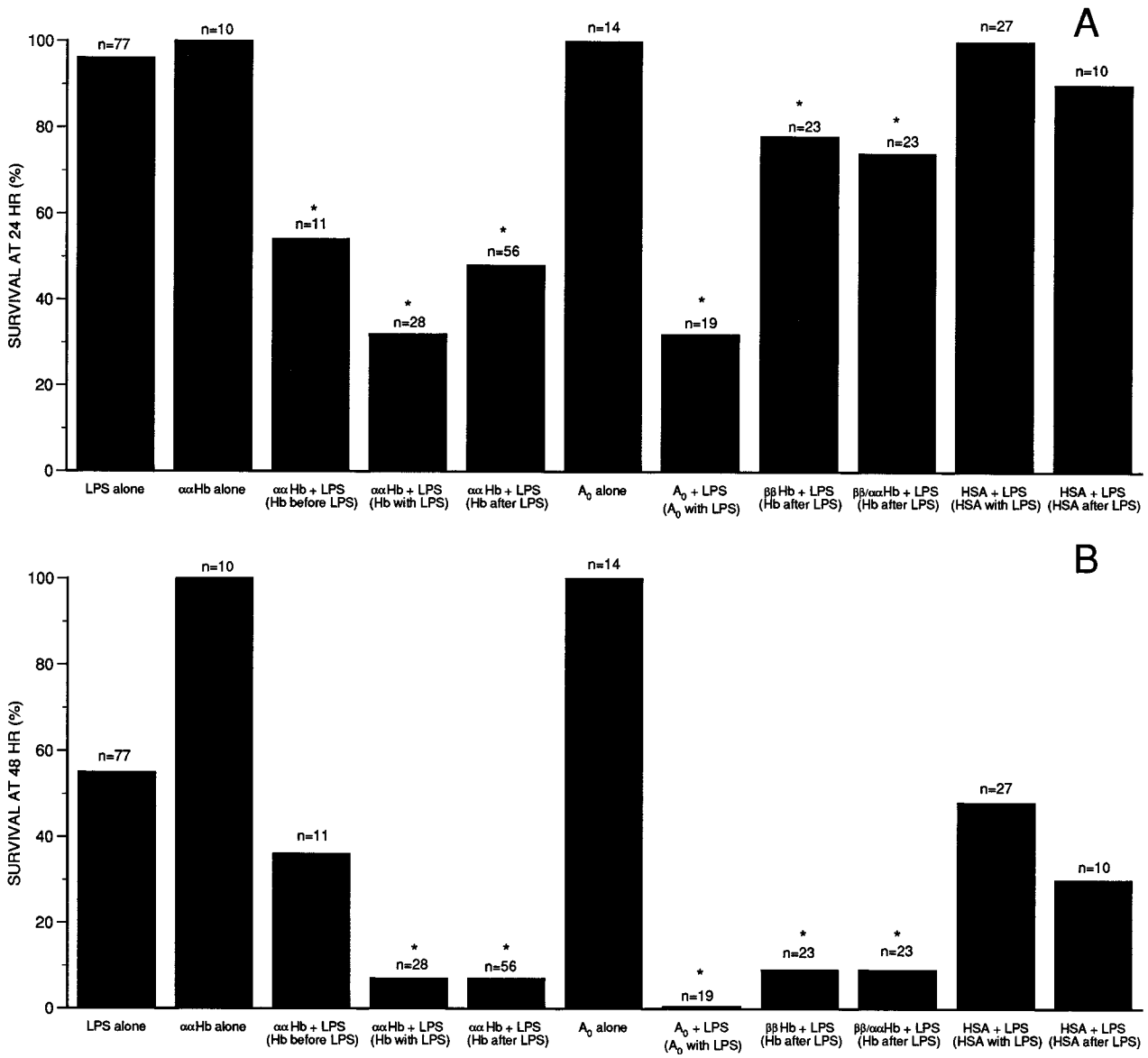

FIG. 3. LPS lethality in mice in the absence and presence of ααHb, ββHb, HbA_0, or HSA. Swiss Webster female mice (28 to 32 g) were injected intraperitoneally with saline or with 0.5 mg of LPS (*E. coli* O55:B5 LPS, in sterile, pyrogen-free saline). Mice also were infused by tail vein with either 0.6 to 0.8 ml of saline, Hb solutions in Ringer's acetate (pH 7.4) (60 mg/mouse), prepared as described in the text, or HSA (60 mg/ml, pH 7.4, in saline with sodium bicarbonate). In various experiments, ααHb was infused either 12 h before, coincident with, or 8 to 10 h after LPS. Bovine fumaryl ββHb and human ββ/ααHb were infused 8 to 10 h after LPS, and HbA_0 was infused coincident with LPS. HSA was infused either coincident with or 8 to 10 h after LPS. Survival was monitored at 24 h (A) and 48 h (B). *, $P < 0.01$ versus LPS alone (Fisher's exact P value).

content of 12 mg of Hb) could be intravenously administered to mice safely; lethality was observed at ≥1.2 mg of hemin. In four independent experiments, 0.48 mg of hemin did not result in enhanced lethality at either of two LPS doses. Experiments to evaluate the ability of heme-free globin to enhance LPS were not considered feasible because of the known instability of free globin chains.

