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Journal of Immunological Methods 212 (1998) 169–185

JOURNAL OF IMMUNOLOGICAL METHODS

A rapid assay of endotoxin in whole blood using autologous neutrophil dependent chemiluminescence

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Received 20 March 1997; revised 17 December 1997; accepted 18 December 1997

Abstract

A rapid (30 min) whole blood assay for the detection of lipopolysaccharide (LPS) is described. This chemiluminescent (CL) assay utilizes the CR_1 and CR_3 receptor-induced oxidant production of polymorphonuclear leucocytes as a detection platform. The differential priming of neutrophils in whole blood by LPS-antibody complexes allows the specificity of the assay to be achieved. Oxidant released in response to complement opsonized zymosan results in luminol oxidation and subsequent light emission. This is dependent on heat labile putative complement proteins in the plasma. The assay consists of a *control* which measures baseline whole blood neutrophil oxidant production. The *test* assay contains murine monoclonal IgM antibody against the Lipid A epitope of LPS and measures the enhanced chemiluminescent response of the neutrophils in the presence of LPS-antibody complexes. Maximal sensitivity of the CL assay is dependent upon optimal antigen–antibody equivalence and duration of pre-incubation with the whole blood sample. The quantification of LPS is possible by inclusion of a positive control containing a maximally reactive LPS dose (800 pg/ml *Escherichia coli* 055:B5 LPS at an antibody concentration of 0.8 μg/assay). The CL assay is insensitive to variations in patient neutrophil concentration over a minimum range of 0.5 to 20×10^9 cells/l. The CL assay is widely reactive with the LPS of many strains of gram negative bacteria but not with the cell wall products of gram positive bacteria or *Candida* and *Aspergillus*. In comparison to acid extraction chromogenic LAL, the CL assay demonstrates superior recovery precision and accuracy in in vitro studies. This was reproducible over a wide range of LPS concentrations (0.017–1.6 EU/ml or 20–2000 pg/ml). This assay may be a clinically useful tool for the diagnosis of infection or endotoxin in patients. © 1998 Published by Elsevier Science B.V.

Keywords: Endotoxin bioassay; Chemiluminescence; Neutrophil activation

1. Introduction

Endotoxemia (lipopolysaccharide or LPS), from the cell wall of gram negative bacteria is an important trigger of septic shock (Van Deventer et al.,

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0022-1759/98/$19.00 © 1998 Published by Elsevier Science B.V.
PII S0022-1759(98)00003-9

1988; Raetz et al., 1991; Casey et al., 1993; Hazinski et al., 1993; Hoffman and Natauson, 1993; Parillo, 1993; Rietschel et al., 1994). Endotoxin complexed to lipopolysaccharide binding protein (LBP) interacts with membrane CD14, triggering a complex cytokine cascade (Beutler et al., 1985; Mathison et al., 1988; Wright et al., 1990; Tobias et al., 1992; Pagin et al., 1995). Despite evidence implicating endotoxin as an important mediator in the pathogenesis of septic shock, its measurement in blood or biological fluids has been difficult (Obayashi et al., 1986, 1987; Roth et al., 1990). The disappointing results from the recent clinical trials of anti-endotoxin treatments (McCloskey et al., 1994; Bone et al., 1995) reflect in part the problem of rapidly identifying patients who might benefit from therapy directed against LPS (Baumgartner et al., 1990; Kuhn et al., 1992; Baumgartner and Glauser, 1993; Warren et al., 1993a,b).

The standard assay for determination of LPS has been the limulus amebocyte lysate (LAL) assay (Levin and Bang, 1968). This assay performs well when endotoxin is not bound by specific receptors (Yoshimi et al., 1995). In plasma and whole blood LPS binds to a number of plasma proteins, including LBP, soluble and cell bound CD14, CD11b/CD18 integrin receptors, high density lipoprotein particles (HDL) and to cellular blood components such as platelets (Pugin, 1996). Various extraction and pretreatment strategies have attempted to release LPS from its binding sites in whole blood (Tamura et al., 1991) or plasma, or to neutralize poorly defined inhibitors or activators, which confound LAL based methodologies (Roth et al., 1990). The results have been generally unsatisfactory.

The limitation of available LPS assays led us to examine an alternative approach for the detection of endotoxin in whole blood. Our goal was to establish a simple and sensitive technique, minimally prone to environmental contamination, that would be amenable to use at the bedside.

We report here the development and standardization of a novel technique for the rapid assay of LPS in whole blood. The assay is based upon CR1 and CR3 receptor triggered oxidant production of blood neutrophils, measured by luminol oxidation and subsequent light emission (Allen, 1986). The detection of endotoxin is dependent upon the formation of antigen–antibody complexes in whole blood and their subsequent detection by complement mediated priming of blood neutrophils. The technique requires few reagents, is simple to perform and minimally susceptible to contamination. It can yield quantifiable results from as little as 10 μl of whole blood in 30 min from the time of sample acquisition.

2. Materials and methods

2.1. Reagents and bacterial products

Luminol (5-amino-2,3-dihydro-1,4-phthalazinedione, free acid), zymosan A (*Saccharomyces cerevisiae*), lipopolysaccharides from *Escherichia coli* (*E. coli*) serotypes (026:B6, 055:B5, 0111:B4), *Salmonella abortus equi*, *S. typhimurium*, *S. enteritidis*, *Klebsiella pneumoniae*, lipoteichoic acids from *Streptococcus sanguis*, *S. pyogenes*, *S. mutans*, *S. faecalis*, *Bacillus subtilis* and *Staphylococcus aureus* were purchased from Sigma (St. Louis, MO). Cell wall extracts of *Neisseria meningitidis*, *Enterobacter cloacae*, *Staphylococcus epidermidis*, *Hemophilus influenzae*, *Aspergillus fumigatus*, *Candida albicans* and *Enterococcus faecalis* were prepared by growing cells on standard culture plates with the appropriate medium (two plates per organism) and transferring the organisms into 25 ml of sterile pyrogen free saline. These suspensions were then autoclaved for 1 h at 120°C to lyse cells and release cell wall components. The cellular debris was removed by centrifugation at $10{,}000 \times g$ for 1 h at 4°C followed by dialysis of the supernatant against sterile water (2×50 volumes) at 4°C overnight to remove the saline. The resultant cell wall extracts were lyophilized and weighed prior to reconstitution. All storage containers used for bacterial, fungal or yeast products were depyrogenated at 300°C for a minimum of 6 h.

2.2. Antibody preparation and purification

A murine hybridoma produced from mice immunized with boiled *E. coli* J5 cells was obtained from the ATCC (Accession number HB9081) and expanded in culture before injection into the peritoneal cavity of pristane primed mice. Peritoneal ascites was collected aseptically, sterile filtered and purified

by sterile gel filtration chromatography on Sephadex G-150 (60 × 2.5 cm column) to yield an enriched IgM monoclonal fraction eluting with the void volume. The antibody was suspended in 50 mM phosphate buffered saline (PBS) at a total protein concentration of 1–2 mg/ml (Bradford, Coomassie Blue protein assay). Polyacrylamide gel electrophoresis (7.5% gel) under denaturing and reducing conditions suggested that an antibody purity of about 80% had been achieved by densitometric estimation. The antibody was stored at 4°C in pyrogen free 1 ml glass vials containing 100 U/ml of Penicillin G and 100 μg/ml Streptomycin sulfate.

