also studied one of the artificial samples (the one with an Fe loading of 1000) in the perpendicular orientation, and no transition was seen at 5 K up to 15 T. Additional experiments with artificial samples of different particle sizes should provide a better understanding of the effects due to the surface spins.

The fabrication of small magnets in a biological host through chemical synthesis presents the opportunity to study nanometer-scale magnetism with a systematic variation of interactions and particle size. Both in the classical regime of intermediate fields and temperatures and in the quantum regime of low fields and temperatures, correlations are found between the particle size and the magnetic properties of the artificial ferritin consistent with the properties of natural ferritin. In another classical regime of high magnetic fields, the anisotropy of the ferritin films is found to be crucial in competition with the antiferromagnetic exchange to produce the spin flop transition. Complementary measurements on the transition such as antiferromagnetic resonance and specific heat would also be desirable.

The ferritin protein is of interest not only in pure research but in applied technologies as well. In addition to serving as a vesicle for the chemical fabrication of new materials, the ferritin protein could also serve as a biomagnetic coating for imaging structural defects in ferrous materials (*28*). There has also been recent work on the use of other superparamagnetic particles as a refrigerant (*29*). Other applications closer to the original source of ferritin include the enhancement of magnetic resonance images in biological studies (*30*) and in the orientation of biological assemblies (*31*). All of these research and practical applications require specific knowledge of the synthesis and spin interactions of biomagnetic entities.

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E5531, a Pure Endotoxin Antagonist of High Potency

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Shock due to Gram-negative bacterial sepsis is a consequence of acute inflammatory response to lipopolysaccharide (LPS) or endotoxin released from bacteria. LPS is a major constituent of the outer membrane of Gram-negative bacteria, and its terminal disaccharide phospholipid (lipid A) portion contains the key structural features responsible for toxic activity. Based on the proposed structure of nontoxic *Rhodobacter capsulatus* lipid A, a fully stabilized endotoxin antagonist E5531 has been synthesized. In vitro, E5531 demonstrated potent antagonism of LPS-mediated cellular activation in a variety of systems. In vivo, E5531 protected mice from LPS-induced lethality and, in cooperation with an antibiotic, protected mice from a lethal infection of viable *Escherichia coli*.

Despite the availability of an array of potent antibiotics, shock due to Gram-negative bacterial sepsis remains a serious unsolved clinical problem (*1*). It is probable that the antimicrobial, cytolytic properties of antibiotics induce the release of LPS from the outer membrane of Gram-negative bacteria (*2*). In humans, acute inflammatory responses to LPS or lipid A (Fig. 1) or both (*3*) result in the release of cytokines and other cellular mediators, including tumor necrosis factor–α (TNF-α), interleukin-1 (IL-1), IL-6, leukotrienes, and thromboxane A2 from monocytes and macrophages (*4*). At extreme levels, these cytokines and cellular mediators are known to

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trigger many pathophysiological events including fever, shock, disseminated intravascular coagulation (DIC), hypotension, and organ failure (5). The antagonism of LPS-induced cellular activation would likely block the subsequent acute inflammatory response and thereby obviate events leading to septic shock.

Previous work suggested that lipid X (Fig. 1), a monosaccharide biosynthetic precursor of lipid A isolated from a variant of *E. coli* (6), was antagonistic against LPS in vitro (7). Furthermore, lipid X was reported to exert a protective effect in mice and sheep against LPS-induced acute lethal toxicity (8). By using lipid X as a lead compound, substantial efforts to develop a potent endotoxin-antagonist were made by us and others (9), but with only limited success.

