British Journal of Pharmacology (1999) 127, 853–862
http://www.stockton-press.co.uk/bjp

E5531, a synthetic non-toxic lipid A derivative blocks the immunobiological activities of lipopolysaccharide

*,[2]Tsutomu Kawata, [1]John R. Bristol, [1]Daniel P. Rossignol, [1]Jeffrey R. Rose, [2]Seiichi Kobayashi, [2]Hiromitsu Yokohama, [2]Akira Ishibashi, [1]William J. Christ, [2]Koichi Katayama, [3]Isao Yamatsu & [1]Yoshito Kishi

[1]Eisai Research Institute of Boston, Inc., 4 Corporate Drive Andover, MA 01810-2441, U.S.A.; [2]Tsukuba Research Laboratories for Drug Discovery, Eisai Co., Ltd., 1-3, Tokodai 5-chome Tsukuba-shi, Ibaraki 300-2635, Japan and [3]Department of Drug Development, Eisai Co., Ltd., 4-6-10, Koishikawa Bunkyo-ku Tokyo 112, Japan

1 The major pathological responses to Gram-negative bacterial sepsis are triggered by endotoxin or lipopolysaccharide. As endotoxin is shed from the bacterial outer membrane, it induces immunological responses that lead to release of a variety of cytokines and other cellular mediators. As part of a program aimed at developing a therapeutic agent for septic shock, we have developed E5531, a novel synthetic lipopolysaccharide antagonist.

2 As measured by release by tumour necrosis factor-α, human monocytes or whole blood can be activated by lipopolysaccharide, lipid A, and lipoteichoic acid (from Gram-positive bacteria). E5531 potently antagonizes activation by all these agents while itself being devoid of agonistic activity.

3 The inhibitory activity of E5531 was dependent on time of addition. When 10 nM E5531 was added simultaneously with lipopolysaccharide or 1–3 h before addition of lipopolysaccharide, production of tumour necrosis factor-α was inhibited by more than 98%. The addition of E5531 1 h after lipopolysaccharide reduced the efficacy of E5531 by 47%.

4 Antagonistic activity of E5531 was specific for lipopolysaccharide as it was ineffective at inhibiting interferon-γ mediated NO release of RAW 264.7 cells, phorbor 12-myristate 13-acetate stimulated superoxide anion production in human neutrophils, concanavalin A stimulated mitogenic activity in murine thymocytes and tumor necrosis factor-α induced E-selectin expression in human umbilical vein endothelial cells.

5 E5531 as well as MY4, an anti-CD14 antibody, inhibited radiolabelled lipopolysaccharide binding in human monocytes.

6 These results support our contention that E5531 is a potent antagonist of lipopolysaccharide-induced release of tumour necrosis factor-α and other cellular mediators and may be an effective therapeutic agent for human septic shock due to Gram-negative bacteria.

Keywords: E5531; lipopolysaccharide; endotoxin; lipid A; lipoteichoic acid; sepsis; TNF

Abbreviations: Con A, Concanavalin A; ELISA, enzyme linked immunosorbent assay; FBS, foetal bovine serum; FMLP, N-formyl-methionyl-leucyl-phenylalanine; HBSS, calcium and magnesium-free Hanks balanced salt solution; HUVEC, human umbilical vein endothelial cell; IC_{50}, 50% inhibitory concentration; IL, interleukin; IFN-γ, interferon-γ; LPS, lipopolysaccharide; LBP, LPS binding protein; LTA, lipoteichoic acid; PBSA, phosphate buffered saline containing 0.1% bovine serum albumin; PMA, phorbor 12-myristate 13-acetate; [^{125}I]-ASD-LPS, radioiodinated azidosalicylamide dithiopropionated LPS; TNF-α, tumour necrosis factor-α

Introduction

Endotoxin (LPS) is the major constituent of the outer membrane of Gram-negative bacteria. During blood-borne infection in mammals, endotoxin triggers many pathophysiological events such as fever, shock, disseminated intravascular coagulation, hypotension, and death (Bone, 1993; Parrillo *et al*., 1990; Welbourn & Young, 1992). In addition, intestinal tract-derived endotoxin has been implicated as one of the causes of many deleterious clinical manifestations occurring during hepatic diseases and liver transplantation (Lumsden *et al*., 1988; Miyata *et al*., 1989). Most of the pathological activities of LPS are attributed to the glycolipid or lipid A portion of the molecule (Galanos *et al*., 1985; Raetz, 1990). LPS and lipid A stimulate the release of cytokines such as TNF-α, IL-1, IL-6, IL-8, as well as nitric oxide, leukotrienes, thromboxane A2, and other mediators released from monocytes/macrophages. When produced in excess, these cellular mediators cause severe pathological responses and tissue damage that can lead to hypotensive shock, multiorgan failure and death (Biliau & Vandeckercchove, 1991; Brown *et al*., 1989). Various strategies for treatment have been sought in order to protect susceptible patients from the life-threatening effects of Gram-negative septic shock. Efficacies of many potentially therapeutic products such as monoclonal antibodies directed against endotoxin or TNF-α, soluble inactive receptors of TNF-α, or IL-1 receptor antagonist (IL-1ra) have been evaluated in clinical trials and found to be without effect or of doubtful efficacy in septic patients (Glauser *et al*., 1994). On the other hand, Lipid X, a monosaccharide and biosynthetic precursor of lipid A, has been isolated from a variant of *E. coli* and structurally characterized (Bulawa & Raetz, 1984; Nishijima & Raetz, 1979). Investigations into its biological properties have suggested that Lipid X antagonizes the effects of LPS *in vitro* (Danner & Parrillo, 1987; Lam *et al*., 1991). *In vivo* protection

* Author for correspondence; E-mail: t1-kawata@eisai.co.jp

against LPS-induced acute lethal toxicity has also been demonstrated in mice and sheep (Golenbock *et al*., 1987; Proctor *et al*., 1986) but, in canine models of sepsis, lipid X has recently been shown to be ineffective (Danner *et al*., 1993). Other reports have concluded that non-toxic LPS or lipid A obtained from non-pathogenic bacteria such as *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* are more potent LPS antagonists than lipid X *in vitro* (Loppnow *et al*., 1990; Qureshi *et al*., 1991; Takayama *et al*., 1989). However, it is likely that difficulties involved in obtaining sufficient amounts of homogenous material with pharmaceutically acceptable purity and stability would limit the use of bacterial derived compounds as therapeutic antagonists for treatment of endotoxin-related diseases. These findings have motivated us to develop a specific and non-toxic synthetic antagonist for the actions of LPS for the treatment of septic shock. E5531 has been described as a potent *in vitro* and *in vivo* antagonist of LPS (Christ *et al*., 1995; Kobayashi *et al*., 1998). This report demonstrated further *in vitro* biological characterization of E5531 in a variety of experimental systems making it a promising potential therapeutic as an anti-endotoxin drug for the treatment of sepsis and septic shock.