2.3. Turbidimetric detection and ELISA of LPS–anti LPS antibody complexes

Anti-LPS antibody (stock conc. 2 mg/ml) was diluted 10 to 10,000 fold in sterile pyrogen free Hanks Balanced Salt Solution (HBSS) containing 6% w/v polyethylene glycol 6000. LPS from *E. coli* serotype 055:B5 was dissolved in HBSS at concentrations of 100, 50, 25, 10 and 1 μg/ml. The antibody solutions and the LPS solutions were placed into sample microcups on a Cobas Fara centrifugal analyzer (Roche Diagnostics, Mississauga, Ont., Canada) and equivalent 100 μl volumes of each solution were pipetted into the analyzer cuvette array at a temperature of 37°C. Optical density was measured repeatedly at 340 nm at 30 s intervals for a total period of 5 min. Standard 96 well ELISA plates were coated with 5 μg/ml of *E. coli* J5 or 055:B5 LPS (100 μl) in 50 mM carbonate/bicarbonate buffer pH 9.6. The LPS antigens were incubated at 37°C for 2 h and then overnight at 4°C. The plates were subsequently washed three times with PBS/Tween (50 mM phosphate buffered normal saline, 0.05% Tween 20, pH 7.5) and blocked with 2 mg/ml low endotoxin human serum albumin in PBS overnight at 4°C. The plates were incubated with antibodies for 90 min at 37°C and subsequently with rabbit antimouse IgM antibody (100 μl of 1:5000 dilution of 1 mg/ml) labelled with alkaline phosphatase for 60 min. Plates were developed by incubation with *p*-nitrophenylphosphate substrate (100 μl of 1 mg/ml in 100 mM diethanolamine buffer pH 10.6 with 5 mM $MgCl_2$) for 15 min at 25°C and read at 405 nm.

2.4. Acid extraction—chromogenic limulus amebocyte lysate assay of whole blood samples

All reagents used for the LAL assay were prepared from LAL grade water (Sigma). Acid or base solutions for whole blood extraction were prepared from LAL grade water and autoclaved at 120°C for 90 min. The method of Tamura et al. was used for the extraction of whole blood samples (Tamura et al., 1991). Whole blood (100 μl) was extracted with 400 μl of nitric acid in Triton $X-100$ solution (0.66 N acid, 0.25% w/v Triton) with heating at 37°C for 5 min followed by centrifugation to remove the supernatant fluid. The supernatant fluid (200 μl) was neutralized with an equivalent volume of 0.55 N sodium hydroxide and assayed for endotoxin using the Pyrochrome chromogenic LAL reagent and protocol (Associates of Cape Cod, Woods Hole, MA). All LAL assay samples were serially twofold diluted from an initial dilution of 1:10 to a final dilution of 1:320 using LAL grade water in Pyroplate (Associates of Cape Cod) 96-well microtitre plates and assayed according to the manufacturers instructions.

2.5. Chemiluminescence reagents

Buffer for measurement of whole blood or white cell chemiluminescence studies was HBSS (pyrogen free, endotoxin less than 0.005 EU/ml) containing 1.5 mM calcium and 0.9 mM magnesium (Gibco BRL, Grand Island, NY). This buffer (500 ml) was vigorously mixed overnight at 25°C with luminol to yield a saturated solution (150 μM, HBSS–luminol) and then supplemented with 4 U/ml of lithium heparin. To prepare human complement opsonized zymosan, pooled fresh frozen citrate anti-coagulated human plasma was dialyzed against four volumes of 28.5% saturated ammonium sulfate solution for 2 h at room temperature and then against fresh 28.5% saturated ammonium sulfate overnight at 4°C. The precipitate was removed by centrifugation and the supernatant dialyzed against two changes of 10 volumes of HBSS without calcium and magnesium at 4°C. This immunoglobulin depleted serum fraction (< 10% of original IgG and IgM based on nephelometric assay) was then mixed with a half volume of heat activated zymosan A (5 g/l of normal saline) in the presence of 1.3 mM calcium and 0.9 mM magne-

sium for 15 min at room temperature to opsonize the zymosan.(Allen, 1986, 1992) The opsonized zymosan was subsequently washed three times with two volumes of ice cold sterile normal saline and resuspended in its original volume (approximately 3×10^6 particles/μl).

2.6. Chemiluminescent assay for endotoxin

All glass surfaces used for endotoxin assay or storage of reagents for endotoxin assay including assay tubes were depyrogenated by heating to 300°C for at least 6 h. All polystyrene and polyethylene surfaces used for storage of antibodies, HBSS–luminol or blood products were sterile and essentially endotoxin free as determined by chromogenic LAL assay of pyrogen free water left in contact with the surface of interest. All pipette tips used for fluid transfer were sterile and pyrogen free (Diamed, Mississauga, Ont., Canada). Blood samples used for the assay were drawn by venipuncture or through indwelling arterial lines into sterile 3 ml EDTA anticoagulated vacutainer tubes (Becton Dickenson, Franklin Lakes, NJ) which were pretested for LPS content (less than 0.005 EU/ml).

All chemiluminescence experiments utilizing whole blood or cell fractions were assayed in triplicate and the results expressed as the mean counts per minute ± 1 SD. In all assays HBSS–luminol buffer (300 μl) was pre-mixed with 30 μl of antibody solution and subsequently incubated with 10 μl of whole blood or isolated neutrophils in fresh human plasma. After incubation with blood at 37°C for 5 min in a thermostatted aluminum heating block the assay tubes were transferred to the chemiluminometer (E.G. & G. Berthold Autolumat LB953, Wildbad, Germany) for addition of 20 μl of human complement opsonized zymosan. All assays were run at 37°C in the chemiluminometer for 20 min with continuous measurement of light emission from each tube at least every 60 s for a minimum 0.6 s counting window. Chemiluminescence reaction curves and integrals were captured using Axis Cellular Luminescence System Software (version 1.03 from ExOxEmis, San Antonio, TX).

2.7. Isolation of neutrophils

Human blood neutrophils (PMN) from healthy volunteers were purified by dextran sedimentation and centrifugation at $200 \times g$ on 10 ml of Ficoll-Paque (Pharmacia, Uppsala, Sweden). Erythrocytes were lysed with buffered ammonium chloride (0.84%). The remaining neutrophils were washed twice with HBSS (without Ca^{2+} and Mg^{2+}), and resuspended in HBSS (without Ca^{2+} and Mg^{2+}). Neutrophil purification was greater than 91% and viability greater than 96% by trypan blue exclusion.

2.8. Preparation of HL-60 cells

HL-60 cells were obtained from the American Tissue Culture Collection (ATCC# CCL-240 batch F-13617, passage #15). Cells were cultured in RPMI 1640 containing 10% heat inactivated fetal bovine serum (Gibco BRL), 100 U/ml penicillin G and streptomycin sulfate, and 2 mM L-glutamine. Cells were cultured and subsequently frozen at passage 18 in liquid nitrogen at cell counts of 1×10^6/ml. Cryopreserved cells were thawed, recovered for 24 h, and then differentiated for 72 h in 1 μM retinoic acid.

2.9. Preliminary evaluation of the clinical utility of the chemiluminescent LPS assay

A cohort of consecutive patients who were culture positive and admitted to the Medical Surgical Intensive Care Unit at The Toronto Hospital were studied with regard to endotoxin levels over a six-week period. The study protocol was reviewed and approved by the *Committee for Research on Human Subjects* of The Toronto Hospital. The need for informed consent was waived by the Committee. Blood samples were drawn into pyrogen free 7 ml EDTA vacutainer tubes, from an indwelling arterial line, within 6 h of ICU admission. Samples for bacterial cultures were drawn using standard practice for blood, protected lung brushings or suspected abscess sites, within 48 h of ICU admission. Assay personnel were blinded to comparative assay data and patient clinical status. A control group comprising 20 healthy ambulatory volunteers was used for comparative purposes and to act as a negative assay control. These volunteers were bled by antecubital vein puncture and blood collected in pyrogen free 7 ml EDTA vacutainer tubes (Becton Dickenson, Mississauga, Ont., Canada).

2.10. Statistical analysis

Chemiluminescence in whole blood samples was evaluated for changes in Response Factor by repeated measures ANOVA with Bonferroni *t*-test post hoc analysis. Comparisons of chemiluminescence assays was by one-way ANOVA with Bonferroni *t*-test post hoc analysis. Comparison of recoveries between LAL and chemiluminescent endotoxin assay was evaluated using Bland–Altman bias plots (Altman and Bland, 1983; Bland and Altman, 1986).