More recently, nontoxic lipid A molecules derived from *Rhodobacter capsulatus* and *R. sphaeroides* LPSs were shown to exhibit endotoxin-antagonistic properties (*10, 11*). It is tempting to develop a structure-activity relation among the naturally occurring lipid A molecules and their analogs as an aid toward understanding the mechanisms of LPS action at the molecular level, which hopefully would lead to design of a therapeutic antagonist for treatment of endotoxin-related diseases. However, such an approach is critically dependent upon firm structural information and availability of homogeneous materials. A synthetic route to lipid A molecules of the *E. coli*–type has been established by seminal contributions by Shiba and co-workers (*12*). However, Shiba's route is incompatible with the 1,3-ketoamido and olefinic functionalities present in the proposed structures for these nontoxic lipid A molecules. We have successfully developed a general, flexible, and convergent synthetic route to these classes of lipid A molecules (*13*), which has provided us with firm structural information and has insured availability of homogeneous materials for studies of their biological properties. We note that the proposed structure for *R. sphaeroides* lipid A was disproved on comparison of the natural product with the synthetic material (*14*), yet, together with the structure proposed for *R. capsulatus* lipid A (Fig. 1), it served as the basis for the design and synthesis of our crucial lead compound.

Synthetic material of the proposed structure of *R. capsulatus* lipid A (*10*) potently antagonized the release of TNF-α induced by LPS in human monocytes [average inhibitor concentration (IC_{50}) ≈1 nM] and exhibited no agonistic activity, even at concentrations of 100 μM. However, it soon became evident that some structural modifications were needed for the development of a drug candidate; upon storage, facile hydrolytic cleavage of the C-3 or C-3′ acyl groups or both produced agonistic by-products.

In order to overcome this problem, a series of hydrolytically stable analogs bearing ether linkages in place of the naturally occurring acyl linkages at the C-3 or C-3′ positions or both were synthesized. These mono- and diether analogs demonstrated antagonistic activity as potent as the synthetic *R. capsulatus* lipid A but importantly, the C-3 and C-3′ diether analogs were no longer hydrolyzed to produce agonistic by-products. There was still some improvement needed; small amounts of contaminants, apparently arising from chemical interactions involving the C-6′ hydroxyl group made it difficult to obtain these analogs with a satisfactory level of purity.

This problem could be overcome by blocking the C-6′ hydroxyl group. Thus, the fully stabilized endotoxin-antagonist was created; this molecule, E5531 (Fig. 1), is shown with the structurally modified sites on the originally proposed *R. capsulatus* lipid A being highlighted by shadowed boxes. E5531 met our criteria for desired chemical properties including purity, stability, and solubility and was subjected to thorough in vitro and in vivo evaluation as a potential drug candidate.

As hoped, E5531 potently antagonized the release of TNF-α induced by LPSs from a variety of Gram-negative bacteria in hu-

