1991); G. M. Kaminska and J. Y. Niederkorn, *Investig. Ophthalmol. Vis. Sci.* **34**, 222 (1993).
5. A. P. Schachat and R. B. Murphy, Eds., *Retina* (Mosby, Baltimore, MD, ed. 2, 1994).
6. H.-J. Xu *et al.*, *Cancer Res.* **51**, 4481 (1991).
7. PEDF was purified from WERI-Rb-27R (*6*) serum-free conditioned media by sequential steps consisting of dialysis (molecular mass cutoff, 30 kD) against distilled water, 60 to 95% ammonium sulfate precipitation, step elution from lentil lectin Sepharose 4B (Pharmacia) with 0.5 M α-methyl-D-mannopyranoside, and elution from a HiTrap heparin Sepharose column (Pharmacia) with increasing NaCl gradient. Purification was monitored by an endothelial cell migration assay (*26*), and the yield was 17.5%. Edman degradation of proteolytically derived internal peptides of the protein yielded two unambiguous sequences (TSLEDFYLDEERTVRVPMMXD and IAQLPLTGXM) (*27*). A BLAST protein homology search revealed that PEDF contains identical sequences.
8. S. P. Beccerra, in *Chemistry and Biology of Serpins*, F. C. Church *et al.*, Eds. (Plenum, New York, 1997), pp. 223–237.
9. J. Tombran-Tink, G. J. Chader, L. V. Johnson, *Exp. Eye Res.* **53**, 411 (1991); F. R. Steele *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1526 (1993).
10. T. Tanawaki, S. P. Becerra, G. J. Chader, J. P. Schwartz, *J. Neurochem.* **64**, 2509 (1995); Y. Sugita *et al.*, *J. Neurosci. Res.* **49**, 710 (1997).
11. R. J. Pignolo, V. J. Cristofalo, M. O. Rotenberg, *J. Biol. Chem.* **268**, 8949 (1993); J. Tombran-Tink *et al.*, *J. Neurosci.* **15**, 4992 (1995).
12. S. P. Becerra *et al.*, *J. Biol. Chem.* **268**, 23148 (1993); S. P. Becerra *et al.*, *ibid.* **270**, 25992 (1995).
13. D. W. Dawson, O. V. Volpert, P. Gillis, unpublished data.
14. Human PEDF cDNA was engineered by polymerase chain reaction to encode a COOH-terminal hexahistidine tag, cloned into pCEP4 (Invitrogen), and transfected into human embryonic kidney cells. Recombinant PEDF was purified from the conditioned media with the Xpress Protein Purification System (Invitrogen).
15. See supplemental figures, available at www.sciencemag.org/feature/data/1040070
16. For preparation of stromal extract, corneas were freed of associated epithelium and as much of the endothelium as possible, washed extensively in ice-cold phosphate-buffered saline (PBS, pH 7.4), and minced into small fragments that were incubated for 24 hours in PBS containing 0.5 mM phenylmethanesulfonyl fluoride. The extract was filter sterilized, stored at −80°C, and tested in migration assays at a final concentration of 10 μg of protein per milliliter.
17. Y.-Q. Wu and S. P. Becerra, *Investig. Ophthalmol. Vis. Sci.* **37**, 1984 (1996).
18. L. P. Aiello *et al.*, *N. Engl. J. Med.* **331**, 1480 (1994); A. P. Adamis *et al.*, *Am. J. Ophthalmol.* **118**, 445 (1994).
19. N. Ogata *et al.*, *Curr. Eye Res.* **16**, 9 (1997); K. Hayasaka *et al.*, *Life Sci.* **63**, 1089 (1998); S. A. Vinores *et al.*, *J. Neuroimmunol.* **89**, 43 (1998).
20. L. P. Aiello *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10457 (1995); A. P. Adamis *et al.*, *Arch. Ophthalmol.* **114**, 66 (1996); J. M. Provis *et al.*, *Exp. Eye Res.* **65**, 555 (1997).
21. L. E. H. Smith *et al.*, *Invest. Ophthalmol. Vis. Sci.* **35**, 101 (1994).
22. E. A. Pierce, E. D. Foley, L. E. Smith, *Arch. Ophthalmol.* **114**, 1219 (1996).
23. S. E. Connolly *et al.*, *Microvasc. Res.* **36**, 275 (1988).
24. M. A. Goldberg, S. P. Dunning, H. F. Bunn, *Science* **242**, 1412 (1988).
25. C. J. Gulledge and M. W. Dewhirst, *Anticancer Res.* **16**, 741 (1996).
26. Migration assays were performed in quadruplicate for each sample with bovine adrenal capillary endothelial cells or human dermal microvascular endothelial cells (Clonetics, San Diego, CA) as described (*28*). To combine multiple experiments, we first subtracted background migration (Bkgd) toward vehicle (0.1% bovine serum albumin) and then normalized data by setting maximum migration toward inducer alone to 100%. All experiments were repeated two to five times. Statistics were performed on raw data before normalization with the Student's *t* test. Standard errors were converted to percentages.
27. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; D, Asp; E, Glu; F, Phe; G, Gly; I, Ile; L, Leu; M, Met; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; X, any amino acid; and Y, Tyr.
28. P. J. Polverini, N. P. Bouck, F. Rastinejad, *Methods Enzymol.* **198**, 440 (1991).
29. PEDF antipeptide antibody (anti-PEDF) was raised in rabbits against a peptide containing PEDF amino acids 327 to 343, conjugated to Keyhole-limpet hemocyanin, and affinity-purified on a peptide column. Polyclonal antisera against bacterial recombinant PEDF/EPC-1 (anti–EPC-1) [B. R. DiPaolo, R. J. Pignolo, V. J. Cristofalo, *Exp. Cell Res.* **220**, 178 (1995)] and the antiangiogenic protein angiostatin [M. S. O'Reilly *et al.*, *Cell* **79**, 315 (1994)] were gifts. Purchased reagents included neutralizing anti-VEGF (Genzyme, Cambridge, MA), pan antibodies to TGFβ, and all angiogenic inducers (R & D Systems, Minneapolis, MN) except lysophosphatidic acid (Sigma). All proteins and antibodies were extensively dialyzed against PBS before use in biological assays.
30. Human vitreous fluid was withdrawn from three cadaveric eyes (refrigerated within 1.4 to 4.5 hours of death) obtained from individuals without ocular disease. Fluid was frozen until used. Fresh vitreous fluid was obtained from bovine and mouse eyes.
31. We thank A. Mountz for VEGF measurements; B. Kennedy and the Midwest Eye Banks and Transplantation Center for human eye tissue; M. K. Francis and V. Cristofalo for anti–EPC-1; M. O'Reilly and J. Folkman for bovine capillary endothelial cells and angiostatin; and C. Hawkins, R. O'Grady, and Y. Mu for assistance with retinoblastomas. Supported by the National Eye Institute, the Retina Research Foundation, the National Cancer Institute, and the Chicago Baseball Charities.