3. Results

3.1. Antibody reactivity

The ability of the murine monoclonal antibody to bind LPS examined by turbidimetric determination is shown in Table 1. At an antibody concentration of 1 μg protein/ml (in a total volume of 200 μl) the formation of immune complexes with LPS from *E. coli* 055:B5, 1200 pg/EU, could be detected in a dose-dependent manner over a range of LPS from 0.1 to 10 μg. The ability of the murine IgM antibody to bind LPS was also confirmed using microtitre plates precoated with LPS (data not shown).

3.2. Antigen–antibody equivalence

In subsequent experiments the anti-LPS antibody was pre-incubated with whole blood at varying concentrations and then mixed with luminol buffer and human complement opsonized zymosan. In the presence of endotoxin, it was found that an increase in chemiluminescent light emission could be detected above the baseline response to opsonized zymosan. The magnitude of the whole blood chemiluminescent response to LPS was dependent on the ratio of antigen and antibody concentration. Increased levels of endotoxin were detected with higher concentrations of antibody suggesting a zone of concentration equivalence. This is illustrated in Fig. 1 at three different antibody concentrations which span three orders of magnitude in antibody and antigen concentration. These assays were conducted using unoptimized conditions for the detection of each level of endotoxin displayed. High levels of LPS (approximately 200–2000 pg/ml or 0.3–1.7 EU/ml) were best detected at higher antibody concentrations (approximately 2 μg/assay), intermediate levels (20–200 pg/ml, 0.017–0.17 EU/ml) at antibody levels of 0.2 μg/assay and low levels of endotoxin (5–20 pg/ml, 0.004–0.017 EU/ml) at antibody levels of 0.02 μg/assay.

Table 1
Turbidometric detection of LPS

LPS concentration LP(μg/assay)	Absorbance ($\times 10^{-3}$)	1 Standard deviation
0	0.4	0.2
0.1	1.4	0.15
1	1.8	0.16
2.5	2.4	0.2
5	3.0	0.2
10	3.7	0.2

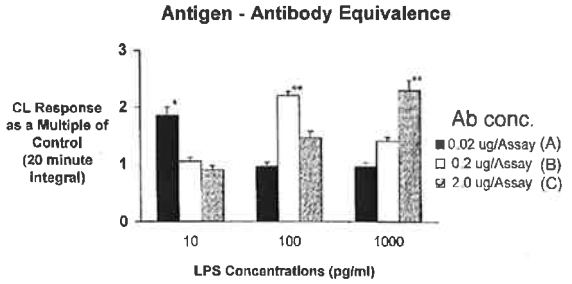

Fig. 1. Dependence of chemiluminescent endotoxin assay on antigen–antibody equivalence. Using blood from four donors LPS concentrations of 10,100 and 1000 pg/ml of whole blood were assayed at increasing antibody concentrations: A = 0.02 μg/assay, B = 0.2 μg/assay and C = 2 μg/assay. Emitted light intensities were expressed as 20 min light integrals normalized to the corresponding assay control containing an equivalent concentration of an irrelevant murine IgM. Values are presented as mean ± 1 SD. Asterisks indicate assays results were significantly different from the other LPS concentrations at the same antibody concentration ($^{*}p < 0.01$, Bonferroni *t*-test, $p < 0.006$ ANOVA) ($^{**}p < 0.03$ Bonferroni *t*-test, $p < 0.006$ ANOVA).

3.3. Studies related to LPS assay dynamics and mechanism

3.3.1. Minimum assay constituents

In order to determine the minimum blood constituents necessary to detect endotoxin the assay was attempted using isolated polymorphonuclear leuko-

cytes and plasma (EDTA anticoagulated) and zymosan (20 μl). Substitution of leukocytes with an equivalent concentration of differentiated HL-60 cells gave similar results (data not shown). Assay sensitivity was enhanced approximately five fold by the inclusion of opsonized zymosan.

The magnitude of chemiluminescence enhancement by endotoxin in the presence of antibody was maximal when an opsonized zymosan concentration of 6.0×10^7 particles/assay was utilized (20 μl of stock mixture).

3.4. Quantification of LPS

To permit quantification of endotoxin in whole blood, a maximum stimulatory concentration of LPS from *E. coli* 055:B5 (determined to be 800 pg/ml or 0.67 EU/ml at an antibody concentration of 0.8 μg/assay) was added to each patient sample in the presence and absence of anti-LPS antibody. A Response Factor was calculated as the difference between the antibody and non antibody dependent 20 min reaction integrals, divided by the difference in antibody and non antibody dependent 20 min reaction integrals of reaction mixtures containing a maximal stimulatory dose of LPS [RF = $\int$(Anti-LPS − Control)/$\int$(Anti-LPS(Max) − Control(Max)].

The averaged standard curve was established with blood obtained from 40 non-endotoxemic donors as displayed in Fig. 2 using this relationship. A simplified kinetic description of the chemiluminescence assay is presented in Appendix A based on equations initially described by Allen (1986). At the antibody concentration employed in the assays depicted in Fig. 2 (0.8 μg protein) a broad plateau of maximum response was achieved over a range of 800 to 2000 pg/ml (0.67–1.6 EU/ml). The range of maximal signal amplification achieved in the antibody containing assay tubes over the control tubes in the 40 samples used for the dose response study was 1.8 to 4.6 fold with a mean amplification of 2.9 fold (SD = 0.9). Since the coefficient of variation (CV) for both the control and antibody containing assay tubes is routinely in the range of 2–4%, this means that the maximal signal amplification to baseline noise ratio is approximately 70 (mean maximal amplification/CV of baseline = 2.9/0.04 = 70, therefore, the mean maximal signal amplification is 70 times larger than 1 CV of the baseline noise).

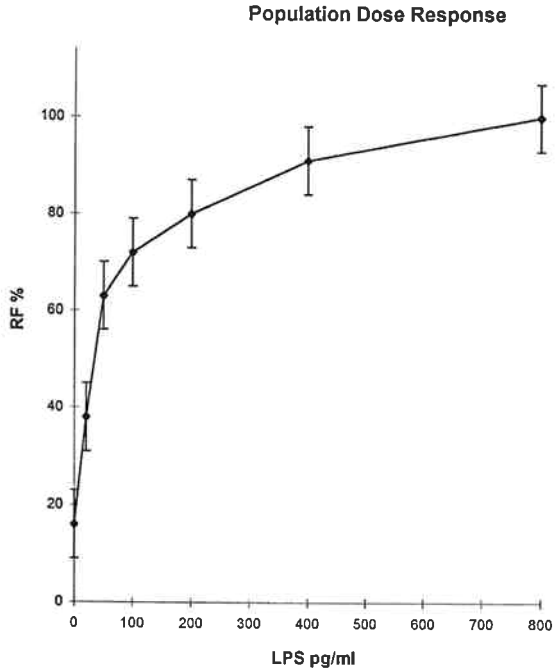

Fig. 2. A standard dose–response curve is shown with $n = 40$ non-endotoxemic donors at LPS concentrations of 0, 20, 50, 100, 200, 400 and 800 pg/ml of *E. coli* 055:B5 endotoxin added to whole blood. Individual data points represent the mean ± SD. The assays were executed with an anti-LPS antibody concentration of 0.8 μg protein/assay with control tubes containing all constituents except antibody. Pre-incubation time with antibody was 5 min at 37°C. RF = $\int$(Anti-LPS − Control)/$\int$(Anti-LPS(Max) − Control(Max).