Endotoxin-resistant mice. LPS was administered to endotoxin-resistant (C3H/HeJ) mice at a dose of 0.5 mg/mouse, corresponding to a 50% lethal dose in normal mice. LPS alone caused no deaths at 48 h in C3H/HeJ mice, and LPS plus Hb caused death in only 14% of these LPS-resistant mice (Fig. 4). For comparison, 48-h mortality in wild-type (Swiss Webster) mice was 94% in mice treated with both LPS and Hb.

Following the demonstration (see above) that Hb enhanced the lethal effects of LPS, we assessed the potential contributions from several possible mechanisms for this result. We considered the possibility that circulating LPS levels were higher in the Hb-treated mice, as well as whether the deleterious systemic response(s) to LPS might be augmented.

Effect of Hb on plasma endotoxin levels. Plasma LPS concentrations became detectably elevated by 30 min after the intraperitoneal administration of LPS and continued to increase for several hours (Fig. 5). Background LPS concentrations in blood obtained from the retro-orbital plexus of untreated animals ranged from undetectable LPS (<0.05 ng/ml) to approximately 2.2 ng/ml. LPS plasma levels at every time point tested were quite variable among mice, but overall, LPS

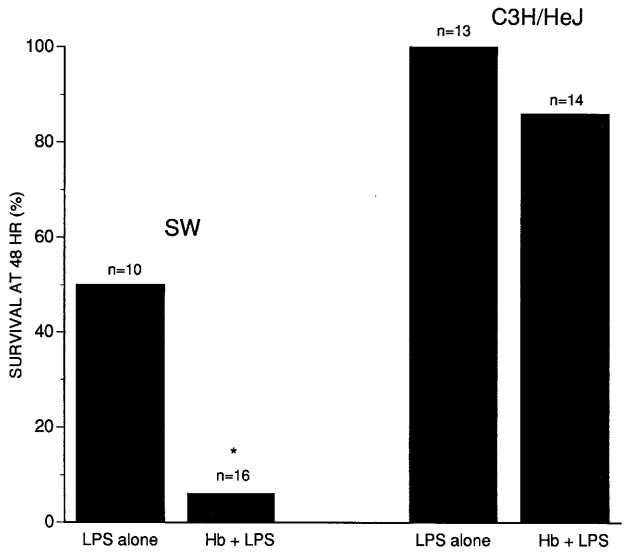

FIG. 4. LPS lethality in Swiss Webster (wild-type) and C3H/HeJ mice in the absence and presence of ααHb. Swiss Webster (SW) or C3H/HeJ (endotoxin-resistant) female mice (28 to 32 g) were injected intraperitoneally with 0.5 mg of LPS (*E. coli* O55:B5 LPS, in sterile, pyrogen-free saline). Immediately following LPS injection, the mice were infused by tail vein with either 0.6 to 0.8 ml of saline or ααHb in Ringer's acetate (pH 7.4) (60 mg/mouse). Survival was monitored at 48 h. *, $P < 0.01$ versus LPS alone (Fisher's exact P value).

concentrations peaked at 4 to 10 h and then gradually declined. LPS concentrations measured with the LAL assay were similar to LPS concentrations obtained by isotopic measurement in separate experiments to determine the levels in plasma of ^{3}H-LPS following intraperitoneal administration. For example, the mean LPS plasma concentrations at 4 h after intraperitoneal injection were 20 μg/ml by LAL measurement and 23 μg/ml by isotopic measurement. Absorption into the blood of the tracer ^{3}H-LPS was the same in both the absence and presence of unlabeled LPS (0.5 mg). Following a nadir at 20 h, plasma LPS levels again rose and were typically greater at 34 and 42 h after LPS injection than at 20 h. Intravenous administration of Hb 8 to 10 h after intraperitoneal injection of LPS did not alter the subsequent range of LPS plasma concentrations (Fig. 5). As demonstrated at the 42-h time point, there also was no relationship between plasma LPS concentrations and Hb doses between 6 and 60 mg/mouse. Furthermore, in both the absence and presence of Hb, there was no relationship between LPS plasma concentration, at any of the time periods examined, and subsequent mortality.