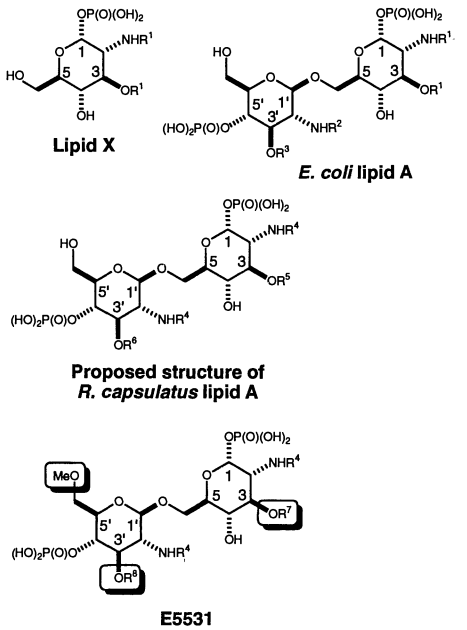

Fig. 1. Chemical structures of *E. coli* lipid A, lipid X, proposed *R. capsulatus* lipid A, and E5531. R^1 = (3*R*)-$COCH_2CH(OH)C_{11}H_{23}$-*n*, R^2 = (3*R*)-$COCH_2CH(OCOC_{11}H_{23}$-*n*)$C_{11}H_{23}$-*n*, R^3 = (3*R*)-$COCH_2CH(OCOC_{13}H_{27}$-*n*)$C_{11}H_{23}$-*n*, R^4 = $COCH_2COC_{11}H_{23}$-*n*, R^5 = (3*R*)-$COCH_2CH$-$(OH)C_7H_{15}$-*n*, R^6 = (3*R*)-$COCH_2CH[OCO$-$(CH_2)_3CH{=}CHC_6H_{13}$-*n*]C_7H_{15}-*n* with the stereochemistry of the olefinic bond being unknown, R^7 = (3*R*)-$CH_2CH_2CH(OH)C_7H_{15}$-*n*, and R^8 = (3*R*)-$CH_2CH_2CH[OCO(CH_2)_3CH{=}CHC_6H_{13}$-*n*]-$C_7H_{15}$-*n* with the stereochemistry of the olefinic bond being *cis*.

Table 1. Antagonism of release of TNF-α induced by LPSs from different species of bacteria. Antagonism of LPS-induced release of TNF-α by E5531 was evaluated in adherent human monocytes-macrophages (1 × 10^6 to 2 × 10^6 per well) in the presence of 1% human serum in RPMI 1640 medium. Addition of E5531 was followed immediately by 10 ng/ml of the indicated LPS. After 3 hours of incubation, media were centrifuged and the resulting supernatants were assayed for TNF-α by an enzyme-linked immunosorbent assay (ELISA). Values given are the average ± SE for three assays, each assay being measured in triplicate.

Source of LPS	TNF-α released (pg/ml)	Antagonism by E5531 (IC_{50} in nM)
Escherichia coli	657 ± 144	1.22 ± 0.66
Klebsiella pneumonia	615 ± 185	1.82 ± 0.79
Pseudomonas aeruginosa	475 ± 119	2.73 ± 1.79
Salmonella minnesota	898 ± 141	0.14 ± 0.06

Table 2. Inhibition of LPS-induced release of cytokines and nitric oxide. Antagonism of LPS by E5531 was tested in adherent monocytes-macrophages (1 × 10^6 to 2 × 10^6 per well) in the presence of 10% human serum in RPMI 1640 for human monocytes, 1% fetal calf serum in complete Williams E medium for RAW 264.7 cells (NO) or was tested in heparinized whole blood. Addition of E5531 was followed immediately by 10 ng/ml of *E. coli* [0111:B4] LPS. After incubation for 24 hours (monocytes-macrophages) or 3 hours (whole blood), media or blood were centrifuged, and the resulting supernatant or plasma was assayed for TNF-α, IL-1β, IL-6, and IL-8 by ELISA. Nitric oxide was tested in RAW 264.7 (murine) cells after a 24-hour incubation as described (*22*). Cytokine levels (in parentheses) are control values obtained from incubations of monocytes-macrophages or whole blood containing LPS only and are in picograms per milliliter for the cytokines and micromolar for NO.

Mediator assayed	IC_{50} for E5531 (nM)	
	Monocytes-macrophages	Whole blood
TNF-α	0.44(782 ±144)	4.7(2400 ± 150)
IL-1β	0.73(523 ± 89)	
IL-6	0.55(3164±692)	2.1(3677 ± 63)
IL-8		1.8(32600±3190)
NO	6.3(23 ± 1)	

man monocytes (Table 1). Inhibitory activity of E5531 was dependent on the dose of LPS used as agonist, as higher doses of LPS required greater concentrations of E5531 for efficacy (*15*). In related studies, E5531 was found to inhibit LPS-induced release of cytokines such as TNF-α, IL-1β, IL-6, and IL-8 in human monocytes and whole blood as well as LPS-induced nitric oxide (NO) generation in a cultured murine macrophage cell line (Table 2). Similar results were observed when E5531 was used to inhibit lipid A–mediated induction of cellular activation (*15*). Most importantly, E5531 was completely devoid of agonistic activity in all human and murine systems tested, even at concentrations 10,000-fold greater than those required for antagonistic efficacy.