3.5. Effects of antibody pre-incubation time on LPS assay

Detection of endotoxin using the chemiluminescent assay was independent of time of pre-incubation of whole blood with assay buffer containing antibody (from 0–15 min). No effect was observed over a period extending from immediate addition of zymosan to the assay mixture following blood injection to a pre-incubation period of blood with antibody extending for 15 min prior to the addition of zymosan at 37°C (data not shown, RM ANOVA $p > 0.05$). As demonstrated in Fig. 3, however, pre-incubation of blood with antibody for 60 min resulted

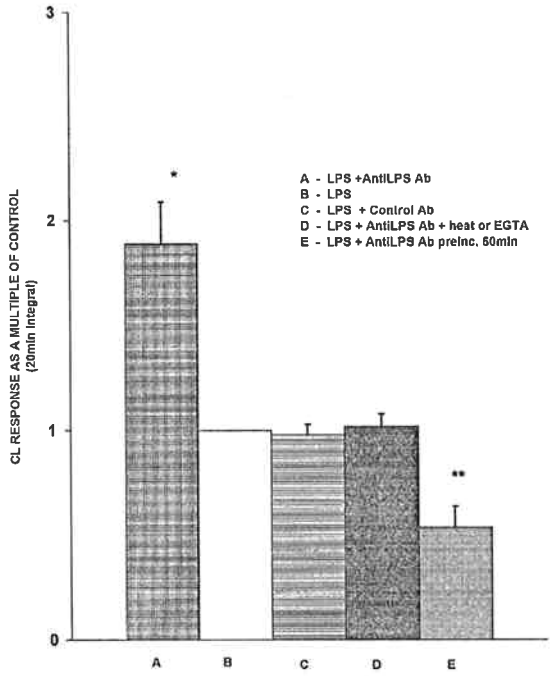

Fig. 3. Blood samples from five donors were assayed for chemiluminescent response to endotoxin (200 pg/ml, *E. coli* 055:B5). Chemiluminescent response was normalized to assay tubes containing LPS as a control (panel B) Light emission is displayed as mean normalized response ± 1 SD. All assays containing antibody employed a concentration of 0.8 μg/assay. Control antibody consisted of an irrelevant murine IgM. In panel D, plasma isolated from whole blood by centrifugation was treated at 56°C for 30 min or with 2 mM EGTA prior to reconstitution with cells. In panel E, anti-LPS antibody was preincubated with whole blood for 60 min prior to the addition of opsonized zymosan. ($^{*}p < 0.0001$ panel A different from panels C and D, Bonferroni *t*-test, $p < 0.0001$ ANOVA.) ($^{**}p < 0.0005$ panel E different from panels C and D Bonferroni *t*-test, $p < 0.0001$ ANOVA.)

in a paradoxical decline in chemiluminescence compared to control.

3.6. Control assays and lability of LPS signal

As indicated in Fig. 3, identical control assays were achieved using either an irrelevant murine IgM at equivalent concentration to anti-LPS antibody (Fig. 3, panel C) or by using no antibody in the control tube (Fig. 3, panel B). Treatment of whole blood with EGTA (2 mM final concentration) or heat treatment of the plasma fraction for 30 min at 56°C abolished the stimulatory effect of LPS–anti-LPS antibody complexes (Fig. 3, panel D). Pre-incubation with anti-LPS antibody for 60 min prior to addition of zymosan resulted in signal attenuation below the control tubes (Fig. 3, panel E).

3.7. Effects of erythrocyte and neutrophil concentrations

The dependence of the chemiluminescent response to LPS–anti-LPS antibody complexes on PMN concentration was tested in assays containing plasma supplemented with increasing concentrations

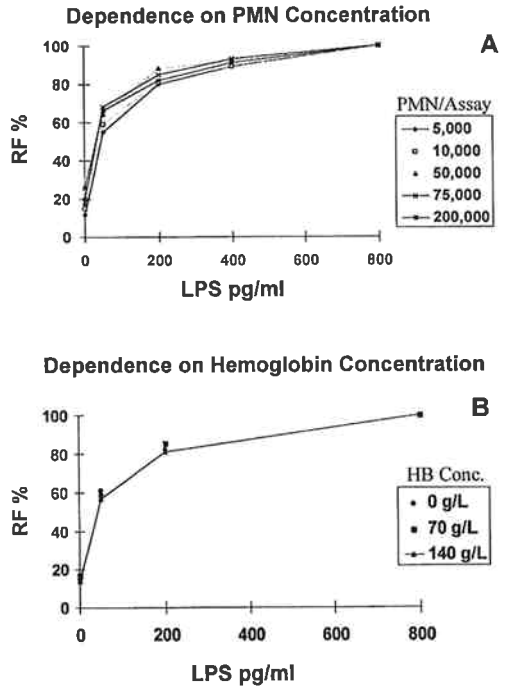

Fig. 4. Dependence of chemiluminescence endotoxin assay on neutrophil and hemoglobin concentration (panels A and B, respectively). Panel A: Utilizing isolated purified neutrophils from three donors assays were performed at neutrophil concentrations of 0.5, 1.0, 5.0, 7.5 and 20×10^9/l equivalent to a range of 5000 to 200,000 cells/assay. Each data point represents a mean of three separate assay determinations. At each LPS concentration no significant difference in response factor was observed as a function of PMN concentration ($p > 0.05$, ANOVA). Panel B: The effect of varying erythrocyte concentration on the endotoxin assay was examined at a fixed neutrophil concentration of 5.0×10^9/l or 5×10^4 cells/assay. Hemoglobin was varied from 0 to 70 and 140 g/l. No significant effect on LPS recovery was observed ($p > 0.05$, ANOVA). Individual data points represent the mean of three replicate assays.

of isolated neutrophils (Fig. 4). Samples were assayed at LPS concentrations of 0, 50, 200, 400, and 800 pg/ml starting with neutrophil concentration of 0.5×10^9 polymorphs/l (5,000 PMN/assay). Subsequent assays were repeated at the same LPS concentrations with neutrophil concentrations of 0.5, 1.0, 5.0, 7.5 and 20×10^9/l (Fig. 4A). At each LPS concentration, no significant difference in response factor was observed as a function of PMN concentration ($p > 0.05$, ANOVA). Using a similar approach, isolated washed erythrocytes were added to assays containing a fixed concentration of neutrophils (5.0×10^9/l or 5×10^4/assay). Assays were performed at LPS concentrations of 0, 50, 200 and 800 pg/ml and erythrocytes were added to give final hemoglobin concentrations of 0, 70 and 140 g/l (Fig. 4B). Again, no significant difference in response factor was observed as a function of hemoglobin and hence, erythrocyte concentration. ($p > 0.05$, ANOVA).

3.8. Sample age

The effects of sample age on endotoxin recovery were studied using cells from ten normal volunteers. Endotoxin was added to EDTA anticoagulated whole blood immediately after harvest and assayed within 15 min of endotoxin addition and again at 90, 120, and 180 min following incubation at room temperature or 37°C. Analysis of Response Factors at endotoxin doses ranging from 50 to 800 pg/ml revealed no difference ($p > 0.05$, paired RM–ANOVA) in endotoxin measurements made up to 90 min, at either 25°C or 37°C, but a detectable decrease in response was present in some samples after 120 min (data not shown). In three samples which showed no deterioration in chemiluminescence response to endotoxin after 3 h of aging, the chemiluminescence response was again evaluated after 12 h of incubation at room temperature and found to be the same as the initial assay. All subsequent chemiluminescence measurements were therefore made within 90 min of sample acquisition.

3.9. Assay precision

The precision of the assay was estimated by making 14 consecutive daily chemiluminescent LPS measurements on a normal volunteer by in vitro addition of LPS at levels of 50 and 400 pg/ml (0.042 and 0.33 EU/ml). At 50 pg/ml LPS the assay had a coefficient of variation (CV) of 12% and at an LPS level of 400 pg/ml a CV of 8%.

3.10. Assay specificity

The specificity of the assay is illustrated in Fig. 5. The assay responded to eight different LPS preparations but not to six separate lipoteichoic acid preparations.