Plasma Hb clearance. In mice which received intraperitoneal LPS 8 to 10 h prior to intravenous Hb infusion, there was a dose-dependent effect of LPS on Hb plasma levels (Fig. 6). Persistence of Hb in the plasma was increased (prolonged half-life [$t_{1/2}$]) at 2, 5, and 10 h after infusion by prior injection of 0.5 mg of LPS ($P < 0.02$ versus Hb levels in the absence of LPS). There also was a trend toward slower clearance of Hb in animals which received 0.1 mg of LPS, although the difference from Hb clearance in control animals was statistically significant only at 2.5 h ($P < 0.05$ versus Hb in the absence of LPS). Interestingly, 12 h after Hb infusion, a time at which a substantial quantity of the Hb had been cleared from the circulation, Hb was not detectable in the peritoneal cavity in animals either with or without prior injection of intraperitoneal LPS.

Particulate carbon clearance. The slower clearance of Hb in endotoxemic mice (see above) suggested that there was a depression of reticuloendothelial cell function. Mice were injected with intraperitoneal LPS only, intravenous Hb only, or intraperitoneal LPS followed by intravenous Hb, and reticuloendothelial cell function was assessed by clearance of intravenously injected carbon particles (India ink). Accelerated par-

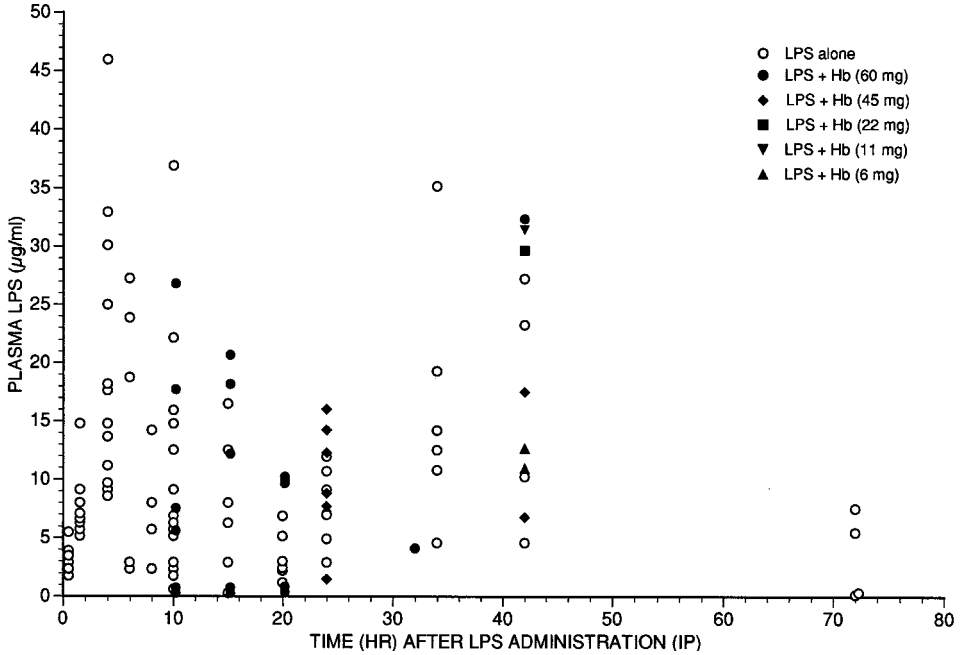

FIG. 5. Plasma LPS concentrations in the absence and presence of ααHb. Swiss Webster female mice (28 to 32 g) were injected intraperitoneally with 0.5 mg of LPS (*E. coli* O55:B5 LPS, in sterile, pyrogen-free saline). Ten hours after LPS injection, mice were infused by tail vein with either 0.6 to 0.8 ml of saline or ααHb. The ααHb dose was 60, 45, 22, 11, or 6 mg/mouse. Blood for LPS levels were serially obtained in EDTA capillary tubes at various time points after LPS injection as described in Materials and Methods. Each data point represents the plasma LPS concentration in a single mouse.