Methods

Materials

Synthesis of E5531 and Lipid X (Figure 1) was performed by Eisai Research Institute (Christ *et al*., 1992; 1995). E5531 is the fully stabilized endotoxin antagonist derived from a non-toxic lipid A, *Rhodobacter capsulatus* lipid A (Figure 1). It contains ether linkages in both the C.3 and C.3′ positions in place of the more labile ester linkages and is derivatized by methylation at the C.6′ position. This material now demonstrated long-term stability during storage, purity greater than 99%. Prior to use, E5531 and Lipid X were dissolved in sterile pyrogen-free water, sonicated by a bath sonifier and diluted by HBSS. LPS from *Escherichia coli* (0111:B4) and LTA from *staphylococcys faecalis* were purchased from Sigma Chem. Co. (St. Louis, MO, U.S.A.). Synthetic lipid A (LA-15-PP) and Lipid IVA were purchased from Daiichi Chem. Co. (Tokyo, Japan). LPS and LTA were dissolved in sterile water at 1 mg ml^{-1} and stored at $-20°C$. Prior to use, LPS and LTA were sonicated and diluted into HBSS. Lipid

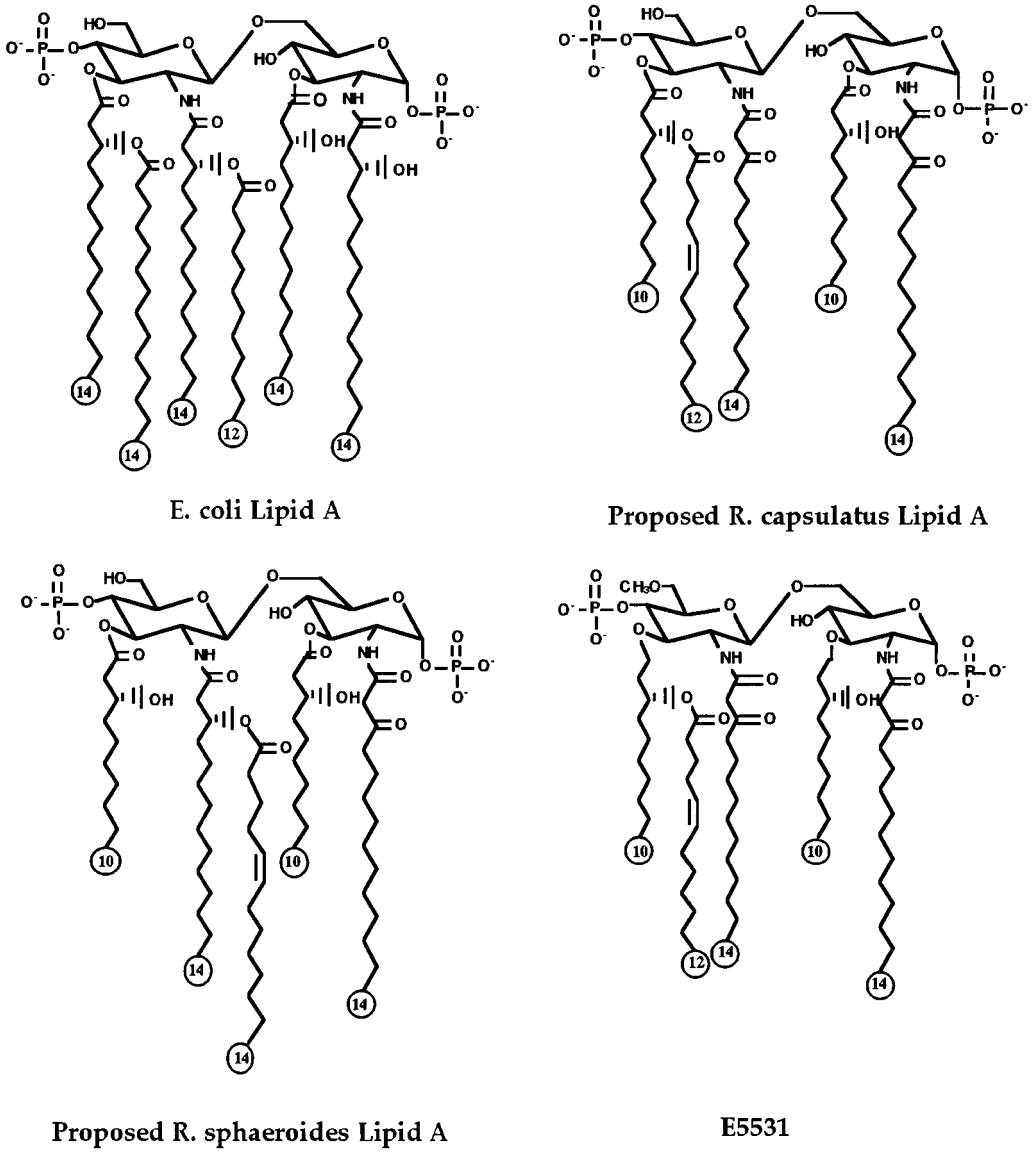

Figure 1 Structures of *E. coli* lipid A, proposed *R. sphaeroides* lipid A, proposed *R. capsulatus* lipid A, and E5531. E5531 is an ether-stabilized form of *R. capsulatus* lipid A.

A and Lipid IVA were dissolved in 0.025% triethylamine, and diluted into HBSS. MY4 and MO2 were purchased from Coulter Immunology (Hialeuth, FL, U.S.A.).

Isolation of human monocytes and neutrophils

Human whole blood was collected aseptically from healthy volunteers into sterile tubes containing heparin (10 Units ml^{-1} blood; LyphoMed Inc., Rosemont, IL, U.S.A.). The volunteers gave written consent to participate in this study. Human monocytes were isolated from the blood by the LeucoPrep system for mononuclear cell isolation (Becton Dickinson, Lincoln Park, NJ, U.S.A.). The harvested monocytes were suspended in HBSS and centrifuged ($600\times$ *g*, 5 min, 4°C). After washing with HBSS, the cells ($1-2\times10^6$ cells ml^{-1}) were resuspended in complete assay medium [RPMI 1640 supplemented with L-glutamine (2 mM), penicillin (80 U ml^{-1}), streptomycin (100 μg ml^{-1}) and 10% heat-inactivated human AB serum] then allowed to adhere onto plastic culture plates for 3 h at 37°C, 5% CO_2. Human neutrophils were isolated by dextran-percoll separation from whole blood. Six ml of 6% dextran solution was added to 20 ml samples of blood, and the blood/dextran mixture was allowed to stand for 30 min at room temperature. The supernatant was mixed with cold HBSS and allowed to stand an additional 20 min on ice, followed by centrifugation ($200\times$ *g*, 10 min, 4°C). The pellet was resuspended into one ml HBSS, and the cell suspension was layered over a Percoll gradient (1.5 ml of each density; 1.04, 1.08, 1.10) and then centrifuged ($600\times$ *g*, 15 min, room temperature). The interface of cells between 1.04 and 1.08 layers was collected and washed by centrifugation ($200\times$ *g*, 10 min, 4°C) three times with HBSS, then the cells were used for superoxide anion determination experiments.

TNF-α assay in monocytes

Human monocytes prepared as described above were incubated in 48-well culture plates at 37°C, 5% CO_2 in RPMI 1640 supplemented with 1% human AB serum in addition to 50 μl of each E5531 and LPS. Whole blood experiments were performed by adding 50 μl of each E5531 and LPS directly to 48-well culture plates containing 400 μl heparinized whole blood. Following incubations for 3 h, the culture plates were centrifuged ($900\times$ *g*, 10 min, 4°C) and the plasma or the culture supernatants were removed then stored at −80°C. TNF-α levels were quantitated using ELISA kit (R&D Systems, Minneapolis, MN, U.S.A.).