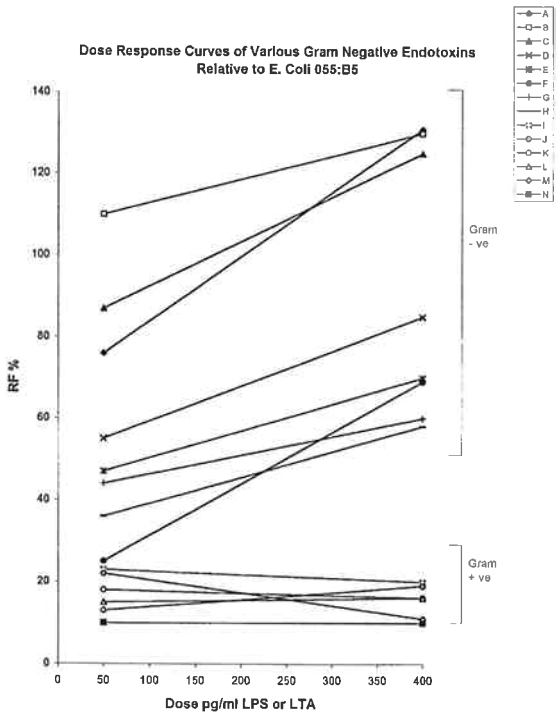

Fig. 5. Response of chemiluminescent endotoxin assay to various gram negative and gram positive cell wall products was tested at two levels (50 and 400 pg/ml of whole blood) using one donor. Bacterial cell wall constituents were standardized against LPS from *E. coli* 055:B5 (1200 pg = 1 EU). All assays were done in triplicate on two separate days and the mean response calculated and plotted as indicated. Gram negative products were phenol extracted LPS preparations and gram positive products were lipoteichoic acid extracts. A = *E. coli* 0111:B4, B = *Serration marcescens*, C = *Klebsiella pneumoniae*, D = *E. coli* 055:B5, E = *E. coli* 026:B6, F = *Salmonella typhimurium*, G = *Salmonella abortus equi*, H = *Salmonella enteritides*, I = *Streptococcus mutans*, J = *Streptoccocus pyogens*, K = *Streptococcus sanguis*, L = *Streptococcus faecalis*, M = *Staphylococcus aureus*, N = *Bacillus subtilis*.

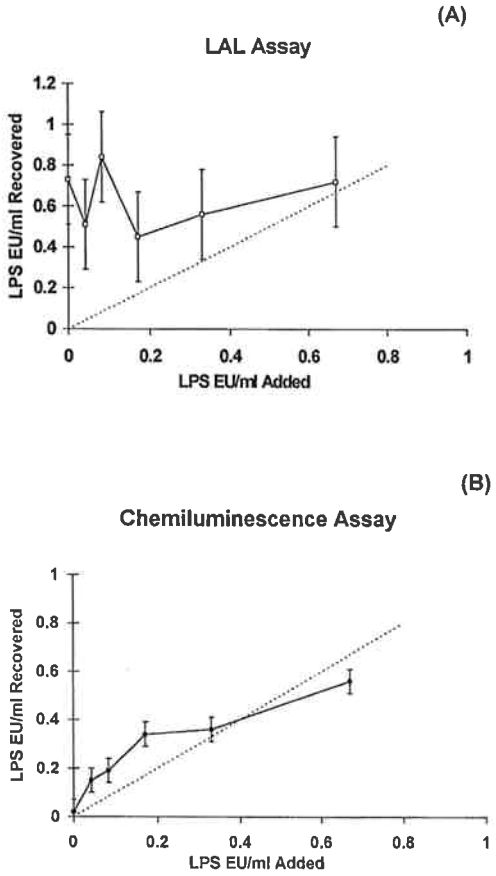

Fig. 6. Recovery of LPS added in vitro to whole blood using the acid extraction chromogenic LAL assay panel A vs. the chemiluminescence based endotoxin assay panel B. *E. Coli* 055:B5 LPS was added to whole blood at concentrations of 0, 50, 100, 200, 400, and 800 pg/ml and incubated for 30 min with gentle agitation at 25°C. This blood, taken from eight separate healthy donors, was then split for assay by the LAL and chemiluminescent procedures. Each data point represents a mean ($n = 8$) ±SEM. Chemiluminescent assay values were interpolated from the standard curve displayed in Fig. 2.

Positive dose responses were also achieved for crude cell wall extracts of *Neisseria meningitis* and *Enterobacter cloacae* (50 and 400 pg/ml, data not shown). The assay did not demonstrate a dose response relationship with cell wall extracts of *Staphylococcus epidermis* or *Staphylococcus aureus*, *Aspergillus fumigatus*, *Candida albicans* and *Enterococcus faecalis* (data not shown). The response of the assay was also tested with live *E. coli* inoculated in vitro into whole blood. The assay was responsive to inocula of 5×10^3 cfu/ml and greater, but was not tested at lower doses of live *E. coli* (data not shown).

3.11. *In vitro recovery studies*

Whole blood samples from eight healthy donors were incubated with *E. coli* 055:B5 endotoxin (1200 pg/EU) at concentrations of 0, 20, 50, 100, 200, 400, 800, and 2000 pg/ml and assayed using the chemiluminescence assay or the chromogenic LAL assay. The recovery data are summarized in Fig. 6. In Fig. 6, the in vitro recovery of LPS added to whole blood is graphically depicted over the range of 0 to 800 pg/ml. The acid extraction whole blood LAL assay showed a much larger positive recovery bias and poorer recovery precision as shown in Fig. 6. In the absence of added LPS, the chemiluminescence based assay showed no false positive results. The sensitivity and specificity of the two assays are summarized in Table 2. Fig. 7 shows a Bland–Altman bias plot in which the difference between observed and expected results is plotted against the expected result (Bland and Altman, 1986). As illus-

Table 2
Recovery studies: Chemiluminescence-based assay vs. acid-extraction chromogenic LAL ($n = 8$ at each dose)

Conc. of LPS added pg/ml	# of subjects in each category		
	< 50 pg/ml	≥ 50, < 200 pg/ml	≥ 200 pg/ml
(A) Chemiluminescence-based assay			
0	8	0	0
20	8	0	0
50	1	7	0
100	0	6	2
200	0	4	4
400	0	3	5
800	0	2	6
2000	0	0	8
(B) Acid-extraction chromogenic LAL			
0	4	0	4
20	2	2	4
50	3	1	4
100	4	0	4
200	4	1	3
400	2	2	4
800	3	0	5
2000	2	2	4

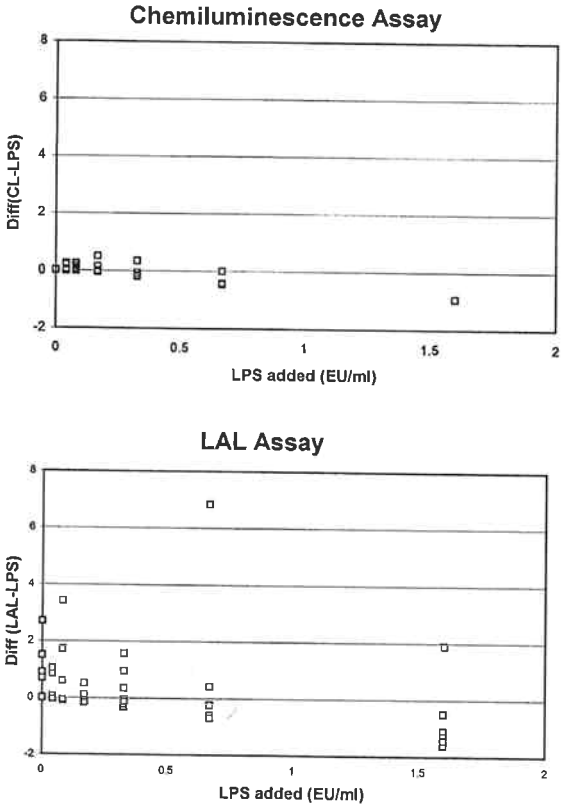

Fig. 7. Analysis of LPS recovery and precision comparing the acid extraction chromogenic LAL whole blood assay to the whole blood chemiluminescence-based assay. Assay performance was evaluated graphically by Bland–Altman Bias plots as shown. This analysis plots the difference between the achieved and expected LPS values for each assay against the expected result. Panel A show the recovery bias of individual data points from $n = 8$ donors assayed by chemiluminescent endotoxin assay at LPS concentrations of 0, 50, 100, 200, 400 and 800 pg/ml (*E. coli* 055:B5). Panel B illustrates the same plot for the LAL-based assay.

trated in the Bland–Altman plots, the chemiluminescence based assay demonstrated less bias and better assay precision.