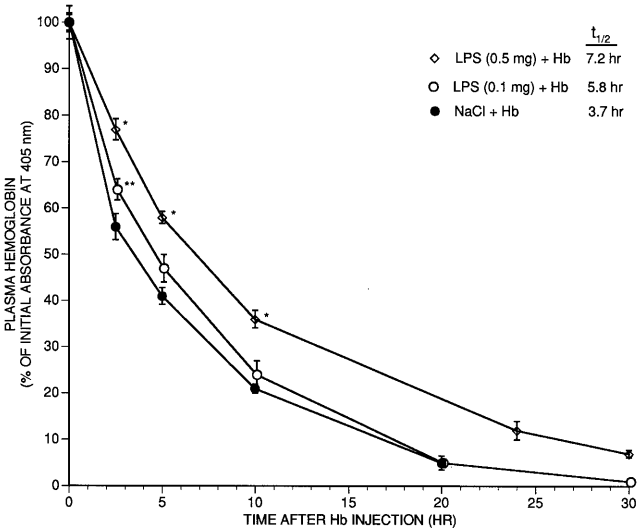

FIG. 6. Hb clearance in mice in the absence or presence of LPS. Swiss Webster female mice (28 to 32 g) were injected intraperitoneally with saline or with 0.1 or 0.5 mg of LPS (*E. coli* O55:B5 LPS, in sterile pyrogen-free saline). Eight to 10 h after injection, mice were infused by tail vein with 0.6 to 0.8 ml of ααHb (60 mg/mouse). Plasma samples were serially obtained with EDTA capillary tubes at various time points after Hb injection. Plasma samples were diluted 1:15 with saline, and absorbances at 405 nm were measured in a plate reader. Initial (t_0) absorbance values at 405 nm, obtained 1 min after Hb infusion, ranged from 1.6 to 2.2, corresponding to plasma Hb concentrations from 4.2 to 5.7 g/dl. Data presented are means ± standard errors for five mice (NaCl), six mice (0.5 mg of LPS), or nine mice (0.1 mg of LPS). *, $P < 0.02$ versus NaCl plus Hb (Mann-Whitney *U* test); **, $P < 0.05$ versus NaCl plus Hb (Mann-Whitney *U* test). $P < 0.05$ for 0.1 mg of LPS plus Hb versus 0.5 mg of LPS plus Hb at 2, 5, and 10 h.

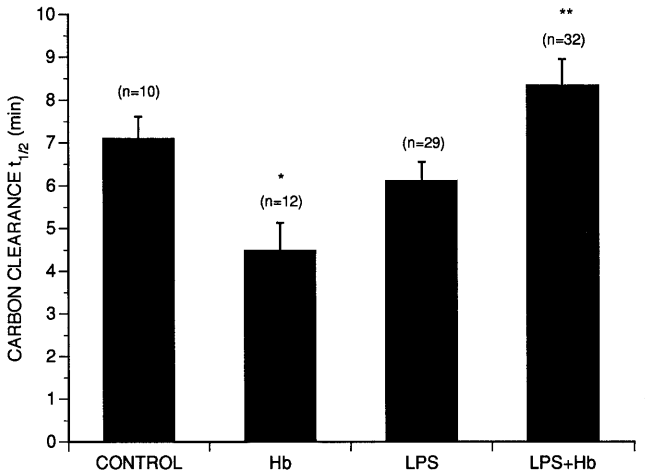

FIG. 7. Carbon particle clearance in mice in the absence or presence of LPS and/or Hb. Swiss Webster female mice (28 to 32 g) were injected intraperitoneally with saline or with 0.5 mg of LPS (*E. coli* O55:B5 LPS, in sterile, pyrogen-free saline). Eight to 10 h after injection, mice were infused by tail vein with 0.6 to 0.8 ml of ααHb (60 mg/mouse) or saline; 10 h later, 0.4 ml of particulate carbon was infused by tail vein. Serial blood samples were obtained with EDTA capillary tubes at various time points (0.5 to 30 min) after carbon injection. Carbon particle clearance rates were calculated for each animal. The results are expressed as the means ± standard errors of the individual $t_{1/2}$ values. *, $P = 0.018$ versus control (Mann-Whitney *U* test); **, $P = 0.005$ versus LPS alone or Hb alone (Mann-Whitney *U* test).

ticle clearance (decreased $t_{1/2}$ of clearance) was observed in animals treated with Hb alone compared to untreated animals ($P = 0.018$), indicating stimulation of reticuloendothelial cell function by Hb (Fig. 7). In animals treated with LPS alone, the mean clearance rate was slightly increased but not significantly different from that in untreated animals ($P = 0.21$). In animals treated with both LPS and Hb, the mean clearance rate was significantly decreased (longer $t_{1/2}$) compared to that of the LPS-treated animals ($P = 0.005$), although clearance was not significantly different from that in untreated animals ($P = 0.19$). Therefore, we were able to demonstrate an association between the synergistic lethality produced by LPS and Hb, compared to the lethality of LPS alone, and decreased reticuloendothelial cell function.

Plasma glucose concentrations. Following intraperitoneal LPS injection in subsequently fasted mice, there was a progressive decrease in plasma glucose levels to a broad nadir between 16 and 32 h, followed by a gradual, partial return toward normal at 48 h (Fig. 8). Plasma glucose levels at each time point after 4 h were considerably lower in LPS-treated mice than in fasted control animals ($P < 0.05$). However, at each of the time points tested, there was no difference in plasma glucose concentrations between animals treated with LPS only and those which received LPS plus Hb.