Superoxide anion assay in neutrophils

Superoxide anion production was determined by a modification of a method utilizing superoxide dismutase-inhibitable reduction of cytochrome C (Markert *et al*., 1984). Neutrophils (4×10^6 cells) suspended in HBSS were incubated with E5531 and lipid A, or PMA (Sigma Chem. Co., St. Louis, MO, U.S.A.) for 1 h in a total volume of 0.2 ml. Then 0.8 ml of HBSS containing Ca^{2+} and Mg^{2+} supplemented with 10^{-6} M FMLP (Sigma Chem. Co., St. Louis, MO, U.S.A.) and 50 μM cytochrome C (horse heart type VI; Sigma Chem. Co., St. Louis, MO, U.S.A.) was added to the cell suspension and incubated for an additional 10 min. After centrifugation ($2000\times$ *g*, 4°C), the absorbance of the supernatant was read at 550 nm with a spectrophotometer (DU-70; Beckman Instruments, Fullerton, CA, U.S.A.). The change in absorbance was converted to nanomoles of cytochrome C reduced and expressed as nanomoles $O_2^-/10^6$ neutrophils.

Mitogenicity assays in splenocytes

All experiments were approved by Eisai Research Institute Animal Care and Use Committee (Andover, MA, U.S.A.). Eight to 12-week-old C3H/HeSnJ male mice (Jackson Laboratories, Bar Harbor, ME, U.S.A.) were housed and cared in our laboratories according to the Guidelines for Care and Use of Laboratory Animals (N.I.H., U.S.A.). The animal room is maintained at 23 ± 1°C, 45 ± 5% relative humidity with a 12 h light/dark cycle. Animals were fed Agway ProLab diet (Agway, Inc., Syracuse, NY, U.S.A.) as solid food and tap water was given *ad libitum*. The spleens from the mice sacrified by CO_2 were pooled and the splenocytes were removed from the spleen capsule with a sterile needle into cold serum-free RPMI 1640. The cells were centrifuged ($600\times$ *g*, 5 min, 4°C), and the pellet resuspended in 2 ml of red blood cell lysing buffer (Sigma Chem. Co., St. Louis, MO, U.S.A.), followed by the addition of serum-free RPMI. The harvested cells were washed in serum-free RPMI solution. The final pellet was resuspended in RPMI 1640 containing 2 mM L-glutamine, 25 mM HEPES, penicillin (80 U ml^{-1}), streptomycin (100 μg ml^{-1}) and 10% heat-inactivated FBS. Mitogenicity was evaluated using a modification of a method (Nakamura *et al*., 1986). Murine splenocytes (100 μl of 8×10^5 cells ml^{-1}) were cultured in 96-well plates along with 50 μl of each E5531 and LPS or Con A (Sigma Chem. Co., St. Louis, MO, U.S.A.). After incubation for 48 h at 37°C, 5% CO_2, the cells were pulsed with [^{3}H]-thymidine (1 μCi ml^{-1}; New England Nuclear, Boston, MA, U.S.A.) then incubated for an additional 5 h. The cells were harvested using a cell harvester (MB-48R; Brandel, Gaithersburg, MD, U.S.A.) and collected onto GF/C filters. After washing the filters ten times with phosphate buffered saline, and the radioactivity was analysed by scintillation spectrometry.

Nitric oxide assay with RAW 264.7 cells

RAW 264.7 cells (American Type Culture Collection, Rockville, MD, U.S.A.) were used for nitric oxide experiments. Cells were maintained in HAM's F12 media supplemented with 10% FBS, L-glutamine (2 mM), penicillin (80 U ml^{-1}) and streptomycin (100 μg ml^{-1}). Cells were seeded into 48-well tissue culture cluster dishes as 2×10^5 cells/well in 0.2 ml of complete assay medium [Williams E medium supplemented with 10% FBS, L-glutamine (2 mM), penicillin (80 U ml^{-1}) and streptomycin (100 μg ml^{-1})]. After plating, the cells were treated with E5531 followed by LPS and incubated for 24 h at 37°C, 5% CO_2. Nitrite, an oxidation product of NO, was assayed in cell culture supernatants (Stuehr & Nathan, 1989). Each 100 μl of cell culture supernatant and Greiss reagent [a freshly prepared 1:1 mixture of 1% sulphanilmide in 5% phosphoric acid/0.1% N-(1-naphthyl) ethylenediamine in distilled water] were combined and absorbance at 540 nm was measured on a microplate reader. The amount of nitrite present was determined by the comparison of absorbances to a standard curve of sodium nitrite.

Assay of E-selection expression in HUVEC

HUVEC were purchased from Sanko Jyunyaku Co. Ltd. (Tokyo, Japan). Cells were seeded on type I collagen-coated 96 well plate (Iwaki Glass Co. Ltd., Tokyo, Japan) and grown to confluence in MCDB 131 medium (Kurorela Kougyo Co. Ltd., Tokyo, Japan) containing 10% FBS, 30 μg ml^{-1} of endothelial cell growth supplement (Becton Dickinson, MA, U.S.A.), 6 IU ml^{-1} heparin (Green Cross, Tokyo, Japan) and

80 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin at 37°, 5% CO_2.

HUVEC were incubated with E5531 in the presence of 100 ng ml^{-1} LPS or 1 ng ml^{-1} TNF-α (Genzyme, Boston, MA, U.S.A.) for 4.5 h. Cells were washed with 100 μl of saline and then fixed with 100 μl of 0.025% glutaraldehyde for 6 min at room temperature. After washing the cells with PBSA, 100 μl of 1 μg ml^{-1} murine anti-E-selectin antibody (R & D Systems, Minneapolis, MN, U.S.A.) was added to each well and incubated for 1 h. After washing the cells twice with PBSA, 100 μl of a 1:1000 dilution of the horseradish peroxidase-conjugated goat anti-mouse IgG (Cappel, Pennsylvania, PA, U.S.A.) was added and incubated for another 1 h. After washing the cells twice with PBSA, 100 μl of 3, 3′, 5, 5′-tetramethylbenzidine substrate solution (Funakoshi, Tokyo, Japan) was added to each well and the plate was incubated. After the colour reaction was stopped by the addition of 100 μl of 1 M phosphoric acid at appropriate time, the absorbance at 450 nm was measured using an ELISA reader.

LPS binding assay in human monocytes

[^{125}I]-ASD-LPS was prepared using a published procedure (Wollenweber & Morrison, 1985). Monocytes (2×10^6 cells in 1 ml) were added to each well of a 24-well plate and incubated for 3 h at 37°C, 5% CO_2. After washing the cells twice with RPMI 1640, remaining adherent cells were overlayed with RPMI 1640 supplemented with 2% human serum. The cells were then treated with 25 μl of inhibitor plus 25 μl [^{125}I]-ASD-LPS ($\sim$60 ng of 18 μCi μg^{-1}) and incubated for 1 h at 37°C, 5% CO_2. Wells were washed three times with one ml of 50 mM Tris buffer (pH 7.4) containing NaCl (150 mM) and 2 mg ml^{-1} bovine serum albumin. One ml of 0.1 N NaOH was added to each well and, after shaking the plate, 950 μl was removed and analysed for radioactivity. Nonspecific binding was evaluated by the addition of 1 mg ml^{-1} of unlabelled LPS added along with the [^{125}I]-ASD-LPS.