15 March 1999; accepted 3 June 1999

HMG-1 as a Late Mediator of Endotoxin Lethality in Mice

Haichao Wang,[1,3]* Ona Bloom,[3] Minghuang Zhang,[3] Jaideep M. Vishnubhakat,[3] Michael Ombrellino,[2,3] Jiantu Che,[3] Asia Frazier,[2,3] Huan Yang,[3] Svetlana Ivanova,[3] Lyudmila Borovikova,[3] Kirk R. Manogue,[3] Eugen Faist,[4] Edward Abraham,[5] Jan Andersson,[6] Ulf Andersson,[7] Patricia E. Molina,[2] Naji N. Abumrad,[2] Andrew Sama,[1] Kevin J. Tracey[2,3]

Endotoxin, a constituent of Gram-negative bacteria, stimulates macrophages to release large quantities of tumor necrosis factor (TNF) and interleukin-1 (IL-1), which can precipitate tissue injury and lethal shock (endotoxemia). Antagonists of TNF and IL-1 have shown limited efficacy in clinical trials, possibly because these cytokines are early mediators in pathogenesis. Here a potential late mediator of lethality is identified and characterized in a mouse model. High mobility group–1 (HMG-1) protein was found to be released by cultured macrophages more than 8 hours after stimulation with endotoxin, TNF, or IL-1. Mice showed increased serum levels of HMG-1 from 8 to 32 hours after endotoxin exposure. Delayed administration of antibodies to HMG-1 attenuated endotoxin lethality in mice, and administration of HMG-1 itself was lethal. Septic patients who succumbed to infection had increased serum HMG-1 levels, suggesting that this protein warrants investigation as a therapeutic target.

Mortality rates for systemic bacterial infection have not declined significantly, despite advances in antibiotic therapy and intensive care. Bacteria do not directly cause lethal shock and tissue injury. Rather, bacterial endotoxin (lipopolysaccharide, LPS) stimulates the acute, early release of cytokines such as TNF and IL-1β from macrophages, and it is these host products that mediate damage (*1*). Macrophages from C3H/HeJ mice do not release TNF and IL-1 when stimulated by LPS; these animals are resistant to LPS lethality (*2*). Normal, LPS-responsive mice can be protected from lethal endotoxemia by therapeutic agents that selectively inhibit cytokine action or prevent cytokine release (*3*).