Specificity of E5531's antagonism of LPS was evaluated by the use of alternative agonists. E5531 was ineffective at inhibiting interferon-γ–induced generation of NO in murine macrophages. In addition, whereas E5531 blocked superoxide production induced by LPS synergism with *N*-formyl-methionyl-leucyl-phenylalanine in human neutrophils, it was ineffective against superoxide production stimulated by phorbol 12-myristate 13-acetate alone (*15*). These results indicated that the antagonistic activity of E5531 is specific for LPS.

Mechanism of action of E5531 was studied by testing its ability to inhibit LPS binding and subsequent activation of nuclear factor κB (NF-κB). In human promonocytic cells (U38) (*16*) stimulated with LPS (33 ng/ml), E5531 (333 nM) blocked NF-κB activation and translocation to the nucleus (*17*). Furthermore, measurement of ^{125}I-labeled LPS binding to human monocytes, monocyte-derived macrophages, and murine macrophages indicated that E5531 was a more potent competitor for cell-surface LPS binding than *E. coli* lipid A (*15, 18*). Taken together, these results suggest that E5531 may antagonize LPS activity at its cell-surface receptor leading to inhibition of transmembrane signal transduction.

In vivo evaluation of E5531 was performed in a murine model. Because of their known low susceptibility to LPS, mice were sensitized (primed) with *Bacillus calmette guerin* (BCG) (*19*). Intravenous injection of 3 μg of *E. coli* LPS into these primed mice induced a rapid increase in plasma TNF-α that reached maximal levels ~1 hour after injection. Mortality subsequently occurred in 100% of these mice (Table 3). In this model, co-injection of E5531 with LPS suppressed the increase in plasma TNF-α and its associated mortality (Table 3). Analysis of E5531 activity in an infection model utilized intraperitoneal infection of *E. coli* in BCG-primed mice. In this model, either E5531 or the β-lactam antibiotic latamoxef gave noticeable but transient protection (Fig. 2). However, co-treatment with the antibiotic and E5531 provided long-lasting protection against mortality. The inability

Table 3. Inhibitory effect of E5531 on LPS-induced increases in plasma TNF-α and lethality in BCG-primed mice. Pathogen-free, 5-week-old male C57BL/6 mice from SLC Inc., Shizuoka, Japan, were primed by an intravenous tail vein injection of 2 mg of BCG (Japan BCG Inc.) 2 weeks prior to the experiments. Inhibition of LPS-induced increases in plasma TNF-α was determined by analyzing TNF-α (ELISA) in plasma samples obtained 1 hour after intravenous injection of 3 μg of *E. coli* LPS plus the indicated amount of E5531. Values are expressed as mean ± SE of plasma values from five mice with the student's *t* test. Mortality was observed 48 hours after intravenous injection of 3 μg/kg of *E. coli* LPS plus the indicated amount of E5531. Each value was obtained from 10 BCG-primed mice. Statistical significance was determined by the χ^2 test.

E5531 (μg/mouse)	Plasma TNF		Mortality (%)
	ng/ml	Inhibition (%)	
0 (control)	582 ± 20	0	100
1	547 ± 45	6	80
3	432 ± 40*	26	20
10	259 ± 28†	55	0*
30	198 ± 24†	66	0*
100	71 ± 18†	88	0*

*$P < 0.01$ versus control. †$P < 0.001$ versus control.