Statistics

Statistical significance was assessed using student's *t*-test. IC_{50} values were calculated using regression analysis from dose-inhibition relationship which the statistical analysis was conducted using the software package, SAS (SAS Institute Japan, Tokyo, Japan).

Results

TNF-α inhibition of E5531 in human whole blood

Therapeutic use of E5531 for human septic shock requires it to be effective against LPS activation of cytokine response in blood. For this reason, we have established assays to measure LPS-induced TNF-α release and evaluate antagonistic activity of E5531 in fresh human blood. In this system, the most reproducible assays involved addition of each agonist and antagonist as 10 fold concentration-solutions, resulting in a heparinized final blood concentration of 80%. Without adding LPS, TNF-α release was undetectable (<15 pg ml^{-1}). Experiments examining dose-response to LPS indicated that saturation of stimulation occurred at concentrations greater than 10 ng ml^{-1}. As shown in Figure 2, response after 3 h incubation with 10 ng ml^{-1} LPS by whole blood was robust and reproducible (release of TNF-α = 3146 ± 466 pg ml^{-1}; n = 15). This response to LPS was potently inhibited by

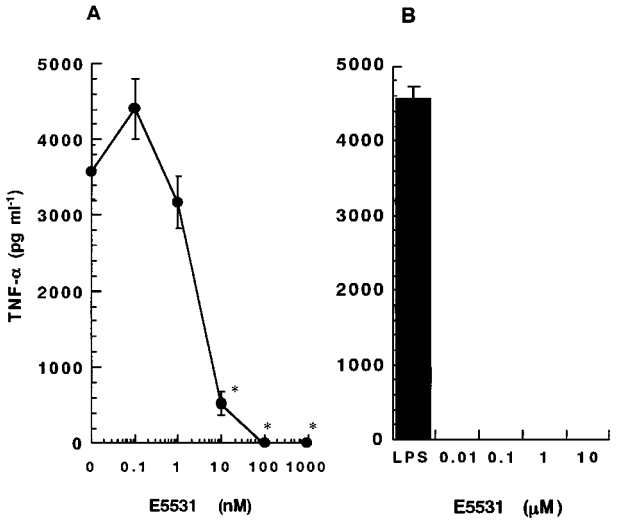

Figure 2 Inhibition of LPS-induced release of TNF-α (A) and agonistic activity (B) of E5531 in human whole blood. Heparinized whole blood was incubated with 10 ng ml^{-1} LPS plus the indicated concentration of E5531 or either of these reagents alone. After 3 h incubation, plasma samples were assayed for TNF-α as described in Methods. Each point represents the mean ± s.e.mean from 15 (A) or three (B) experiments assayed in triplicate. *P<0.01 versus LPS alone.

E5531 (IC_{50} = 2.6 ± 0.2 nM; n = 15) and inhibition was complete at concentrations greater than 100 nM. No TNF-α release measurable when E5531 was added alone at up to 10 μM concentrations, while LPS (10 ng ml^{-1}) increased TNF-α to 4575 ± 161 pg ml^{-1} in these same assays. This indicates that E5531 possesses no agonistic activity. (Figure 2).

E5531, Lipid X and Lipid IVA inhibition of LPS, lipid A and LTA-induced production of TNF-α from human monocytes

E5531 inhibited human monocyte response to *E. coli* LPS more potently than that of whole blood. At 10 ng ml^{-1} LPS, the IC_{50}s for inhibition of TNF-α production by E5531, Lipid X and Lipid IVA were 0.13, 3347 and 71 nM, respectively (Table 1). This means that E5531 was approximately 26,000 and 500 fold more potent than Lipid X and Lipid IVA, respectively. The lipid A moiety of LPS is known to be the toxicophore of the endotoxin molecule (Galanos *et al*, 1985). Using 10 ng ml^{-1} *E. coli* lipid A as an agonist in this system, E5531 demonstrated an IC_{50} of 0.16 nM indicating that the activity of E5531 is against lipid A, the most agonistic portion of the LPS molecule (Table 1). In addition, it is known that LTA, an endotoxin-like product from Gram positive bacteria, *S. faecalis*, shows biological action through CD14 dependent pathway similar to LPS (Hattor *et al*., 1997). LTA also stimulated TNF-α production at 100 ng ml^{-1} and E5531 was potently antagonistic against 100 ng ml^{-1} LTA with 0.08 nM of IC_{50} (Table 1).

Time dependence of E5531 efficacy in human monocytes

In order to determine the antagonistic activity of E5531 relative to time of LPS exposure, we have assayed the ability of E5531 to inhibit LPS-induced cellular activation when added before and after LPS (Figure 3). When 10 nM E5531 was added simultaneously with LPS or 1–3 h before addition of LPS, production of TNF-α was inhibited by more than 98%.

Nearly complete inhibition was still observed if addition of E5531 was delayed 15 or 30 min after addition of LPS (98 and 83% inhibition, respectively). The addition of E5531 1 h after LPS reduced the efficacy of E5531 by 47%. These results suggest that E5531 can effectively inhibit LPS-induced cellular activation when added as late as 30 min after the agonist. After this time, cellular events initiated in response to LPS were less-effectively blocked by addition of E5531. This suggests that antagonism by E5531 occurs during or close to the initiation of early receptor recognition events or trans-membrane transduction of LPS-induced signalling.

Effect of E5531 on IFN-γ-potentiated TNF-α production in human monocytes

The *in vivo* septic response is likely to include generation of IFN-γ which has been shown to potentiate cellular response to LPS (Adams & Hamilton, 1987). Therefore, we investigated the possibility that IFN-γ may affect the ability of E5531 to block LPS-induced TNF-α production. As shown in Figure 4, when human monocytes were preincubated with 100 Units ml^{-1} of human IFN-γ for 24 h, TNF-α release was augmented 4 fold in response to LPS stimulation [770±49 pg ml^{-1} (LPS alone) to 3212±204 pg ml^{-1} (LPS±IFN-γ)]. To examine the ability of E5531 to antagonize this IFN-γ-potentiated LPS response, human monocytes were pre-incubated with or without IFN-γ for 24 h followed by the addition of LPS (10 ng ml^{-1}) along with varying concentrations of E5531. The treatment of monocytes with IFN-γ did not affect the antagonistic activity of E5531. The IC_{50}s for E5531 were 0.18 nM in cultures pre-incubated with IFN-γ and 0.25 nM in control incubations without IFN-γ. In both cases, 10 nM E5531 inhibited 100% of the TNF-α production stimulated by LPS.

Table 1 Antagonistic activities of E5531, Lipid X and Lipid IVA in TNF-α production by LPS, lipid A and LTA in human monocytes

Stimulant	*Concentration* (ng ml^{-1})	*Compound*	*IC_{50}* (nM)	n
E. coli LPS	10	E5531	0.13±0.04	13
		Lipid X	3347±466	12
		Lipid IVA	71±30	4
E. coli Lipid A	1	E5531	0.033±0.01	3
S. faecalis LTA	100	E5531	0.061±0.03	3

Adherent human monocytes were incubated for 3 h with the indicated amount of *E. coli* LPS, *E. coli* lipid A or *S. faecalis* LTA followed by addition of E5531, Lipid X or Lipid IVA. After 3 h incubation, the supernatant was assayed for TNF-α as described in Methods. *n* means number of experiments assayed in triplicate.