Blood cultures. We considered the possibility that the combination of LPS and Hb had damaged the normal barriers to bacteria entering the bloodstream from the gastrointestinal tract, thus resulting in bacterial sepsis. Blood cultures were obtained from 12 mice which had received both intraperitoneal LPS and intravenous Hb at 26 or 36 h after LPS, times at which mice prominently exhibited the effects of endotoxemia and were beginning to die. Only 1 of 12 blood samples was shown to contain gram-negative bacteria; the remaining cultures remained sterile for 30 days. Therefore, the development of bacterial sepsis was not a significant mechanism of lethality in these mice.

Tissue histology. Histologic evaluation failed to demonstrate any evidence of inflammation, hemorrhage, necrosis, or thrombosis in sections of liver, kidney, lung, brain, ovary, uterus, heart, or skeletal muscle from mice in any treatment group (LPS, Hb, or LPS-Hb). Adrenal glands were also normal, with no evidence of hemorrhage or necrosis. Karyorrhexis, i.e., de-

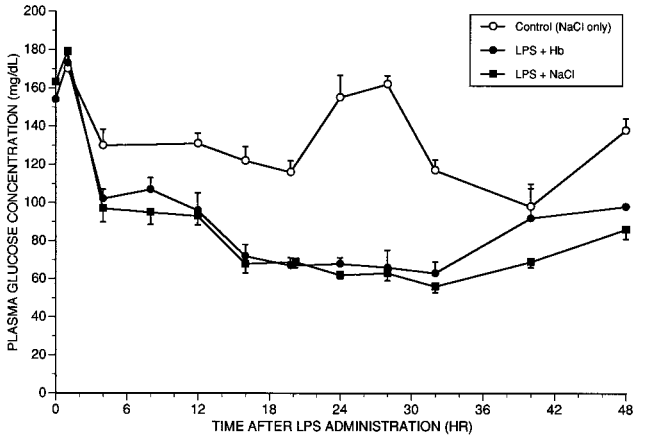

FIG. 8. Plasma glucose concentrations in the absence or presence of ααHb. Swiss Webster female mice (28 to 32 g) were simultaneously injected intraperitoneally with 0.5 mg of LPS (*E. coli* O55:B5 LPS, in sterile, pyrogen-free saline) and intravenously with either 0.6 to 0.8 ml of saline (■; $n = 10$) or ααHb (60 mg/mouse) (●; $n = 13$). All animals were fasted after the beginning of the experiment. Blood for glucose levels was serially obtained in EDTA capillary tubes at various time points after LPS injection. Fasted control mice (○; $n = 8$) received intravenous saline only. Data are expressed as the means ± standard errors of the combination of two independent experiments, each of which demonstrated the differences shown.

struction of nuclei with resultant nuclear fragmentation products, was noted in the splenic white pulp of LPS-treated mice and was even more prominent in LPS-Hb-treated mice; however, there was no acute inflammation (observations not shown).

DISCUSSION

The basis for our studies was the recent recognition that Hb is an LPS-binding protein that, in vitro, enhances many biological activities of LPS (11, 12, 23, 25). A major anticipated use of cell-free Hb as an erythrocyte substitute would be for the emergency treatment of patients with hemorrhage. Associated medical conditions in these patients will likely include severe trauma, gastrointestinal tract ischemia, and bacterial infection. Because of the common occurrence of endotoxemia during these underlying medical conditions, we hypothesized that infusion of Hb into endotoxemic animals might be detrimental. Our studies, which involved large numbers of animals and many independent lethality assays, demonstrated that mortality was significantly greater in mice which received both LPS and Hb than in mice treated with LPS alone. For the majority of our lethality assays, the experimental conditions were purposely chosen such that Hb was given as a bolus infusion into animals with preexisting endotoxemia, after LPS had been absorbed into the bloodstream from an extravascular site (in these studies, the peritoneal cavity) over a prolonged period of time. However, increased toxicity also was observed both in experimental models in which LPS was administered to animals coincident with Hb or after prior Hb infusion and clearance of approximately half of the infused Hb. This is important because endotoxemia in sick patients is characteristically intermittent and prolonged in many clinical scenarios, and the infusion of solutions of Hb into patients with preexisting, coincident, or subsequent endotoxemia is likely in clinical practice. Enhanced lethality was directly related to the infused dose of Hb; synergism was clearly observed with intravenous Hb infusions that generated clinically useful initial plasma Hb concentrations of $\geq$3 g/dl. The synergistic effect of Hb on LPS mortality was a generalized property of all Hb preparations tested, including non-cross-linked HbA_0, α-α-cross-linked preparations, and β-β-cross-linked preparations, but was not observed with the control protein albumin. Therefore, our studies extend the previous observations of White et al. (30) that the intravenous administration of both Hb and LPS was more toxic than administration of LPS alone.