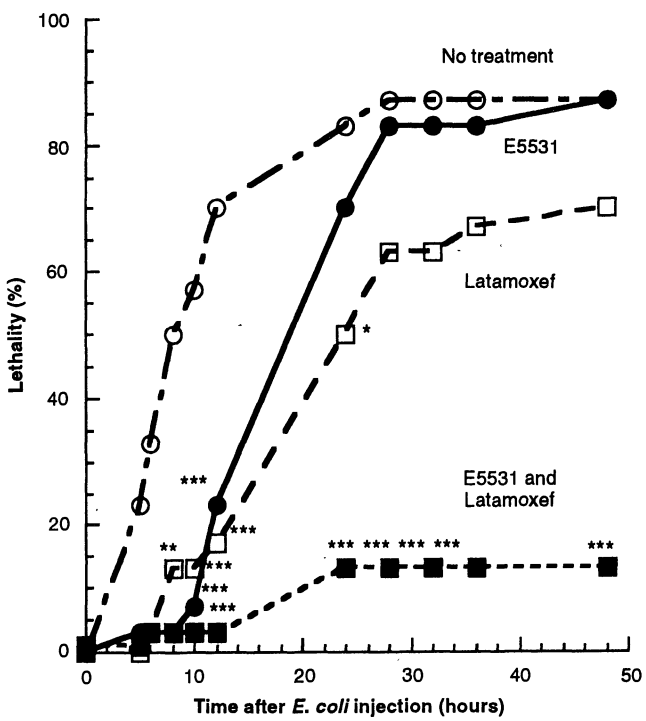

Fig. 2. E5531 and antibiotic cooperate to suppress lethality in a peritoneal infection model. BCG-primed mice were infected by intraperitoneal (IP) injection of *E. coli* [2.0×10^7 colony-forming units (CFU)]. One hour later, 5% glucose solution alone (-○-) or 600 μg latamoxef (-□-) or 100 μg E5531 (-●-) or 600 μg latamoxef plus 100 μg E5531 (-■-) was intravenously injected through the tail vein, and the incidence of death was monitored at the times indicated. Statistical significance: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ versus control (two-sided Fisher exact test). At 4 hours, $P < 0.05$ for E5531 or latamoxef only. In the E5531/latamoxef co-treatment group, $P < 0.05$ at 4 hours, $P < 0.01$ at 6 hours, and $P < 0.001$ at 8 and 10 hours. Seven days after IP infection with *E. coli*, only 4 of 30 mice co-treated with E5531 and latamoxef died, whereas 26 out of 30 (no treatment), 26 out of 30 (E5531 alone), and 21 out of 30 (latamoxef alone) died.

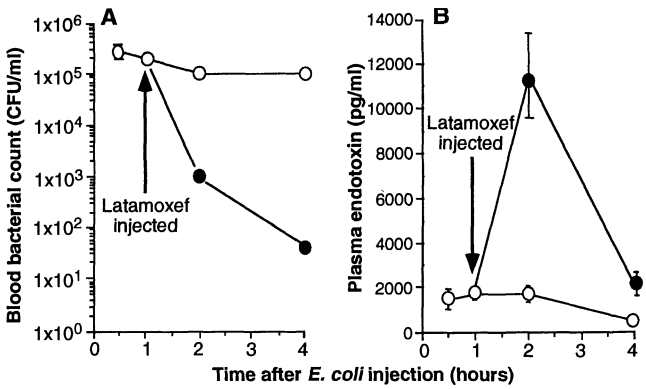

Fig. 3. Bacterial and endotoxin levels in plasma during peritoneal infection. BCG-primed mice ($n = 4$) were infected IP with *E. coli* [8.6×10^6 CFU in (**A**) and 2.3×10^7 CFU in (**B**)]. One hour later, mice were intravenously administered 5% glucose alone (-○-) or 600 μg latamoxef (-●-) in 5% glucose. Blood bacterial counts (A) were determined by plating plasma dilutions on modified Drigalski agar (BTB agar E-MA84, Eiken Chemical Co., Tokyo, Japan) or endotoxin levels (B) were determined by an endotoxin-specific colorimetric limulus test (Toxicolor System–ENDOSPECY: ESTEST, Seikagaku Kogyo Tokyo, Japan). E5531 did not affect blood bacterial counts.

of the antibiotic alone to cause sustained suppression of lethality in this model is likely due to host inflammatory response to endotoxin released from bacteria killed by antibiotic (*20*); indeed, administration of antibiotic dramatically decreased blood bacterial counts, but plasma endotoxin level concomitantly increased in these animals (Fig. 3). This model of intraperitoneal infection may thus be representative of the clinical situation for human sepsis (*2*).