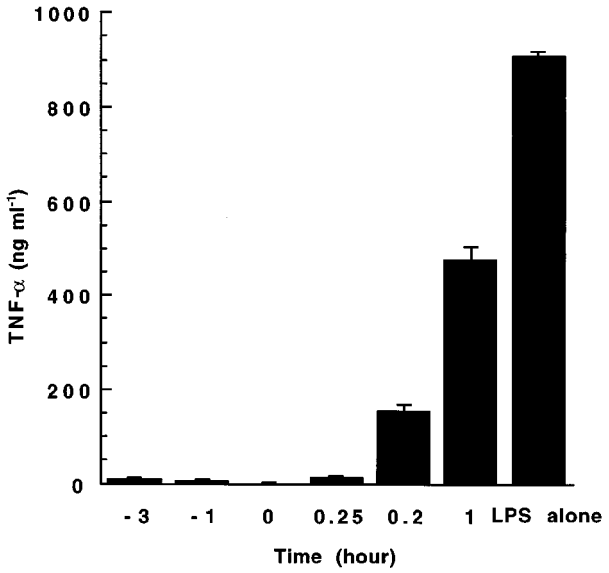

Figure 3 Time course for the addition of E5531. At the times indicated, E5531 was added to adherent human monocytes at 10 nM. At time zero, 10 ng ml^{-1} *E. coli* LPS was added and the cultures were incubated for an additional 3 h and assayed for TNF-α as described in Methods. Each column represents the means±s.e.mean of triplicate and the result is representative of two similar experiments assayed in triplicate.

E5531 inhibition of lipid A-enhanced superoxide anion production by FMLP in human neutrophils

LPS and lipid A can prime neutrophils to potentiate FMLP-mediated superoxide anion production (Danner & Parrillo, 1987). Using human neutrophils, we investigated the effect of E5531 on this priming activity of lipid A (Figure 5). Superoxide anion release in response to 1 μM FMLP increased approximately 7.7 fold from 0.70±0.04 to 5.38±0.40 nmol O_2^- 10^6 $cells^{-1}$ by pretreatment with 50 nM lipid A. When tested at concentrations from 10 pM−10 μM, E5531 inhibited the ability of lipid A to potentiate FMLP-stimulated superoxide anion production with an IC_{50} of 174 nM. As a control, PMA alone (10 nM) stimulated superoxide anion production from 0.70±0.04 to 4.85±0.20 nmol 10^6 $cells^{-1}$; this stimulation was unaffected by E5531 (Figure 5). Based on these results, E5531 specifically antagonized only lipid A priming of human neutrophils.

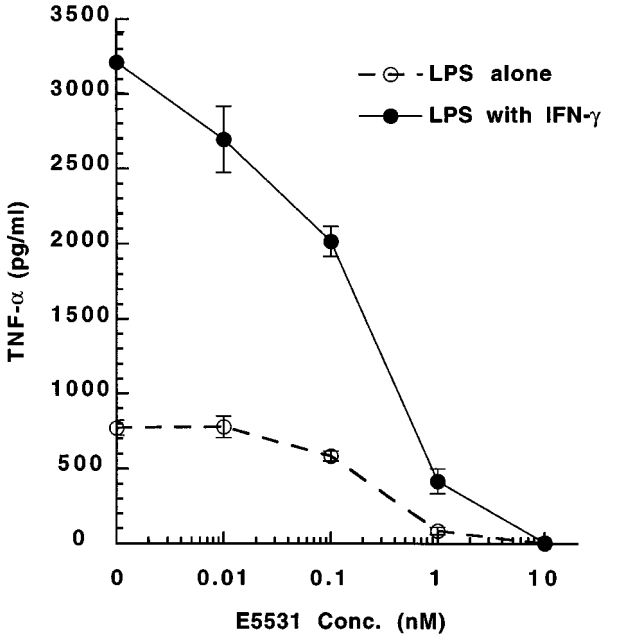

Figure 4 Inhibition of IFN-γ potentiated TNF-α production by E5531. Human monocytes were incubated with or without 100 U ml^{-1} of IFN-γ for 24 h then treated with the indicated concentration of E5531 and 10 ng ml^{-1} *E. coli* LPS. After an additional 3 h incubation, supernatant samples were assayed for TNF-α as described in Methods. Each point represents the means ±s.e.mean of triplicate and the result is representative of two similar experiments assayed in triplicate.

E5531 inhibition of LPS-induced mitogenicity in murine splenocytes

The specificity of E5531 activity against LPS was evaluated by testing inhibitory activity against LPS and Con A-induced mitogenicity (Figure 6). Both 30 ng ml^{-1} LPS and 3 μg ml^{-1} Con A stimulate the incorporation of [^{3}H]-thymidine into murine splenocytes *in vitro*, indicating that both agents are mitogenic in this system. As expected for an antagonist with a high degree of specificity, E5531 dose-dependently antagonized the mitogenic effect of LPS (IC_{50} = 72 nM) but not that of Con A. In addition, the activity of E5531 decreased with increasing concentrations of LPS (data not shown), but demonstrated nearly complete suppression of stimulation by LPS when used at 1 μM. These results indicate that E5531 is a potent antagonist of LPS-induced mitogenicity in murine splenocytes *in vitro*.

Effects of E5531 on LPS-induced NO release

LPS-stimulated release of NO from murine macrophages, and this activity is also potentiated by IFN-γ (Rose *et al*., 1995). In our assay system, release of NO from RAW 264.7 cells induced by 10 ng ml^{-1} LPS is increased 160% by the addition of 1 Unit ml^{-1} murine IFN-γ and 210% by 10 Units ml^{-1} IFN-γ (data not shown). Addition of higher levels of IFN-γ (100 Units ml^{-1}) alone resulted in significant stimulation of NO release ($\sim$18 μM nitrite in 24 h; Figure 7). This stimulation due to IFN-γ alone was not blocked or enhanced by E5531, even when added at concentrations up to 1000 nM. These data also indicate that E5531 did not induce NO production when added to cells in the presence of 100 Units ml^{-1} of IFN-γ.

LPS alone (10 ng ml^{-1}) induced $\sim$21 μM nitrite. This induction was blocked completely by E5531 (Figure 7). LPS (10 ng ml^{-1}) in combination with 100 Units ml^{-1} of IFN-γ induced total NO levels of 34 μM. In this case, E5531 inhibited NO induction to the level seen induced by IFN-γ alone (Figure 7). These results indicate that even in the presence of very high concentrations of IFN-γ, E5531 can still inhibit LPS-mediated cellular activation. In addition, the lack of inhibition of IFN-γ-induced NO release suggests that E5531 antagonistic activity is specific for LPS.

E5531 inhibition of LPS-induced E-selectin expression in HUVEC

In membrane CD14 negative HUVEC, LPS stimulation is mediated through binding with soluble CD14. We investigated whether E5531 inhibits LPS-induced E-selectin expression in the system (Figure 8).