To date, the only major known contraindication to the assay was PMN non-reactivity due to high doses of exogenously administered corticosteroids. Patients on significant corticosteroid therapy (50–100 mg, Solucortef iv., t.i.d. for at least one day) failed to respond to LPS–antibody complexes based on our preliminary studies on blood samples from septic patients in the intensive care unit. This inhibitory effect was reproduced in vitro in our laboratory by the addition of hydrocortisone to whole blood (20 μg/ml) and likely involves the inhibition of neutrophil signal transduction pathways resulting in priming of oxidant burst activity. Cyclosporin at clinical doses achieved in liver and kidney transplant patients has no inhibitory effect on the assay and we have been successful in measuring endogenous endotoxin in patients on standard chemotherapy regimens (cyclophosphamide/methotrexate/5-fluoro-uracil, cyclophosphamide/epirubicin/5-fluoro-uracil) who have presented to the emergency department with fever and rigors. In 12 ICU patients with physiological and clinical symptoms indicative of systemic inflammatory response syndrome but no detectable LPS under baseline conditions, a dose response relationship which was co-incident with Fig. 2 was obtained. Four of these 12 patients were subsequently classified as septic on the basis of positive cultures for gram positive organisms. In three sepsis patients with pre-existing endotoxemia which subsequently resolved, a dose response relationship co-incident with Fig. 2 was also achieved when blood obtained one day after resolution of endotoxemia was exogenously incubated in vitro with increasing concentrations of LPS up to 800 pg/ml.

3.12. Detection of endotoxin in patients with severe sepsis and highly primed neutrophils

In order to confirm the validity of the assay in patients with highly primed neutrophils, we examined the assay response in two severely septic ICU patients (multiple organ dysfunction score (MODS) score > 15). Based on the opsonin receptor reserve assay (Stevens et al., 1994) using a maximal C5a challenge both patients had opsonin receptor reserves of $< 5\%$ and therefore their opsonin receptors (CR_1 and CR_3) were almost completely expressed on the neutrophil surface under ambient conditions. (Normal CR_1 and CR_3 opsonin receptor reserve is $> 60\%$ in normal non septic patients or ambulatory volunteers.)

The first patient had a necrotic bowel (confirmed on autopsy two days after the assay was done) and elevated whole blood endotoxin > 400 pg/ml. As shown in Fig. 8 (panels A and B) this patient was still able to further prime the neutrophil respiratory burst activity with LPS–anti-LPS immune com-

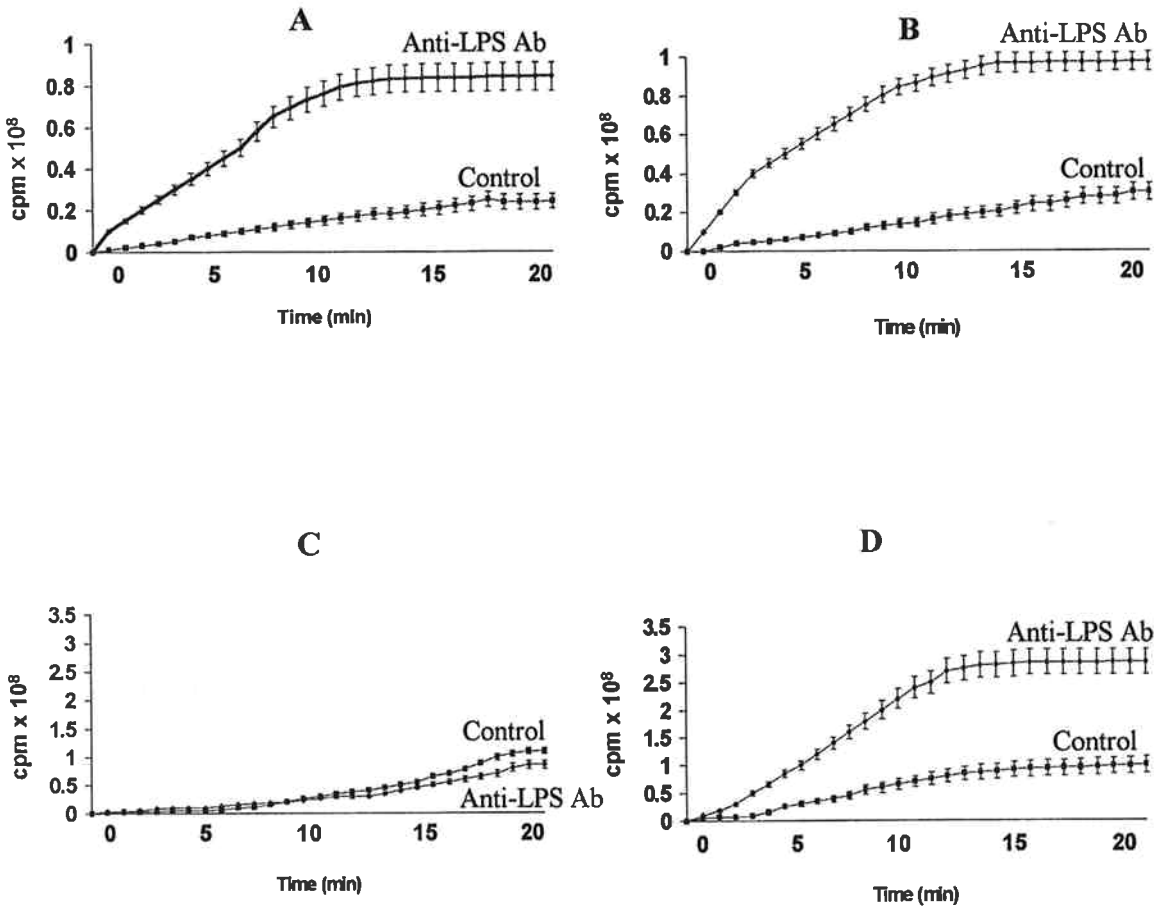

Fig. 8. Detection of endotoxin in septic patents with highly primed neutrophils was explored in two ICU patients. Chemiluminescence curves containing circles (●) represent test assay tubes containing anti-LPS antibody and curves containing squares (■) represent control tubes containing all assay constituents except anti-LPS antibody. Panels A and B represent chemiluminescent endotoxin assays without (A) and with (B) an exogenous dose of *E. Coli* 055:B5 endotoxin (800 pg/ml) in a patient with necrotic bowel and high endogenous endotoxin levels (> 400 pg/ml). This patient had baseline neutrophil chemiluminescence which was 80 × higher than ambulatory controls on a per neutrophil basis. Panels C and D represent an endotoxin assay in a patient with gram positive pneumonia. Panel C: no exogenous endotoxin added. Panel D: 800 pg/ml of endotoxin added. This patient had no detectable endogenous endotoxin or evidence of gram negative infection by culture. The patients baseline neutrophil chemiluminescence was approximately 25 × higher than ambulatory controls on a per neutrophil basis.

plexes despite a baseline state of PMN activation which was approximately 80 times higher than control neutrophils from normal volunteers.

The second patient had no detectable endogenous endotoxin (Fig. 8, panel C) but was able to respond to a maximal exogenous dose of endotoxin as shown in panel D. This patient had a gram positive infection (streptococcal pneumonia) and a state of neutrophil activation which was 25 times higher than neutrophils from normal healthy volunteers.