Translating these pathogenic insights into clinical therapy has proved difficult, in part because these "early" mediators (TNF and IL-1) are released within minutes after LPS exposure (*4*). Thus, even a minimal delay in treatment directed against TNF or IL-1 is ineffective (*3, 5*). Paradoxically, LPS-responsive mice treated with lethal doses of LPS succumb at latencies of up to 5 days, long

[1]Department of Emergency Medicine and [2]Department of Surgery, North Shore University Hospital–New York University School of Medicine, Manhasset, NY 11030, USA. [3]The Picower Institute for Medical Research, Manhasset, NY 11030, USA. [4]Department of Surgery, Klinicum Grosshadern, Ludwig-Maximilians University, Munich, Germany. [5]Division of Pulmonary Sciences and Critical Care Medicine, University of Colorado Health Sciences Center, Denver, CO 80262, USA. [6]Department of Infectious Disease, Karolinska Institute, Huddinge University Hospital, Stockholm, Sweden. [7]Department of Rheumatology, Astrid Lindgren's Children's Hospital, Karolinska Institute, Stockholm, Sweden.

*To whom correspondence should be addressed. E-mail: hwang@picower.edu

after serum TNF and IL-1 have returned to basal levels. Moreover, mice deficient in TNF die within several days of LPS administration (*6*), suggesting that mediators other than TNF might contribute causally to endotoxin-induced death.

To identify potential "late" mediators of endotoxemia, we stimulated murine macrophage-like RAW 264.7 cells with LPS and analyzed the conditioned culture medium by SDS–polyacrylamide gel electrophoresis (PAGE). LPS stimulation for 18 hours induced the appearance of a 30-kD protein that was not apparent at earlier time points. The NH_2-terminal sequence of this late-appearing factor (Gly-Lys-Gly-Asp-Pro-Lys-Lys-Pro-Arg-Gly-Lys-Met-Ser-Ser) was identical to murine HMG-1, a 30-kD member of the high mobility group (HMG) nonhistone chromosomal protein family (*7, 8*). Based on the HMG-1 sequence in GenBank (accession no. M64986), we designed primers and isolated HMG-1 cDNA after polymerase chain reaction (PCR) amplification. Recombinant HMG-1 (rHMG-1) protein was expressed in *Escherichia coli*, purified to homogeneity, and used to generate polyclonal antibodies (*9*).

Immunoblot analysis revealed that large amounts of HMG-1 were released from RAW 264.7 cells in a time-dependent manner (Fig. 1A), beginning 6 to 8 hours after stimulation with LPS. Cell viability, as judged by trypan blue exclusion and lactate dehydrogenase release, was unaffected by LPS concentrations that induced the release of HMG-1, indicating that HMG-1 release was not due to cell death. HMG-1 mRNA levels were unaffected by LPS treatment (Fig. 1B), indicating that HMG-1 release is unlikely to be linked to increased transcription of the gene. Stimulation of RAW 264.7 cells for 18 hours with TNF (5 to 100 ng/ml) or IL-1β (5 to 100 ng/ml) also induced HMG-1 release in a cytokine dose-dependent manner. In contrast, stimulation with interferon-γ (IFN-γ) alone did not induce HMG-1 release, even at concentrations up to 100 U/ml; however, IFN-γ increased by three- to fivefold the amount of HMG-1 released by stimulation with either TNF or IL-1 (*10, 11*). Pulse labeling experiments with ^{35}S-methionine revealed that most of the HMG-1 released during the first 12 hours after TNF and IFN-γ stimulation was derived from a preformed protein pool. Radioactivity was incorporated into newly synthesized HMG-1 from 12 to 36 hours after macrophage stimulation (*10, 11*).

We next examined the inducible release of HMG-1 from other cell types. LPS triggered HMG-1 release from human primary peripheral blood mononuclear cells and primary macrophages from LPS-sensitive mice (C3H/HeN), but not from macrophages from LPS-resistant C3H/HeJ mice (*11, 12*). Human primary T cells, rat adrenal (PC-12) cells, and rat primary kidney cells did not release HMG-1 after stimulation with LPS, TNF, or IL-1β. Like other macrophage products (for example, TNF, IL-1β, and macrophage migration inhibitory factor), HMG-1 lacks a classical secretion signal sequence, so the mechanism of release remains to be determined.

To determine if HMG-1 was released systemically during endotoxemia in mice, we measured serum HMG-1 levels after LPS administration. Serum HMG-1 was readily detectable 8 hours after administration of a median lethal dose (LD_{50}) of LPS and was maintained at peak, plateau levels from 16 to 32 hours after LPS treatment (Fig. 1C). About 20 to 50 μg of HMG-1 was released into the murine circulation within 24 hours after endotoxin administration [assuming a distribution half-life ($t_{1/2}$) of 3 min and an elimination $t_{1/2}$ of 20 min]; this is comparable to the quantity of TNF and IL-1 released by LPS treatment. The kinetics of HMG-1 appearance in the blood of LPS-treated mice differs from that of previously described lethal LPS-induced mediators.