Both 100 ng ml^{-1} LPS and 1 ng ml^{-1} TNF-α significantly induced E-selectin expression on HUVEC, 2.56 and 7.12 fold, respectively. E5531 inhibited LPS-induced E-selectin expression in a dose-dependent manner with an IC_{50} of 1.5 nM and inhibited the expression 96.2% at 100 nM. On the other hand, E5531 did not measurably inhibit TNF-α-induced E-selectin expression even at a concentration up to 100 nM, suggesting that the effect of E5531 was specific for LPS.

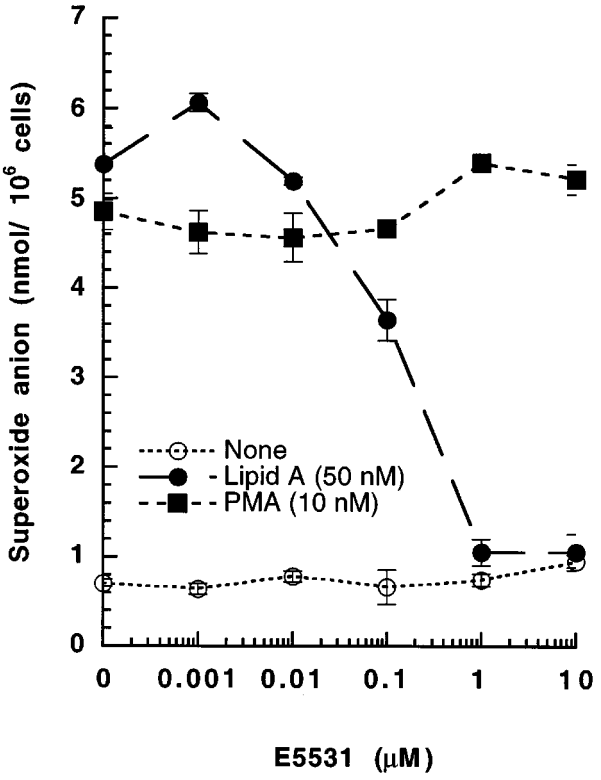

Figure 5 Inhibition of FMLP-stimulated superoxide anion production by E5531 in lipid A primed human neutrophils. Human neutrophils were incubated for 1 h with either 50 nM lipid A or 10 nM PMA plus the indicated concentration of E5531. After the incubation, superoxide anion produced by 1 μM FMLP and 10 nM PMA was measured as described in the Methods. Each point represents the means$\pm$s.e.mean of triplicate and the result is representative of three similar experiments assayed in duplicate or triplicate.

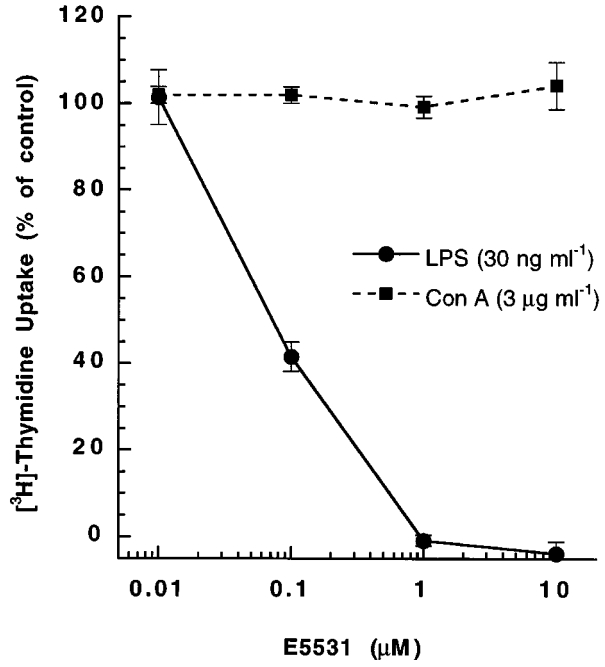

Figure 6 Effects of E5531 on the mitogenic activities of LPS and Con A. Mouse splenocytes (4×10^5 cells well^{-1}) were incubated with the indicated concentration of E5531, and 30 ng ml^{-1} of LPS or 3 μg ml^{-1} of Con A. After 48 h incubation, 0.5 μCi of [^{3}H]-thymidine was added to each well. After an additional 5 h incubation, the radioactivity incorporated into cells was determined. [^{3}H]-thymidine uptake of basal, E5531 (10 μM), LPS (30 ng ml^{-1}) and Con A (3 μg ml^{-1}) alone were 5001$\pm$88, 3633$\pm$177, 14,980$\pm$686 and 94,799$\pm$3638 d.p.m., respectively. Each point represents the means$\pm$s.e.mean of quadruplicate and the results is representative of three similar experiments assayed in duplicate or quadruplicate.

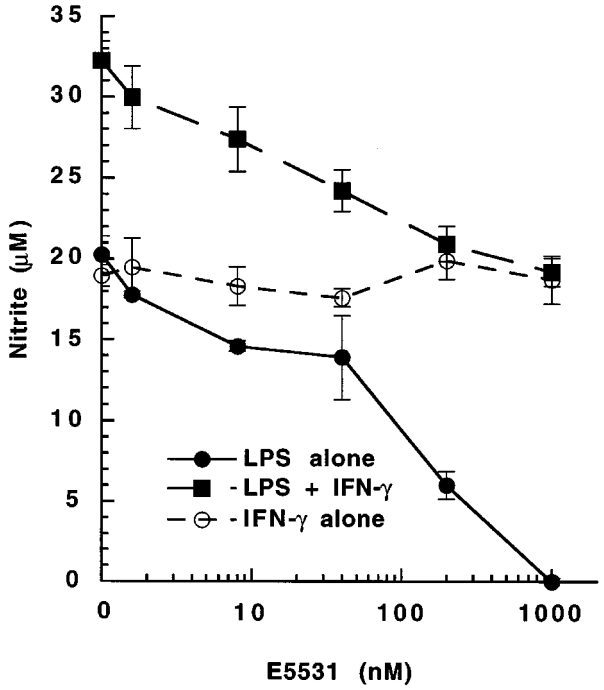

Figure 7 E5531 inhibition of LPS and IFN-γ-induced production of NO. RAW 264.7 cells were incubated in the presence or absence of 10 ng ml^{-1} LPS either with or without 100 Unit ml^{-1} IFN-γ plus the indicated concentration of E5531. After 24 h, culture supernatants were assayed for nitrite as described in Methods. Each point represents the mean ± s.e.mean of triplicate and the result is representative of three similar experiments assayed in duplicate or triplicate.