Although Hb alone was well tolerated, it was not certain that the synergistic mortality observed in mice treated with both Hb and LPS was caused solely by an LPS-dependent mechanism. Specifically, it was possible that the process of LPS-induced oxidation of Hb which we recently demonstrated (13) is deleterious in vivo and that Hb-related reactions (e.g., formation of methemoglobin and hemichromes or release of toxic free iron and/or free heme) contribute to mortality. To investigate this possibility, we studied mortality produced by Hb and LPS in a strain of mouse (C3H/HeJ) which is relatively insensitive to LPS, requiring approximately 20 to 50 times more LPS than wild-type mice to obtain a 50% lethal dose (22, 27) and thereby making possible the detection of any Hb-based pathologic effects. There was only a low level of toxicity in C3H/HeJ mice given both LPS and Hb, indicating that any LPS-induced products of Hb oxidation that might have been generated in vivo were not sufficient to account for the high mortality in normal mice that received both LPS and Hb. These results suggested that mortality in the normal endotoxin-sensitive mice was due to LPS-related rather than Hb-related pathophysiological responses.

A series of experiments was performed to evaluate several possible mechanisms for the observed synergistic toxicity of Hb and LPS. The indication that mortality was based on LPS-related pathophysiological responses (rather than Hb-related oxidative processes) and the LPS-binding property of Hb suggested that alteration by Hb of LPS absorption and/or clearance, leading to higher LPS blood levels, might provide a possible mechanism for Hb enhancement of LPS mortality. Therefore, we evaluated whether Hb infusion 8 to 10 h after LPS administration (the standard conditions used to obtain the data presented in Fig. 1 and 2) resulted in LPS plasma levels that were higher than in the absence of Hb. However, at all time points examined, the ranges of plasma LPS concentrations were not different between mice which received LPS only and those which received LPS plus Hb. Therefore, there was no evidence that Hb infusion influenced either the continuing absorption of LPS or its clearance, and accordingly, enhanced mortality could not be correlated with plasma LPS levels. Interestingly, in both groups there was a nadir in the plasma LPS concentrations at 20 h after intraperitoneal injection, followed by increased LPS levels in several mice at 34 and 42 h. Many of these animals appeared extremely toxic at these late time points. We speculate that the high LPS levels that developed later than 24 h after administration of LPS might result from a damaged or inflamed peritoneum that had become more permeable to the injected endotoxin, thus permitting increased entry of LPS into the circulation. Perhaps continued absorption of the intraperitoneally injected LPS in association with a decreased intravascular clearance rate accounts for the elevated levels. Alternatively, at the later time points, the high LPS levels might reflect translocation of gut-derived LPS into the circulation. Interestingly, translocation of live bacteria from the gastrointestinal tract into the bloodstream, a process which could potentially result in lethal bacterial sepsis, was a rare event.

The $t_{1/2}$ of a bolus infusion of Hb was approximately doubled in mice pretreated with LPS (0.5 mg). This prolonged persistence of Hb in the presence of LPS suggested that intravascular clearance mechanisms were abnormal in these mice. Since the cross-linked preparation of Hb used in these experiments is primarily cleared by tissue macrophages (with negligible formation of dimers which could lead to renal clearance), our results suggested that the combination of LPS and Hb might have resulted in depression of normal reticuloendothelial system clearance mechanisms. Attenuated killing of microorganisms by Kupffer cells has previously been proposed as a mechanism for enhanced mortality from gram-negative bacteria in the presence of Hb (14, 15). Furthermore, sensitivity of animals to LPS (e.g., increased pyrogenic response) has previously been associated with depressed reticuloendothelial cell function (e.g., after Thorotrast blockade) (1), although other studies have found an association between sensitivity to LPS (e.g., enhanced mortality) and stimulated reticuloendothelial system function (e.g., after glucan [6] or glycerol trioleate [26] administration). We reasoned that if initial uptake of LPS and Hb saturated these cells sufficiently to subsequently decrease their particle clearance capacity, the cells might not function normally, and depressed reticuloendothelial cell function then could contribute to the observed enhancement by Hb of LPS-induced mortality. To further examine this possibility, we studied the influence of LPS alone, Hb alone, and LPS plus Hb on reticuloendothelial cell function as measured by clearance of particulate carbon. Hb alone stimulated carbon clearance, a result in agreement with the previously reported effect of po-

lymerized Hb on reticuloendothelial cell function (20). Intraperitoneal LPS alone had no demonstrable effect in our study of reticuloendothelial cell function, although high doses of LPS administered intravenously are known to depress the reticuloendothelial cell system (2). However, the combination of Hb and LPS produced a depression in reticuloendothelial cell function compared to the effect of LPS alone, suggesting that reticuloendothelial system dysfunction may contribute to the observed synergistic mortality that followed administration of Hb plus LPS. Nevertheless, it remains uncertain whether a direct toxic effect of Hb and LPS on the reticuloendothelial cells, or rather a decrease in hepatic and splenic perfusion, resulted in the depressed reticuloendothelial cell function associated with the combined administration of LPS and Hb.