Passive immunization of unanesthetized mice with a single dose of antibodies to HMG-1 (anti–HMG-1) 30 min before a lethal dose (LD_{100}) of LPS did not prevent LPS-induced death (Fig. 2A). Based on the kinetics of HMG-1 accumulation in serum (Fig. 1C), and the relatively short biological half-life of antibodies to cytokines (*3, 13*), we reasoned that complete neutralization of a late-appearing mediator might require repeated dosing. Administration of anti–HMG-1 in two doses (one 30 min before LPS and one 12 hours after LPS) increased the survival rate of the mice to 30%. With three doses of antiserum (−30 min, +12 hours, +36 hours), 70% of the treated mice survived, as compared with 0% survival in controls treated with three matched doses of preimmune serum ($P < 0.05$). No late death occurred over 2 weeks, indicating that anti–HMG-1 did not merely delay the onset of LPS lethality, but provided lasting protection.

To investigate whether antibody treatment could be delayed until after administration of LPS, we injected anti–HMG-1 beginning 2 hours after LPS (followed by additional doses at 12 and 36 hours after LPS). This delayed treatment conferred significant protection against an LD_{100} of LPS (Fig. 2B). Preimmune

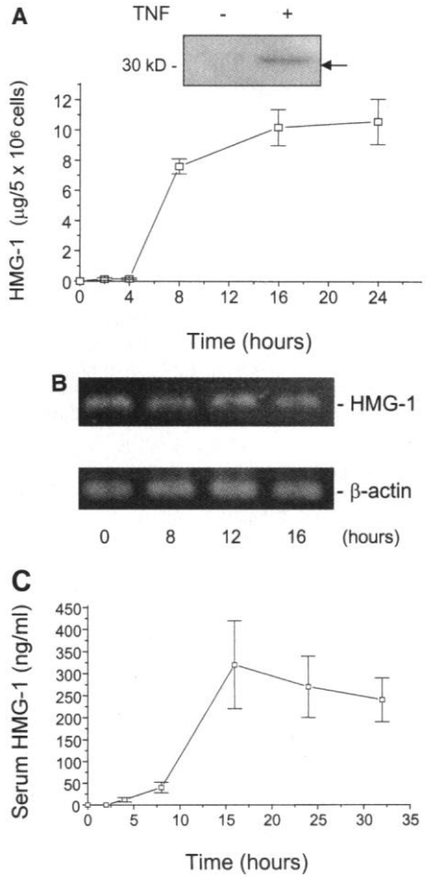

Fig. 1. (A) Release of HMG-1 from cultured macrophages after stimulation with LPS. Murine macrophage-like RAW 264.7 cells (American Type Culture Collection, Rockville, Maryland) were cultured in RPMI 1640 medium, 10% FBS, and 1% glutamine. When 70 to 80% confluence was reached, cells were resuspended in serum-free OPTI-MEM I medium and seeded onto tissue culture plates (5×10^6 cells per well). After 2 hours, RAW 264.7 cells were treated with LPS (*E. coli* 0111:B4, 100 ng/ml) and proteins in the cell-conditioned medium were fractionated by SDS-PAGE, excised from Coomassie- stained SDS-PAGE gels, and subjected to NH_2-terminal sequencing analysis (Commonwealth Biotechnologies, Richmond, Virginia). Polyclonal antisera against purified recombinant HMG-1 were generated in rabbits (Biosynthesis, Lewisville, Texas); immunoblotting showed that antiserum reacted with native HMG-1 released by RAW cells (inset). HMG-1 levels were measured by optical intensity of bands on immunoblots with NIH 1.59 image software, with reference to standard curves generated with purified rHMG-1. Data are shown as the mean ± SE (n = 3). **(B)** Expression of HMG-1 mRNA in macrophages. Murine macrophage-like RAW 264.7 cells were cultured in RPMI 1640, 10% FBS, and 1% glutamine, and stimulated with LPS (1 μg/ml) for 0, 8, 12, and 16 hours as indicated. Total RNA was isolated with the SV Total RNA Isolation System (Promega) and levels of HMG-1 mRNA were determined by reverse transcriptase (RT)–PCR with the Access RT-PCR System (Promega; β-actin primers, 5′-TCATGAAGTGTGACGTTGACATCCGT-3′ and 5′-CCTAGAAGCATTTGCGGTGCACGATG-3′; and HMG-1 primers, 5′-ATGGGCAAAGGAGATCCTA-3′ and 5′-ATTCATCATCATCATCTTCT-3′). **(C)** Accumulation of HMG-1 in serum of LPS-treated mice. Male Balb/C mice (20 to 23 g) were treated with LPS [10 mg/kg, intraperitoneally (ip)]. Serum was assayed for HMG-1 by immunoblotting; the detection limit is ~50 pg. Data are shown as the mean ± SE (n = 3).

serum–treated controls all developed lethargy, piloerection, and diarrhea before death, whereas anti–HMG-1–treated mice remained well groomed and active, had no diarrhea, and were viable. To clarify that anti–HMG-1 protected mice from LPS lethality, we purified the immunoglobulin G (IgG) fraction from anti–HMG-1 and administered it to mice exposed to an LD_{100} of LPS (*11, 14*). The highest dose of anti–HMG-1 IgG tested, 5 mg per mouse, conferred complete protection against an LD_{100} of LPS, whereas all control mice given comparable doses of rabbit IgG died (Table 1). Treatment with anti–HMG-1 IgG (2 mg per mouse) significantly reduced serum HMG-1 levels, whereas no reduction was observed after treatment with a lower dose of antibodies (0.5 mg per mouse) or with control IgG (5 mg per mouse). Antiserum against a chemically synthesized peptide corresponding to the first 12 amino acids of HMG-1 also significantly attenuated the lethality of endotoxemia in mice (*15*).