We have developed a potent endotoxin antagonist E5531 by organic synthesis. In vitro studies showed E5531 to be a pure and specific LPS antagonist, while in vivo experiments demonstrated that E5531 protected BCG-primed mice from lethality induced by LPS and also death caused by viable *E. coli* infection. That these remarkable protective effects have been extended to humans (*21*) suggests that E5531 may be clinically useful in the treatment of Gram-negative sepsis and septic shock.

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Stretching of a Single Tethered Polymer in a Uniform Flow

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The stretching of single, tethered DNA molecules by a flow was directly visualized with fluorescence microscopy. Molecules ranging in length (L) from 22 to 84 micrometers were held stationary against the flow by the optical trapping of a latex microsphere attached to one end. The fractional extension x/L is a universal function of $\eta v L^{0.54 \pm 0.05}$, where η and v are the viscosity and velocity of the flow, respectively. This relation shows that the DNA is not "free-draining" (that is, hydrodynamic coupling within the chain is not negligible) even near full extension (~80 percent). This function has the same form over a long range as the fractional extension versus force applied at the ends of a worm-like chain. For small deformations (<30 percent of full extension), the extension increases with velocity as $x \sim v^{0.70 \pm 0.08}$. The relative size of fluctuations in extension decreases as $\sigma_x/x \cong 0.42 \exp(-4.9\, x/L)$. Video images of the fluctuating chain have a cone-like envelope and show a sharp increase in intensity at the free end.

The deformation of polymers in hydrodynamic flows is a fundamental and still incompletely resolved problem in polymer physics (*1*, *2*). The major difficulty in theoretical descriptions of polymer chain dynamics is the hydrodynamic coupling within the chain—the motion of one part of the chain perturbs the surrounding flow and modifies the hydrodynamic force exerted on another part. Here we present results of the stretching of single, tethered DNA molecules in a uniform fluid flow.

Direct observation and controlled deformation of individual DNA molecules gives insights into the previously inaccessible regime of single polymer dynamics (*3*–*6*). Earlier, we observed the relaxation of stretched DNA molecules in dilute and concentrated polymer solutions, using optical tweezers (*7*) and fluorescence microscopy (*3*, *5*, *6*). The present experiment addresses the balance of forces between hydrodynamic drag on a deformed polymer and its entropic elasticity. In earlier work, Smith *et al.* measured the extension of single DNA molecules stretched by a force applied at the ends (*4*). They attached one end to a surface and exerted a combination of magnetic and hydrodynamic forces on a magnetic microsphere at the other end (*4*). Because the forces exerted on the microsphere were larger than the hydrodynamic drag due to the DNA, Smith *et al.* measured primarily the elastic force, a static property of a polymer in solution. Theoretical calculations (*8*, *9*) show quantitative agreement between the elasticity data and the force law for a worm-like chain and give an approximate formula (*8*) of

$$\frac{FA}{k_B T} = \frac{1}{4}\left(1 - \frac{x}{L}\right)^{-2} - \frac{1}{4} + \frac{x}{L} \qquad (1)$$

where F is the force applied across the ends, A is the persistence length, x is the extension, L is the length of the polymer, and $k_B T$ is the thermal energy.

We made our measurements by optically trapping a microsphere attached to one end of a DNA molecule while the other end remained free. In this way we were able to investigate the hydrodynamic interaction between the polymer and the fluid. The chain was positioned away from any surface, and we elongated it in a uniform (nonshearing) flow by translating the fluid past the trapped, stationary microsphere (Fig. 1A). By determining the scaling properties of the system as a function of polymer length and solvent viscosity, we investigated the effects of hydrodynamic coupling. As Zimm showed (*10*), the hydrodynamic coupling within a polymer near equilibrium causes, in the simplest case, the total hydrodynamic drag to scale as $F_{drag} \sim L^{0.5}$. If hydrodynamic cou-

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