3.13. Clinical utility of the chemiluminescent LPS assay

In order to investigate the potential clinical utility of the chemiluminescent LPS assay for the detection of gram negative bacterial infection, we compared the results of the LPS assay with that of standard microbiological culture in the routine hospital laboratory on culture positive patients admitted to the intensive care unit over a consecutive six-week pe-

Table 3
Clinical utility of a chemiluminescent assay for endotoxin

Patient group	Mean endotoxin level[a] [SD]	Significance
(A) Normal controls ($n = 20$)	Not detected	
(B) Gram positive ($n = 4$)	29.4 [17.6]	B vs. C: $p < 0.0002$
(C) Gram negative ($n = 13$)	455.3 [296.9]	

[a] Endotoxin levels are in pg/ml. Means were evaluated by unpaired *t*-test.
Patients in group A were a control group of 20 ambulatory healthy hospital employees with no evidence of infection.
Patients in groups B and C comprised all culture positive patients admitted to a medical surgical intensive care unit (MSICU) over a six-week period.
Endotoxin levels were drawn from an arterial line within 6 h of admission to the MSICU. Culture data pertains to infections identified ±48 h of MSICU admission.

riod. The study was conducted in a blinded fashion so that neither party analyzing the samples was aware of the patients clinical status, bacterial culture or LPS results. As summarized in Table 3 the mean levels of LPS in a healthy control group were compared to patients with documented gram positive and gram negative infections. In the control group endotoxin was not detected and low levels were detected in the patients with gram positive infection (mean = 29 pg/ml, SD = 17.6). In contrast, patients with culture documented gram negative infection had high mean levels of LPS (mean = 455 pg/ml, SD = 297). Only one patient with gram negative infection had LPS levels between 40–100 pg/ml. All other patients had LPS levels in excess of 200 pg/ml. Therefore every patient with gram negative infection had detectable LPS levels.

4. Discussion

The application of whole blood neutrophil mediated chemiluminescence to the investigation of phagocytic cell function, pioneered by Allen (Allen et al., 1972, Allen, 1986, 1992, 1993; Stevens et al., 1994) and others (Stevens et al., 1978; DeChatelet et al., 1982), has not yet been widely adopted for clinical use (Stevens et al., 1994). Whole blood chemiluminescence is simple, sensitive, and allows neutrophil function to be studied without a need for cell purification.

The most widely used technique for endotoxin assay has been the chromogenic LAL assay procedure. Although readily performed in non-biologic fluid, the presence of poorly characterized inhibitors of the limulus reaction has limited its reliability in blood or plasma (Roth et al., 1990). LAL procedures for the detection of endotoxin in whole blood or plasma require multiple sample manipulations increasing the risk of environmental contamination. Total assay time often exceeds 90–120 min. The chemiluminescence based endotoxin assay procedure can be executed with a 10 μl whole blood sample, with minimal sample manipulation and can be completed in 30 min. The chromogenic LAL method uses sample pre-dilution and heat treatment or extraction with strong acids to dissociate LPS from its specific binding sites on LBP, CD14, HDL particles and other receptors (Obayashi et al., 1986, 1987; Roth et al., 1990; Tamura et al., 1991).

The chemiluminescence assay combines the specificity of a murine monoclonal antibody against the Lipid A epitope of endotoxin (Parent et al., 1992; Wood et al., 1992; Seelen et al., 1995) with the sensitivity of whole blood chemiluminescence (Allen, 1986, 1992, 1993).

As shown in Fig. 1 the sensitivity of the whole blood CL based endotoxin assay was dependent upon optimal antigen–antibody equivalence. The detection of low levels of endotoxin was best achieved with lower concentrations of antibody and increasing concentrations of endotoxin were detected with parallel increases in the concentration of antibody. In order to span the range of endotoxin concentrations which we have detected in critically ill patients, we chose an intermediate concentration of antibody (0.8 μg/assay) which gave a maximal plateau response at 800 pg/ml (0.67 EU/ml) (Fig. 2). The CL-based assay can be performed with different concentrations of antibody depending upon the clinical and analyti-

cal requirements for sensitivity and range of detection. A lower concentration of antibody can be used to provide a high sensitivity screening assay which is capable of detecting low endotoxin levels with a high degree of sensitivity below 100 pg/ml. Such an assay can be combined either simultaneously or sequentially with an assay which utilizes a higher antibody concentration to discriminate higher endotoxin concentrations in the range of 200–1000 pg/ml.

As demonstrated in Fig. 2, the dose–response relationship is hyperbolic in nature as has been described by others (Zhang et al., 1995) for the interaction of immune complexes with neutrophils in whole blood. The recent studies by Zhang et al. (1995) utilizing defined immune complexes and a variety of chimeric mouse–human antibodies which bind to polymorphs via Fc receptors have shown similar dose–response characteristics to those defined in the chemiluminescent LPS assay. At an antibody concentration of 0.8 μg/assay all subjects and patients reached a maximal chemiluminescent response at an LPS concentration of 800 pg/ml (0.67 EU/ml). This response plateau was nominally maintained from endotoxin levels of 800–2000 pg/ml, minimizing the possibility of signal loss due to antigen excess and the formation of suboptimal antigen–antibody complexes. Each unknown sample was assayed directly with antibody and additionally antibody with an exogenously added LPS concentration of 800 pg/ml to give the upper limit of response. This approach allowed us to verify each patients ability to respond to LPS–antibody complexes and hence eliminated false negative results. Inclusion of a maximal positive control with each sample as a setpoint for the individual's own maximal response allows the assay to compensate for inter-individual variations in neutrophil reactivity.

Studies related to the minimum constituents required to support the chemiluminescent assay of LPS have shown that plasma, neutrophils and complement opsonized zymosan are requisite components. As shown in Fig. 3, heat treatment of plasma to inactivate complement proteins or chelation of calcium with EGTA quenched the increased CL signal initiated by LPS–antibody interaction. Studies utilizing an equimolar concentration of irrelevant monoclonal murine IgM as a control antibody to measure baseline chemiluminescent response have shown that an equivalent baseline response is achieved in the absence of exogenously added antibody (Fig. 3). For this reason the baseline control for each sample is assayed without added antibody to minimize assay complexity and expense.

Under the assay conditions described the chemiluminescent detection of LPS was independent of variations in neutrophil and red cell concentration (Fig. 4) over the range of clinical interest ($0.5–20 \times 10^9$/l PMNs and 0–140 g/l haemoglobin). The assay was also insensitive to variations in pre-incubation time with antibody ranging from immediate addition of antibody and opsonized zymosan to a 15-min incubation with antibody at 37°C.

The reactivity of the chemiluminescence assay to a variety of common gram negative endotoxins relative to *E. coli* 055:B5 endotoxin is summarized in Fig. 5. The chemiluminescence assay was broadly reactive with many pathogenic endotoxins including extracts of *Neisseria meningitis* and *Enterobacter cloacae*. It did not react with all gram positive cell wall extracts tested as well as extracts of the fungal species *Aspergillus fumigata* and *Candida albicans*. At a dose of 400 pg/ml the response of the gram negative endotoxins tested varied over an approximate 2.2 fold range from a minimum response factor of 58% to a maximum of 130% relative to *E. coli* 055:B5 LPS which displayed mid-range reactivity on a per weight basis.