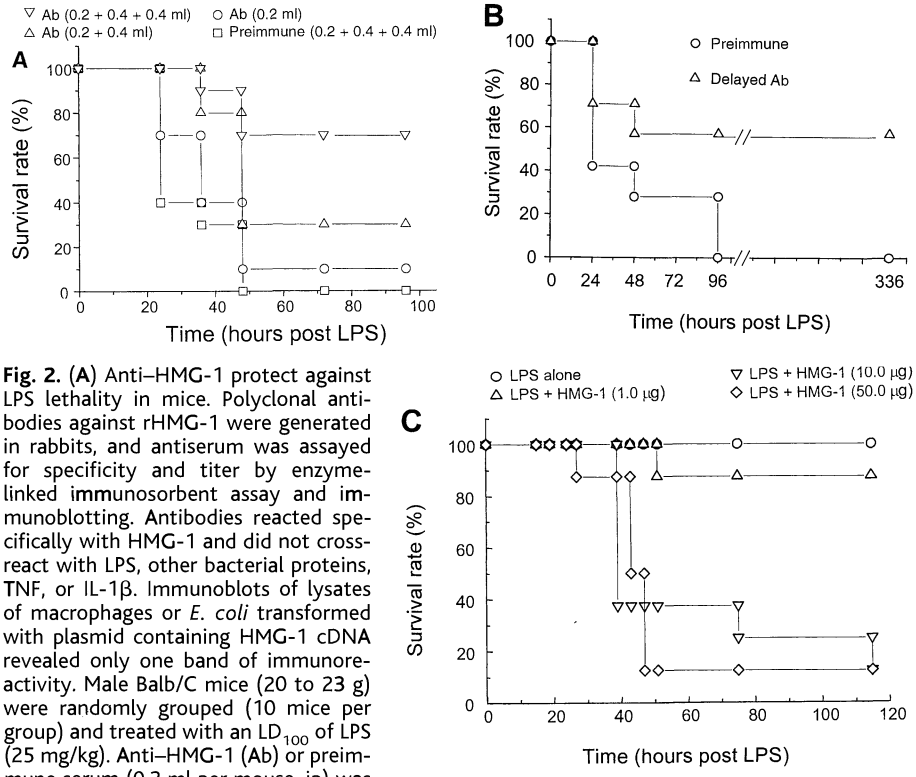

Fig. 2. (**A**) Anti–HMG-1 protect against LPS lethality in mice. Polyclonal antibodies against rHMG-1 were generated in rabbits, and antiserum was assayed for specificity and titer by enzyme-linked immunosorbent assay and immunoblotting. Antibodies reacted specifically with HMG-1 and did not cross-react with LPS, other bacterial proteins, TNF, or IL-1β. Immunoblots of lysates of macrophages or *E. coli* transformed with plasmid containing HMG-1 cDNA revealed only one band of immunoreactivity. Male Balb/C mice (20 to 23 g) were randomly grouped (10 mice per group) and treated with an LD_{100} of LPS (25 mg/kg). Anti–HMG-1 (Ab) or preimmune serum (0.2 ml per mouse, ip) was administered 30 min before LPS. Additional doses of preimmune (0.4 ml, ip) or anti–HMG-1 (0.4 ml, ip) were administered at 12 and 36 hours after LPS as indicated. (**B**) Delayed administration of anti–HMG-1 protects against LPS lethality in mice. Male Balb/C mice (20 to 23 g) were randomly grouped (seven mice per group) and treated with an LD_{100} of LPS. Anti–HMG-1 or preimmune serum (0.4 ml per mouse) was administered at 2, 24, and 36 hours after LPS. (**C**) Administration of rHMG-1 is lethal to mice. Recombinant HMG-1 was purified and LPS content determined by the Limulus Amoebocyte Lysate Test (Bio-Whittaker, Walkersville, Maryland). Purified rHMG-1 protein contained <2.5 ng of LPS per microgram of rHMG-1. Male Balb/C mice (20 to 23, 10 animals per group) were injected with a nonlethal dose of LPS (3.1 mg/kg, ip). Purified rHMG-1 protein was administered intraperitoneally in the doses indicated at 2, 16, 28, and 40 hours after LPS.