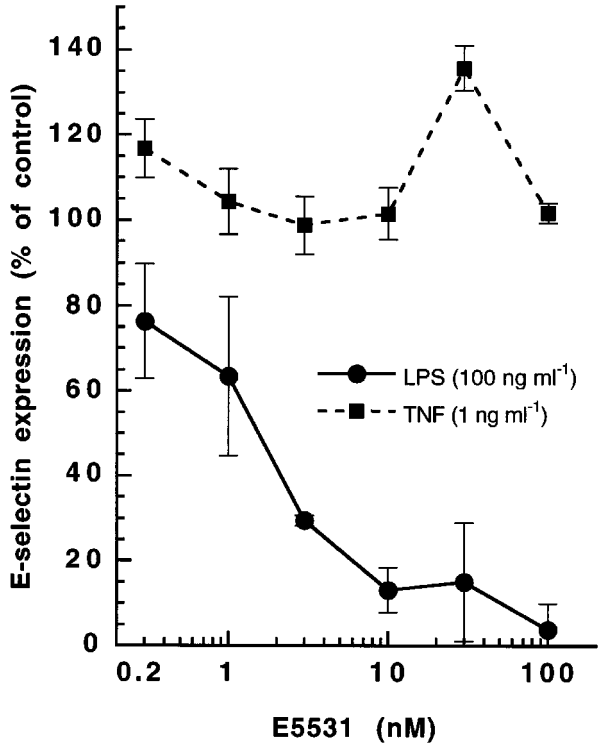

Figure 8 HUVEC were incubated with E5531 in the presence of LPS or TNF-α for 4.5 h. E-selectin expression was measured by ELISA after fixing with glutaraldehyde as described in Methods. Each point represents the means ± s.e.mean of triplicate and the result is representative of two similar experiments assayed in triplicate. LPS and TNF-α stimulated absorbance of 0.131 to 0.335 and 0.083 to 0.591, respectively.

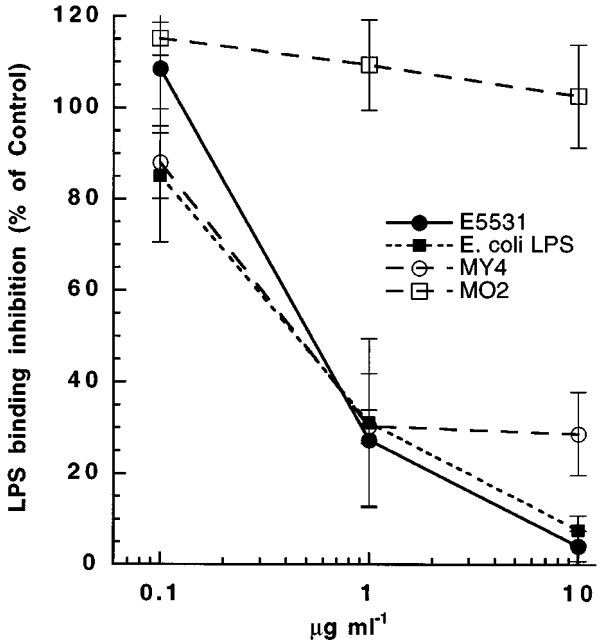

Figure 9 E5531 and other competitors inhibition of [^{125}I]-ASD-LPS binding. Human monocytes were incubated for 1 h with 60 ng of 18 μCi μg^{-1} [^{125}I]-ASD-LPS and one of the indicated reagents in RPMI containing 2% human serum. The cells were washed three times in washing buffer followed by the addition of 0.1 N NaOH to solubilize the radioactive substrate. Aliquots of the mixture were analysed using a gamma counter. Each point represents the means ± s.e.mean of three experiments assayed in duplicate.

Inhibition of LPS binding by E5531 in human monocytes

The ability of E5531 to inhibit the binding of LPS to human monocytes was evaluated. An examination of time dependence of LPS binding determined that maximum specific binding of [^{125}I]-ASD-LPS occurred after 1 h incubation with monocytes and remained constant to human monocytes for up to 3 h thereafter.

E5531 dose-dependently inhibited binding of [^{125}I]-ASD-LPS with an IC_{50} of 0.52 μg ml^{-1}. Under these conditions, complete inhibition was demonstrated when the compound was used at 10 μM (Figure 9). Unlabelled *E. coli* LPS inhibited [^{125}I]-ASD-LPS binding with an IC_{50} of 0.43 μg ml^{-1} to human monocytes. MY4, a monoclonal antibody directed against the CD14 receptor, also inhibited LPS binding in this assay system. However, MO2, another monoclonal antibody directed against a different epitope of CD14, did not inhibit LPS binding. Before the binding experiment, we have confirmed that MY4 also inhibited LPS-induced TNF-α production, but MO2 did not inhibit it (data not shown). The results showed that E5531 inhibited CD14 mediated LPS binding.

Discussion

Cellular activation of hematopoietic cells by endotoxin results in release of a variety of cytokines and other cellular mediators likely to play a major role in induction of septic shock. The antagonistic potency of E5531 against LPS-induced cellular activation could be demonstrated by assaying any of a variety of cytokines in several systems (Christ *et al*., 1995). Here, we measured release of TNF-α in human whole blood and monocytes (Figures 2A, 3 and Table 1). These results suggest

that E5531 effectively suppresses TNF-α production in response to LPS or lipid A. In addition, E5531 also antagonizes priming human neutrophils by LPS or lipid A (Figure 5). E5531 inhibited mitogenicity of LPS in murine thymocytes (Figure 6) and LPS-induced nitric oxide production in a murine macrophage cell line (Figure 7). Furthermore, E5531 inhibited LPS-induced E-selectin expression in HUVEC (Figure 8). Taken together, antagonism of this wide variety of cytokines (Christ *et al*., 1995) and other biological responses as well as inhibition of NF-κB activation by LPS (Christ *et al*., 1992) demonstrates that E5531 antagonizes activation of cells by LPS.

Biosynthetic precursors of lipid A, as well as non-toxic lipid As from different Gram-negative bacteria, can antagonize the toxic activities of LPSs from organisms such as *E. coli* and *S. minnesota* (Loppnow *et al*., 1990; Qureshi *et al*., 1991; Rose *et al*., 1995; Takayama *et al*., 1989). Based on our research and other published reports (Danner & Parrillo, 1987; Lam *et al*., 1991), Lipid X, a biosynthetic monosaccharide precursor of lipid A, was only a weak LPS antagonist. On the other hand, lipid IV_A, a disaccharide precursor of lipid A biosynthesis has demonstrated greater potency as an antagonist of LPS in human whole blood (Kobach *et al*., 1990). Related studies from us and other laboratories suggest that other synthetic and biologically-derived analogues of bacterially-derived non-toxic lipid As from both *R. capsulatus* and from *R. sphaeroides* can more potently antagonize the toxic effects of LPS (Loppnow *et al*., 1990; Lynn & Golenbock, 1992; Qureshi *et al*., 1991; Rose *et al*., 1995; Takayama *et al*., 1989). However, despite their antagonistic properties, some bacterially-derived materials also exhibited agonistic activity in human and/or animal model systems (Rose *et al*., 1995). The activity of E5531 places it among the most potent antagonists of LPS ever reported. In monocytes, E5531 was approximately 26,000 fold more active than Lipid X and 500 fold more active than Lipid IV_A (Table 1) and demonstrates similar or better *in vitro* antagonistic activity when compared to results previously reported for *R. Sphaeroides* lipid A (Rose *et al*., 1995).

E5531 was tested at concentrations up to 10 μM and found to be completely devoid of agonistic activity in human whole blood (Figure 2B). In addition, E5531 was not agonistic in LPS-sensitive mice *in vivo* even when administered in the presence of a LPS-potentiating agent such as BCG (Kobayashi *et al*., 1998).