Since we had shown previously that cellular responses to LPS, in vitro, are enhanced by LPS (11, 23, 25), we thought it possible that Hb might result in increased mortality by augmenting the hypoglycemia associated with LPS. Animals were fasted during this experiment to ensure that the sicker LPS-Hb group of mice did not have caloric deprivation different from that of the LPS-only group of mice. The two groups demonstrated similar levels of hypoglycemia in excess of that found in fasted control mice, with no effect of Hb. Interestingly, both groups also demonstrated partial recovery of glucose levels after 30 h, and therefore it is not certain the extent to which hypoglycemia contributed to mortality in either group of mice.

Finally, since both Hb and LPS are cleared primarily via Kupffer cells and splenic macrophages, and these cells are major sources of pathologic inflammatory mediators, it is possible that Hb and LPS cleared by an altered reticuloendothelial system are processed slowly, leading to their prolonged interaction with reticuloendothelial cells and resultant enhancement of deleterious host cytokine responses. We have begun to study the influence of Hb on the production of tumor necrosis factor (TNF) as a likely candidate for such a cytokine-mediated mechanism. TNF is produced early in the response to LPS and is believed to be a major mediator of the diverse pathophysiologic responses to LPS (3, 21, 28). In preliminary experiments, we have observed higher plasma TNF levels in mice which received both LPS and Hb than in mice treated with LPS only. These preliminary findings need to be confirmed and expanded, and additional investigations will be required to assess whether differences in production of other proinflammatory and/or anti-inflammatory cytokines provide additional mechanisms for the synergistic toxicity of Hb and LPS.

In conclusion, LPS-related mortality is greatly increased by a variety of forms of cell-free Hb. However, the mechanism for this effect remains unclear. This finding is of clinical interest because of the likely presence of endotoxemia in many patients who would be transfused with solutions of Hb. It is also possible that an interaction between Hb and LPS contributes to the Hb-enhanced mortality associated with gram-negative bacterial sepsis which has recently been demonstrated with both native (unmodified) and polymerized pyridoxalated Hb (8).

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REFERENCES

1. **Beeson, P. B.** 1947. Tolerance to bacterial pyrogens. II. Role of the reticuloendothelial system. J. Exp. Med. **86:**39–44.
2. **Benacerraf, B., and M. M. Sebestyen.** 1957. Effect of bacterial endotoxins on the reticuloendothelial system. Fed. Proc. **16:**860–867.
3. **Beutler, B., I. W. Milsark, and A. Cerami.** 1985. Passive immunization against cachectin/tumor necrosis factor protects mice from lethal effect of endotoxin. Science **229:**869–871.
4. **Bucci, E., A. Razynska, H. Kwansa, H. Matheson-Urbaitis, M. O'Hearn, J. A. Ulatowski, and R. C. Koehler.** 1996. Production and characteristics of an infusable oxygen carrying fluid based on hemoglobin intramolecularly crosslinked with sebacic acid. J. Lab. Clin. Med. **128:**146–153.
5. **Christensen, S. M., F. Medina, R. M. Winslow, S. M. Snell, A. Zegna, and M. A. Marini.** 1988. Preparation of human hemoglobin A_0 for possible use as a blood substitute. J. Biochem. Biophys. Methods **17:**145–154.

5a. **Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council.** 1996. Guide for the care and use of laboratory animals. National Academy Press, Washington, D.C.