To determine if HMG-1 was toxic, we administered highly purified rHMG-1 to unanesthetized Balb/C mice (10 to 50 μg per mouse). Within 2 hours, the mice developed signs of endotoxemia, including lethargy, piloerection, and diarrhea. At higher doses (500 μg per mouse), three of five mice died at 18, 30, and 36 hours after rHMG-1 administration. Toxicity and lethality were not observed in control mice treated with a protein fraction purified from *E. coli* transformed with a plasmid devoid of HMG-1 cDNA (*9*), indicating that the toxicity we observed was specific to HMG-1. To exclude the possibility that endotoxin contamination of HMG-1 preparations mediated lethality, we injected rHMG-1 into LPS-resistant mice. rHMG-1 (500 μg per mouse) was lethal within 16 hours both to C3H/HeJ (n = 4) and C3H/HeN (n = 3) mice, indicating that HMG-1 itself is toxic even in the absence of LPS signal transduction. When sublethal doses of rHMG-1 were injected into Balb/C mice together with sublethal doses of LPS, the combined challenge was lethal to 90% of the mice, as compared with 0% lethality in mice exposed to LPS or HMG-1 alone (Fig. 2C). Thus, HMG-1 itself mediates lethality in both LPS-sensitive and LPS-resistant mice.

Animal models of human sepsis, including the murine endotoxemia model used here, have inherent limitations (*16*). As an initial step in determining whether HMG-1 participates in the pathogenesis of human sepsis, we studied 8

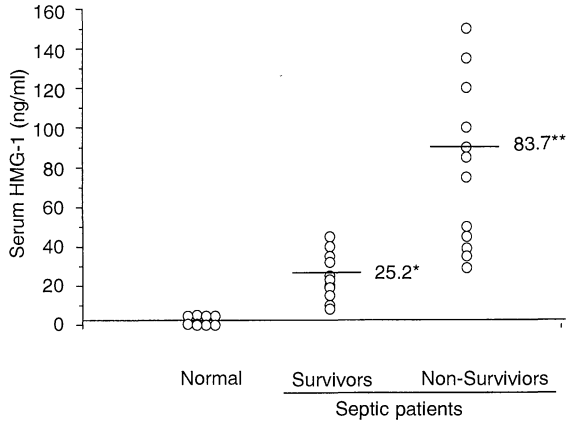

Fig. 3. Increased serum HMG-1 levels in human sepsis. Serum was obtained from 8 healthy subjects and 25 septic patients infected with Gram-positive [*Bacillus fragilis* (one patient), *Enterococcus faecalis* (one patient), *Streptococcus pneumoniae* (four patients), *Listeria monocytogenes* (one patient), or *Staphylococcus aureus* (two patients)], Gram-negative [*E. coli* (seven patients), *Klebsiella pneumoniae* (one patient), *Acinetobacter calcoaceticus* (one patient), *Pseudomonas aeroginosa* (one patient), *Fusobacterium nucleatum* (one patient), *Citrobacter freundii* (one patient)], or unidentified pathogens (five patients). Serum was fractionated by SDS-PAGE, and HMG-1 levels were determined by immunoblotting analysis with reference to standard curves of purified rHMG-1 diluted in normal human serum; the detection limit is ~50 pg. *P < 0.05 versus normal. **P < 0.05 versus survivors.

Table 1. Protection against LPS lethality by anti–HMG-1 IgG. Balb/C mice (male, 20 to 23 g, three to six mice per group) were injected intraperitoneally with IgG purified from anti–HMG-1 or control rabbit IgG 30 min before injection of an LD_{100} of LPS. All mice were then treated with additional doses of anti–HMG-1 IgG or control IgG at 12 and 24 hours after LPS. Serum HMG-1 levels were determined by immunoblots (under denaturing conditions) 14 hours after LPS challenge (n = 3 per group). ND, not determined.

Dose of HMG-1–immunoreactive IgG (mg/mouse)	Serum HMG-1 (ng/ml)	Survival 2 weeks after LPS (%)
0	1003 ± 180	0 (0/6)
0.5	1070 ± 20	17 (1/6)
2.0	415 ± 240	50 (3/6)*
5.0	ND	100 (3/3)*

*P < 0.05 versus treatment with control rabbit IgG.

normal subjects and 25 critically ill septic patients with bacteremia and sepsis-induced organ dysfunction. HMG-1 was not detectable in the serum of normal subjects, but significant levels were observed in critically ill patients with sepsis (Fig. 3), and these levels were higher in patients who succumbed as compared to patients with nonlethal infection.

HMG-1 is a highly conserved protein with >95% amino acid identity between rodent and human (*17–20*). It has previously been characterized as a nuclear protein that binds to cruciform DNA (*21*), and as a membrane-associated protein termed "amphoterin" that mediates neurite outgrowth (*19, 20*). Extracellular HMG-1 interacts directly with plasminogen and tissue type plasminogen activator (tPA), which enhances plasmin generation at the cell surface; this system plays a role in extracellular proteolysis during cell invasion and tissue injury (*19*). In addition, HMG-1 has been suggested to bind to the receptor for advanced glycation end products (RAGE) (*22*).