Only minor differences in potency of E5531 are observed for inhibition of both LPS-induced TNF-α and NO with or without the potentiating effects of IFN-γ (Figures 4 and 7). However, no antagonism to other stimulants was observed. For example, neither IFN-γ-induced NO production (Figure 7), PMA-stimulated superoxide anion production (Figure 5), Con A-stimulated mitogenicity (Figure 6) or TNF-α-induced E-selectin expression (Figure 8) were inhibited. These results indicate that E5531 is a specific antagonist for LPS or lipid A.

In *in vitro* assays, plasma components had only minor inhibitory effects on the activity of E5531. Comparison of the ability of E5531 to block TNF-α release in monocytes (1% serum) and whole blood (80% plasma) indicated differences in potencies of approximately 20 fold (IC_{50}s = 0.13 nM in Table 1 and 2.6 nM in Figure 2, respectively). At the present time, it is unclear how plasma components affect the activity of E5531, however, it is possible that the relatively large amount of proteins in blood may non-specifically adsorb the compound or some proteins may specifically bind E5531, rendering it less active. Recent studies demonstrate that LPS binds to a variety of proteins including transferrin (Berger *et al*., 1991), lactoferrin (Appelmelk *et al*., 1994), lysozyme (Takada *et al*., 1994), bactericidal/permeability increasing protein (Marra *et al*., 1992) and even albumin (Wollenweber & Morrison, 1985). It may be possible that E5531 binds to one or more of these same proteins. In addition, it is well-known that lipoproteins such as HDL inactivate LPS after LBP binds LPS and transfer it to HDL (Emancipator *et al*., 1992; Vosbeck *et al*., 1990; Weinstock *et al*., 1992). These lipoproteins may also contribute to reduce the activity of E5531 in blood, although it retains its potent LPS antagonistic activity.

The exact mechanism of E5531 activity in blocking the biological activities of LPS has yet to be elucidated. However, evidence indicating that E5531 is a lipid A receptor antagonist include: (1) inhibitory activity of E5531 was only seen with LPS and was ineffective against IFN-γ, PMA, Con A or TNF-α (Figures 5, 6, 7 and 8); (2) antagonistic activity of E5531 was dependent on added LPS concentration: when LPS concentration increased from 10 ng ml^{-1} to 1000 ng ml^{-1}, activity of E5531 decreased more than 100 fold (Christ *et al*., 1995); and (3) E5531 antagonism decreased with time after addition of LPS: the addition of E5531 1 h after LPS reduced antagonism to 47% inhibition (Figure 3). These results suggest that E5531 is a type of 'competitive' receptor antagonist. E5531 was also a potent antagonist against lipid A as well as LPS (Table 1), indicating that E5531 antagonizes the lipid A, toxicophore of LPS. Lipid A is also a common structural feature of LPSs from a variety of Gram negative bacteria (Raetz, 1990). Clearly, antagonism of the activity of this common toxicophore is a probable explanation for E5531 activity against a wide variety of LPSs (Christ *et al*., 1995). E5531 also antagonized LTA which is a product from Gram positive bacteria (Table 1). However, it is likely that the biological activity of LTA is also through LPS receptor or CD14. Therefore, this result also supports the hypothesis that E5531 is a competitor of natural CD14 ligands.

Working on the assumption that E5531 blocked the interaction of LPS with cells, binding studies were performed to determine if this is the mechanism by which E5531 antagonizes cellular activation (Figure 9). In these experiments, unlabelled *E. coli* LPS and E5531 effectively competed for available binding sites for [^{125}I]-ASD-LPS on human monocytes with IC_{50} value of 0.52 μg ml^{-1} (0.34 μM) and 0.43 μg ml^{-1}, respectively. Assuming LPS has an average molecular weight of 30,000 kDa, its IC_{50} is 0.014 μM. Thus, E5531 demonstrated 24 fold less affinity than LPS. MY4, an antibody of CD14 which inhibits the biological activity of LPS potently suppressed LPS binding, supporting the inhibited LPS binding that is through CD14.

LPS binding to its receptor requires recognition by at least two elements, LBP (Mathison *et al*., 1992; Tobias *et al*., 1989) and CD14 (Frey *et al*., 1992). LBP disaggregates LPS micelles and functions as a LPS carrier protein which can transfer LPS to CD14 and/or to other LPS binding molecules such as lipoproteins. The LPS/LBP complex binds to CD14 resulting in activation of a variety of endotoxin-sensitive cells. Membrane-bound CD14 is a glycerophosphatidylinositol anchored protein. Therefore, it is hypothesized that CD14 functions as the ligand binding protein of a multimeric LPS receptor involved in cell signalling. It is likely that E5531 functions by interacting at the same site(s) where LPS or lipid A binds. Alternatively, E5531 may competitively block LPS/LBP binding by forming complexes with LBP. The E5531/LBP complex or E5531 alone may block LPS binding to CD14 and antagonize LPS-triggered biological responses. These possibilities are currently under investigation. Recently, TLR-2, one of human Toll-like receptors, has been proposed as a signalling receptor for LPS (Yang *et al*., 1998) and TLR-4 has been

found to be the source of genetic mutation in two LPS-resistant mouse mutants (Poltorak *et al*., 1998). Therefore, it would be of interest to determine if E5531 inhibits LPS binding to either TLR-2 or TLR-4.

Ten years of clinical trials testing anti-cytokine therapies and endotoxin binding have yielded disappointing results (Glauser, 1996). We know that multiple cytokines and other cellular mediators are induced by endotoxin and involved in the septic response, making it doubtful that inhibiting the action of a single cytokine will be effective. In addition, some cytokines may be important immunomodulators required for defense against infection. This would require timely administration of anti-cytokine therapy that may otherwise block their beneficial action. Blocking the entire cytokine cascade by inhibiting effects of endotoxin altogether, has been previously attempted, with two anti-lipid A monoclonal antibodies. These failed in clinical trials, probably due to rather low intrinsic binding affinities for the lipid A component of bacterial endotoxin (Awarren *et al*., 1993). On the other hand, it has been recently reported that rBPI21, a recombinant amino-terminal fragment of human bactericidal/permeability-increasing protein can kill meningococci and bind to and clear bacterial endotoxin with high affinity. This material was effective in children with severe meningococcal sepsis (Giroir *et al*., 1997). The latter result suggests that anti-endotoxin therapy is effective in one form of bacterial sepsis. Like rBPI21, the synthetic compound described herein is a potent inhibitor of the activity of endotoxin but even more effective in an endotoxin challenge test in human volunteers (Lynn *et al*., 1995; Rossignol *et al*., 1999). The ability of E5531 to potently block endotoxin activity represents a novel point for interfering with the sepsis cascade. E5531 is now in phase II studies to evaluate efficacy in sepsis patients.

In conclusion, E5531 is a potent and synthetic lipid A antagonist that effectively inhibits LPS-induced cellular activation as measured by release of cytokines such as TNF-α and other cellular mediators such as nitric oxide and superoxide anion. E5531 is specifically antagonistic against LPS and is both non-toxic and non-agonistic. It is possible that the potent and specific antagonist activity of E5531 may serve as a beneficial therapeutic agent in the treatment of Gram-negative septic shock and other clinical indications associated with endotoxin.

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(Received November 11, 1998
Revised March 15, 1999
Accepted March 17, 1999)