The recovery of *E. coli* 055:B5 LPS added to the whole blood of eight normal volunteers at levels of 0, 20, 50, 100, 200, 400, 800 and 2000 pg/ml is summarized in Figs. 6 and 7. Endotoxin recovery was studied over a range of 0–2000 pg/ml on the basis of preliminary clinical studies in ICU patients which validated the efficacy of the chemiluminescent assay in detecting gram negative infections. All endotoxin concentrations in the recovery study for the chemiluminescence based assay were determined on the basis of the whole blood standard curve displayed in Fig. 2. As demonstrated in Fig. 6, the chemiluminescence based assay displayed a progressive increase in endotoxin recovery with addition of increasing concentrations of endotoxin to whole blood. The same pattern was not apparent in the whole blood LAL based assay in Fig. 6. At an LPS cut-off value of 50 pg/ml the CL assay had a

sensitivity of 98% and a specificity of 100% compared to 63% sensitivity and 38% specificity for the LAL assay (Table 2). As indicated in Figs. 6 and 7, the CL assay showed less positive bias or over recovery of added LPS at endotoxin concentrations of 50, 100, and 200 pg/ml than the LAL assay. We attribute the over recovery of the chemiluminescent assay to a left shift in the dose–response curve for these recovery studies due to the use of anti-LPS antibody from a later preparation than the one used to generate the dose response in Fig. 2. The standardization of the assay due to batch to batch variation in IgM concentration in antibody preparations has been improved but not yet fully optimized.

The improved precision and accuracy of the chemiluminescent assay compared to the acid extraction chromogenic LAL is also clearly emphasized in Fig. 7 which displays the Bland–Altman Bias plots for both assays. In this analysis the individual assay results are subtracted from the amount of LPS added to each sample and plotted against the known LPS concentration added. The chemiluminescent assay gives a tighter spread along the zero bias line.

In conjunction with preliminary clinical studies conducted in the ICU we confirmed that dose–response curves co-incident with the relationship shown in Fig. 2 were achieved in 12 ICU patients with systemic inflammatory response syndrome (four of these patients had confirmed gram positive infection). Comparable dose response curves were also achieved in three patients with endotoxemia after their endotoxemia had resolved and exogenous endotoxin was added to a maximal concentration of 800 pg/ml. In a small clinical study designed to assess whether the chemiluminescent LPS assay was useful as a predictor of gram negative infection we compared culture confirmed bacterial infections to the LPS assay (Table 3). Patients with culture documented gram positive infections had a low mean blood endotoxin level (29.4 pg/ml) as expected, while patients with culture documented gram negative infection had elevated mean blood endotoxin levels (455.3 pg/ml, $p < 0.0002$ vs. gram positive patients). We were able to detect endotoxin in every patient with gram negative infection and only one such patient had an LPS level between 40–100 pg/ml. All other patients with gram negative infection had LPS levels in excess of 200 pg/ml. All healthy ambulatory control patients with no evidence of infection had non detectable LPS levels. The sensitivity of the assay for the detection of gram negative infection ranged from 100% with an assay cutoff value of 40 pg/ml (13 LPS values above the cutoff out of 13 patients with gram negative infection) to 92% with a cutoff of 100 pg/ml (12 LPS values above the cutoff). In comparison, Hurley (1994) using meta-analysis has suggested that chromogenic or gelation LAL has a sensitivity of 54% for the detection of gram negative bacteraemia. Our promising initial diagnostic sensitivity suggests that the assay proposed may be of clinical utility in detecting gram negative infection and also endotoxemia due to other causes. The blood LPS compartments accessed by our assay remain to be elucidated in future studies.

Endotoxin tolerance has been shown to have suppressive effects on neutrophil cytokine synthesis (McCall et al., 1993), systemic activation (Barroso-Aranda et al., 1994) and oxidant production (Leone et al., 1992). In our preliminary patient studies, pre-existing endotoxemia which resolved or persistent low level endotoxemia of several days duration did not prevent the typical response to exogenous in vitro LPS addition. The detailed effects of endotoxin tolerance on the LPS assay described have not, however, been specifically studied in a large patient population.

As indicated in Fig. 8, the CL endotoxin assay is functional in patients with highly activated neutrophils. Both patients had baseline PMN activation which ranged from 25 to 80 times above those of normal patients based on chemiluminescent response/neutrophil. In these two patients the opsonin receptor reserve as defined by Stevens et al. (1994) was less than 5% indicating near maximal extrusion of CR_1 and CR_3 receptors under ambient baseline conditions. These results suggest that highly-activated PMN in patients with severe sepsis (MODS > 15) still have sufficient reserve to allow further activation by LPS–anti-LPS complexes.

In summary, we describe a rapid and simple whole blood bio-assay for the detection of endotoxin. It can be used to detect and quantify lipopolysaccharide with a detection sensitivity at the 20 pg/ml (0.017 EU/ml) level, or less if required, and is specific for LPS from most gram negative pathogens. The assay utilizes 10 μl of whole blood which is

premixed with a luminol physiological buffer containing either anti-LPS antibody or no antibody. The reaction is initiated with 20 μl of complement opsonized zymosan and the assay is completed following a 20 min incubation and read period. The detection of LPS is based on the differential activation of PMN in the antibody containing tubes compared to the control (no antibody) tubes. This assay will provide a more rapid, reliable and sensitive method for the detection of endotoxin in whole blood. The basic platform technology of this assay is sufficiently generic to allow the detection of other bacterial and fungal products in whole blood.

Appendix A. Equations describing the quantification of LPS based on the chemiluminescence assay

As previously formalized by Allen (1986), the chemiluminescent response observed in whole blood in the presence of a lumiphor can be described by the following relationships:

$$\mathrm{CL} = a[P_{ox}], \tag{1}$$

where CL is the light emission expressed as counts observed per unit time, a is the proportionality constant and $[P_{ox}]$ is the concentration of neutrophil oxidants produced (under the conditions employed in our case, the predominant oxidant reacting with the lumiphor luminol is HOCl, hypochlorous acid). This equation is based on the assumption that the chemiluminescent substrate or lumiphor is non rate limiting and that the reaction is zero order with regard to the concentration of luminol.

$$[P_{ox}] = \mathrm{d}[P_{circ}][S_{opsn}], \tag{2}$$

where $[P_{circ}]$ is the concentration of surface opsonin receptors (CR1 and CR3) on the circulating phagocytes and $[S_{opsn}]$ is the concentration of complement opsonized zymosan. Allen has shown that the acceleration phase of phagocyte oxygenation shows a zero order dependence on opsonized zymosan when it is present at an excess and non rate limiting concentration. Under these conditions $[P_{ox}] = \mathrm{d}[P_{circ}]$.

$$V_o = \mathrm{d}[P_{ox}]/\mathrm{d}t = k_i[P_{circ}], \tag{3}$$

where V_o is the rate of oxidant production or the slope of the CL light emission over time when zymosan is in excess. In the presence of antigen–antibody complexes, the rate of oxidant production is:

$$V_x = k_i[P_{circ}][F(\mathrm{AgAb})], \tag{4}$$

where $[F(\mathrm{AgAb})]$ is an unknown function of the concentration of antigen–antibody complex. In a chemiluminescent LPS assay each analysis is composed of tubes containing specific antibody and control samples which contain no antibody or an irrelevant antibody of the same isotype. The control tubes will therefore give a baseline response defined by Eq. (3). In each unknown LPS analysis set, one group of tubes contains an exogenous dose of LPS which gives a maximal chemiluminescent response. In these tubes the rate of light emission is given by:

$$V_{max} = k_m[P_{circ}][F(\mathrm{AgAb})_{max}] \tag{5}$$

for the antibody containing tubes and $V_{om} = k_m[P_{circ}]$ for the control tubes.

The response factor (RF) is calculated by subtracting the rate of maximal light emission in the unknown antibody containing sample V_x from the rate in the control sample V_o. This difference is then divided by the analogous difference derived from the samples containing a maximum stimulatory concentration of exogenous LPS mixed with and without anti-LPS antibody (i.e., $V_{max} - V_{om}$). The equation for RF therefore becomes:

$$\mathrm{RF} = \frac{(V_x - V_o)}{(V_{max} - V_{om})} = \frac{k_i[P_{circ}]\{F(\mathrm{AgAb}) - 1\}}{k_m[P_{circ}]\{F(\mathrm{AgAb})\mathrm{max} - 1\}} \tag{6}$$

This relationship predicts that the response factor should be independent of opsonin receptor concentration $[P_{circ}]$ and hence also of phagocyte or neutrophil concentration.

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