As with other inflammatory mediators such as TNF and IL-1, there may be protective advantages of extracellular HMG-1 when released in nontoxic amounts. Macrophages release HMG-1 when exposed to the early, acute cytokines, indicating that HMG-1 is also positioned as a mediator of other inflammatory conditions associated with increased levels of TNF and IL-1 (for example, rheumatoid arthritis and inflammatory bowel disease). Indeed, in most inflammatory scenarios, LPS is probably not the primary stimulus for HMG-1 release; it seems more likely that TNF and IL-1 function as upstream regulators of HMG-1 release. The delayed kinetics of HMG-1 release suggest that serum HMG-1 levels may be a convenient marker of disease severity. Moreover, the observations that HMG-1 itself is toxic, and that anti–HMG-1 prevents LPS lethality, point to HMG-1 as a potential target for therapeutic intervention.

References and Notes

1. K. J. Tracey *et al.*, *Science* **234**, 470 (1986); C. A. Dinarello, *FASEB J.* **8**, 1314 (1994); D. C. Morrison and J. L. Ryan, *Annu. Rev. Med.* **38**, 417 (1987); L. L. Moldawer, *Crit. Care Med.* **22**, S3 (1994).
2. B. Beutler, N. Krochin, I. W. Milsark, C. Luedke, A. Cerami, *Science* **232**, 977 (1986); J. W. Killion and D. C. Morrison, *Infect. Immun.* **54**, 1 (1986); A. Poltorak *et al.*, *Science* **282**, 2085 (1998).
3. K. J. Tracey *et al.*, *Nature* **330**, 662 (1987); M. Bianchi *et al.*, *J. Exp. Med.* **83**, 927 (1996); H. R. Alexander, G. M. Doherty, C. M. Buresh, D. J. Venzon, J. A. Norton, *J. Exp. Med* **173**, 1029 (1991); G. Wakabayashi, J. A. Gelfand, J. F. Burke, R. C. Thompson, C. A. Dinarello, *FASEB J.* **5**, 338 (1991).
4. D. G. Hesse *et al.*, *Surg. Gynecol. Obstet.* **166**, 147 (1988); H. R. Michie *et al.*, *N. Engl. J. Med.* **318**, 1481 (1988).
5. M. P. Fink, *Crit. Care Med.* **23**, 989 (1995); E. Abraham, *Chest* **113** (suppl.), 224S (1998).
6. M. W. Marino *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 8093 (1997); F. Amiot, C. Fitting, K. J. Tracey, J. M. Cavaillon, F. Dautry, *Mol. Med.* **3**, 864 (1997).
7. M. E. Bianchi, M. Beltrame, G. Paonessa, *Science* **243**, 1056 (1989); J. M. Walker, K. Gooderham, J. R. Hastings, E. Mayes, E. W. Johns, *FEBS Lett.* **122**, 264 (1980); L. Einck and M. Bustin, *Exp. Cell Res.* **156**, 295 (1985).
8. HMG-1 has also been termed "amphoterin" [J. Parkkinen *et al.*, *J. Biol. Chem.* **268**, 19726 (1993)]. HMG-1 and amphoterin are the same protein; we use the name "HMG-1" to reflect the original description of this protein as the first member of the HMG protein family.
9. HMG-1 was cloned by DNA amplification of the 648–base pair (bp) open reading frame from Rat Brain Quick-Clone cDNA (5 ng; Clontech, Palo Alto, CA) with the following primers: 5′-CCCGCGGATCCTCGAGGGAAGGATGGGCAAAGGAGATCCTA-3′ and 5′-CCCGCAAGCTTATTCATCATCATCATCTTCT-3′ (PCR at 94°C for 1 min, 56°C for 2 min, 72°C for 45 s; 30 cycles). The 680-bp PCR product was digested with Bam HI and Hind III and subcloned into the Bam HI–Hind III cloning sites of the pCAL-n vector (Stratagene, La Jolla, CA). The recombinant plasmid was transformed into *E. coli* BL21(DE3)pLysS (Novagen, Madison, WI), and positive clones were confirmed by DNA sequencing of both strands. Transformed cells were induced with isopropyl-D-thiogalactopyranoside, and rHMG-1 protein was purified with a calmodulin-binding resin column (Stratagene). As controls for experiments involving administration of rHMG-1 to mice, we purified proteins from *E. coli* BL21(DE3)pLysS that had been transformed with a plasmid that lacks the HMG-1 cDNA insert (pCAL-n). The amount of control material administered to mice was normalized to the number of *E. coli* that produce 0.5 mg of rHMG-1.
10. H. Wang and K. J. Tracey, unpublished observations.
11. Supplementary data can be found on *Science* Online at www.sciencemag.org/feature/data/1037699.shl.
12. Macrophages were obtained from the peritoneal cavity of LPS-sensitive (C3H/HeN and Balb/C) or LPS-resistant (C3H/HeJ) mice 4 days after intraperitoneal injection with 2.0 ml of thioglycollate broth (4%; Difco, Detroit, MI). Macrophages were pooled from four mice, resuspended into RPMI 1640, 10% fetal bovine solution (FBS), and 1% glutamine, and plated at a density of 4×10^6 cells per well in six-well Falcon Primaria tissue culture plates. After 24 hours, the culture medium was replaced with serum-free OPTI-MEM-I medium, and LPS (1 μg/ml) was added. The level of HMG-1 in the culture medium was determined 18 hours later by immunoblotting. HMG-1 was not detectable in culture medium of LPS-stimulated C3H/HeJ murine macrophages; HMG-1 levels reached 1 μg/10^6 cells in the culture medium of LPS-stimulated C3H/HeN murine macrophages.
13. L. B. Hinshaw *et al.*, *Circ. Shock* **30**, 279 (1990); C. Nathan and M. Sporn, *J. Cell Biol.* **113**, 981 (1991); D. G. Remick *et al.*, *Am. J. Pathol.* **136**, 49 (1990); R. E. Walker *et al.*, *J. Infect. Dis.* **174**, 63 (1996).
14. IgG was purified from anti–HMG-1 by Protein G–Sepharose HiTrap affinity chromatography (Pharmacia Biotech). Purified IgG fractions were desalted by ultrafiltration through Centricon-10 (Millipore), followed by two washes with 1× phosphate-buffered saline. The specificity of IgG was confirmed by immunoblot analysis of macrophage lysates, which revealed one band of 30 kD. Anti–HMG-1 IgG did not cross-react with LPS, TNF, IL-1, or bacterial proteins on immunoblots.
15. H. Wang and K. J. Tracey, unpublished observations.
16. M. P. Fink and S. O. Heard, *J. Surg. Res.* **49**, 186 (1990); K. J. Tracey, and E. Abraham, *SHOCK* **11**, 224 (1999); E. A. Deitch, *ibid.* **9**, 1 (1998).
17. K. L. Lee *et al.*, *Nucleic. Acids Res.* **15**, 5051 (1987); S. Ferrari, L. Ronfani, S. Calogero, M. E. Bianchi, *J. Biol. Chem.* **269**, 28803 (1994); S. Ferrari, P. Finelli, M. Rocchi, M. E. Bianchi, *Genomics* **35**, 367 (1996).
18. J. F. Maher and D. Nathans, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 6716 (1996).
19. J. Parkkinen *et al.*, *J. Biol. Chem.* **268**, 19726 (1993).
20. M. Salmivirta, H. Rauvala, K. Elenius, M. Jalkanen, *Exp. Cell Res.* **200**, 444 (1992).
21. M. E. Bianchi and M. Beltrame, *Am. J. Hum. Genet.* **63**, 1573 (1998); D. Landsman and M. Bustin, *Bioessays* **15**, 539 (1993); M. Bustin and N. K. Neihart, *Cell* **16**, 181 (1979).
22. O. Hori *et al.*, *J. Biol. Chem.* **270**, 25752 (1995); A. M. Schmidt *et al.*, *Diabetes* **45**, S77 (1996).