6. **Crafton, C. G., and N. R. Di Luzio.** 1969. Relationship of reticuloendothelial functional activity to endotoxin lethality. Am. J. Physiol. **217:**736–742.
7. **Eaton, J. W., P. Brandt, and J. R. Mahoney.** 1982. Haptoglobin: a natural bacteriostat. Science **215:**691–693.
8. **Griffiths, E., A. Cortes, N. Gilbert, P. Stevenson, S. MacDonald, and D. Pepper.** 1995. Haemoglobin-based blood substitutes and sepsis. Lancet **345:** 158–160.
9. **Hau, T., and R. L. Simmons.** 1980. Mechanisms of the adjuvant effect of hemoglobin in experimental peritonitis. III. The influence of hemoglobin on phagocytosis and intracellular killing by human granulocytes. Surgery **87:** 588–592.
10. **Hau, T., R. D. Nelson, V. D. Fiegel, R. Levenson, and R. L. Simmons.** 1977. Mechanisms of the adjuvant action of hemoglobin in experimental peritonitis. J. Surg. Res. **22:**174–180.
11. **Kaca, W., R. I. Roth, A. Ziolkowski, and J. Levin.** 1994. Human hemoglobin increases the biological activity of bacterial lipopolysaccharides in activation of limulus amebocyte lysate and stimulation of tissue factor production by endothelial cells. J. Endotoxin Res. **1:**243–252.
12. **Kaca, W., R. I. Roth, and J. Levin.** 1994. Hemoglobin: a newly recognized lipopolysaccharide (LPS) binding protein which enhances LPS biological activity. J. Biol. Chem. **269:**25078–25084.
13. **Kaca, W., R. I. Roth, K. D. Vandegriff, G. C. Chen, F. A. Kuypers, R. M. Winslow, and J. Levin.** 1995. Effects of bacterial endotoxin on human cross-linked and native hemoglobins. Biochemistry **34:**11176–11185.
14. **Kaye, D., and E. W. Hook.** 1963. The influence of hemolysis on susceptibility to Salmonella infection: additional observations. J. Immunol. **91:** 518–527.
15. **Kaye, D., and E. W. Hook.** 1963. The influence of hemolysis or blood loss on susceptibility to infections. J. Immunol. **91:**65–75.
16. **Langermans, J. A. M., and W. K. Bleeker.** 1995. Haemoglobin-based blood substitutes and infection. Lancet **345:**863–864.
17. **Langermans, J. A. M., M. E. B. van Vuren-van der Hulst, and W. K. Bleeker.** 1996. Safety evaluation of a polymerized hemoglobin solution in a murine infection model. J. Lab. Clin. Med. **127:**428–434.
18. **Lee, J. T., D. H. Ahrenholz, R. D. Nelson, and R. L. Simmons.** 1979. Mechanisms of the adjuvant effect of hemoglobin in experimental peritonitis. V. The significance of the coordinated iron component. Surgery **86:**41–48.
19. **Litwin, M. S., C. W. Walter, P. Ejarque, and E. S. Reynolds.** 1963. Synergistic toxicity of gram-negative bacteria and free colloidal hemoglobin. Ann. Surg. **157:**485–493.
20. **Marks, D. H., J. Patressi, and I. H. Chaudry.** 1985. Effect of pyridoxalated stabilized stroma-free hemoglobin solution on the clearance of intravascular lipid by the reticuloendothelial system. Circ. Shock **16:**165–172.
21. **Michie, H. R., K. R. Manogue, D. R. Spriggs, A. Revhaug, S. O'Dwyer, S. M. Wolff, and D. W. Wilmore.** 1988. Detection of circulating tumor necrosis factor after endotoxin administration. N. Engl. J. Med. **318:**1481–1486.
22. **Morrison, D. C.** 1986. C3H/HeJ mouse strain: role in elucidation of host response to bacterial endotoxins, p. 23–28. *In* P. Bonventre (ed.), Microbiology. American Society for Microbiology, Washington, D.C.
23. **Roth, R. I.** 1994. Hemoglobin enhances the production of tissue factor by endothelial cells in response to bacterial endotoxin. Blood **83:**2860–2865.
24. **Roth, R. I.** 1996. Hemoglobin enhances the binding of bacterial endotoxin to human endothelial cells. Thromb. Haemostasis **76:**258–262.
25. **Roth, R. I., J. Levin, K. W. Chapman, M. Schmeizl, and F. R. Rickles.** 1993. Production of modified crosslinked cell-free hemoglobin for human use: the

role of quantitative determination of endotoxin contamination. Transfusion **33:**919–924.
26. **Stuart, A. E., and G. N. Cooper.** 1962. Susceptibility of mice to bacterial endotoxin after modification of reticuloendothelial function by simple lipids. J. Pathol. Bacteriol. **83:**245–254.
26a. **Su, D., R. I. Roth, and J. Levin.** 1995. Hemoglobin enhances the mortality from bacterial endotoxin. Blood **86**(Suppl. 1)**:**445a.
27. **Sultzer, B. M.** 1968. Genetic control of leucocyte responses to endotoxin. Nature **219:**1253–1254.
28. **Tracey, K. J., B. Beutler, S. F. Lowry, J. Merryweather, S. Wolpe, I. W. Milsark, R. J. Hariri, T. J. Fahey III, A. Zentella, J. D. Albert, G. T. Shires, and A. Cerami.** 1986. Shock and tissue injury induced by recombinant human cachectin. Science **234:**470–474.
29. **Ulatowski, J. A., E. Bucci, T. Nishikawa, A. Razynska, M. A. Williams, R. Takeshima, R. J. Traystman, and R. C. Koehler.** 1996. Cerebral O_2 transport with hematocrit reduced by cross-linked hemoglobin transfusion. Am. J. Physiol. **270**(Heart Circ. Physiol. 39)**:**H466–H475.
30. **White, C. T., A. J. Murray, D. J. Smith, J. R. Greene, and R. B. Bolin.** 1986. Synergistic toxicity of endotoxin and hemoglobin. J. Lab. Clin. Med. **108:** 132–137.
31. **Winslow, R. M., and K. W. Chapman.** 1994. Pilot-scale preparation of hemoglobin solutions. Methods Enzymol. **231:**3–16.

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