23. We thank C. Dang for technical assistance; J. Eaton, J. Roth, B. Sherry, M. Bukrinsky, and M. Symons for critical reading of the manuscript; and D. Prieto for administrative assistance.

11 December 1998; accepted 3 May 1999

Ploidy Regulation of Gene Expression

Timothy Galitski,[1] Alok J. Saldanha,[1,2] Cora A. Styles,[1] Eric S. Lander,[1,2] Gerald R. Fink[1,2]*

Microarray-based gene expression analysis identified genes showing ploidy-dependent expression in isogenic *Saccharomyces cerevisiae* strains that varied in ploidy from haploid to tetraploid. These genes were induced or repressed in proportion to the number of chromosome sets, regardless of the mating type. Ploidy-dependent repression of some G_1 cyclins can explain the greater cell size associated with higher ploidies, and suggests ploidy-dependent modifications of cell cycle progression. Moreover, ploidy regulation of the *FLO11* gene had direct consequences for yeast development.

Changes in the number of chromosome sets occur during the sexual cycle, during metazoan development, and during tumor progression. Organisms with a sexual cycle double their ploidy upon fertilization and reduce their ploidy by half at meiosis. In the development of almost all plants and animals, specialized polyploid and polytene cell types arise though endocycles, cell cycles lacking cell division (*1*). Aberrant cell cycle control during tumor progression is thought to result in polyploidy and altered cell behavior (*2*).

Cells of different ploidy typically show very different developmental, morphological, and physiological characteristics. However, a lack

[1]Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02142, USA. [2]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA.

*To whom correspondence should be addressed. E-mail: fink@wi.mit.edu