0273-2289/96/6003—0189$08.50

Detection of Endotoxin Using an Evanescent Wave Fiber-Optic Biosensor

E. A. JAMES, K. SCHMELTZER, AND F. S. LIGLER*

Naval Research Laboratory, BioMolecular Science and Engineering, Washington, DC 20375-5348

Received August 8, 1995; Accepted September 18, 1995

ABSTRACT

The lipopolysaccharide endotoxin is the most powerful immune stimulant known and a causative agent in the clinical syndrome known as sepsis. Sepsis is responsible for more than 100,000 deaths annually, in large part due to the lack of a rapid, reliable, and sensitive diagnostic technique. This study describes the detection of LPS from *E. coli* at concentrations as low as 10 ng/mL, in 30 s using an evanescent wave fiber-optic biosensor. Polymyxin B, covalently immobilized onto the surface of the fiber-optic probe, selectively bound fluorescently labeled LPS. Unlabeled LPS was detected in a competitive assay format using labeled LPS for signal generation. The competitive assay format worked in both buffer and plasma with similar sensitivities. This method can be used with other LPS capture molecules such as antibodies, lectins, or antibiotics, to simultaneously detect LPS and to determine the LPS serotype. The LPS assay using the fiber-optic biosensor is applicable to both clinical and environmental testing.

Index Entries: Endotoxin; lipopolysaccharide; polymyxin B; fiber-optic; immunoassay; biosensor.

INTRODUCTION

Lipopolysaccharide (LPS) is found in the outer cell membrane of Gram-negative bacteria and is the primary bacterial surface antigen. Also known as endotoxin, LPS is of significant clinical importance and can

*Author to whom all correspondence and reprint requests should be addressed.

result in the syndrome known as sepsis. Individuals suffering from physical trauma (*1*), burns (*2*), infection (*3*), and hemodialysis (*4*), are particularly susceptible for developing sepsis, which can result in mortality rates of greater than 30%. Sepsis has also been implicated in sudden infant death syndrome (SIDS) (*5*). With the rapid evolution of antibiotic resistant bacteria, there has been a concomitant increase in sepsis and related problems. Early diagnosis is an important component in effective medical intervention.

LPS and airborne bacteria are also significant contaminants in certain industrial settings, particularly those generating organic dust (*6,7*) and waste management. Also, there is a relationship between LPS levels and "sick building syndrome" (*8*).

LPS consists of a polysaccharide portion and a unique lipid moiety known as lipid A. The lipid A region is structurally conserved and characteristic of all Gram-negative bacteria. It is also the toxic component of LPS and is responsible for eliciting immunologic and humoral responses. In addition, LPS is a known mitogen and can act in synergy with concanavalin A to stimulate an immune response.

Accurate and rapid determination of LPS concentration has been difficult to establish. Most LPS assays are based on the enzyme-linked immunosorbant assay (ELISA) or the Limulus amebocyte lysate (LAL) assay. The difficulties in LPS detection are a result of both the chemical nature of the LPS and the interfering substances found in plasma. Neither LPS nor lipid A adsorb well to the ELISA plates (*9*). In most instances, it is necessary to complex the LPS to another molecule (such as bovine serum albumin (BSA) that does absorb to ELISA plates. It has been shown that LPS complexed with BSA is biologically active and binds to polymyxin B (*9–11*).

Polymyxin B (PMB) is a naturally occurring antibiotic, synthesized by the bacteria, Bacillus polymyxa. It is a decapeptide, unique in that it contains both D- and L-amino acids and has 5 or 6 DAB (α,γ-diaminobutyric acid) residues. Chemically, it is amphipathic and contains several reactive amino groups from the DAB and a fatty acid tail. It inhibits the growth of both Gram-negative and Gram-positive bacteria although Gram-negative bacteria are more sensitive (*12*). PMB has broad reactivity and will bind to many species or serotypes of LPS (*13*). PMB interacts nonspecifically with the LPS from a variety of serotypes, thus it is possible to use it as a general probe for LPS. Established binding constants of LPS for PMB are on the order of $10^8\ M^{-1}$ (*14*). PMB is known to bind to the inner core saccharide region (KDO) and Lipid A moiety of LPS by ionic and hydrophobic mechanisms without any covalent bond formation and the binding of PMB to LPS has been shown to be a 1:1 (*15*).

The LAL assay has been shown to be very sensitive for the detection of LPS, but is also very sensitive to interfering substances. The LAL assay detects a class of molecules that possess endotoxic characteristics, making this a comparative toxicity assay (*16*). Indeed the LAL assay response varies depending on the type or source of LPS being tested. In samples

such as plasma or blood there are several factors known to interfere with the LAL reaction. The reaction is inhibited or enhanced by antibiotics, hormones, heavy metals, and some amino acids, and plasma protein (*17*) resulting in both false positives and false negatives. As a result, assays using LAL reactions while sensitive, are generally difficult to quantitative (*3,18*).

This paper describes a rapid and sensitive method for the detection and quantitation of LPS in PBS and plasma that is unaffected by substances that interfere with the ELISA or LAL assays. The method is simple and is used to determine LPS concentration in the low ng/mL range.

MATERIALS AND METHODS

Reagents

Polymyxin B (PMB)

Polymyxin B Sulfate (PMB) was purchased from Sigma Chemicals, (St. Louis, MO). PMB with a minimum of 6000 USP units per mg was used for immobilization onto the fiber-optic probe.

LPS

Escherichia coli serotype O128:B12 LPS and tetramethyl rhodamine isothiocyanate (TRITC) labeled O128:B12 LPS were purchased from Sigma Chemical. These LPS preparations were isolated for Sigma phenol extraction procedure (*19*). Protein concentration was less than 1%. The method of Skelly et al. (*20*) was used by Sigma for the TRITC-LPS conjugation.

Biosensor Fiber-Optics

The technique of evanescent wave fiber-optic measurements has been described in detail elsewhere (*21*). Briefly, the excitation light at 514 nm is supplied by a 50 mW argon-ion laser (Omnichrome, Chino, CA). The laser light is focused onto the end of a fiber-optic probe. Fluorescence returning up the fiber is discriminated from the excitation light by KV550 filter (Schott Glass Technologies, Duryea, PA) and detected using a photodiode, (Fig. 1). The change in fluorescence emission is converted into an electrical signal which is measured.

Fiber Preparation

A plastic clad, fused silica fiber with a core diameter of 200 μm was used throughout the experiments. The cladding from the distal 12 cm of the fiber was stripped off and the fiber dipped in hydrofluoric acid (HF) for 1 min to remove residual cladding. The exposed core was tapered by immersion in HF acid at computer-controlled rate to form a combination taper. The tapering decreased the original 200 μm core diameter to 55 μm at the tip (22).

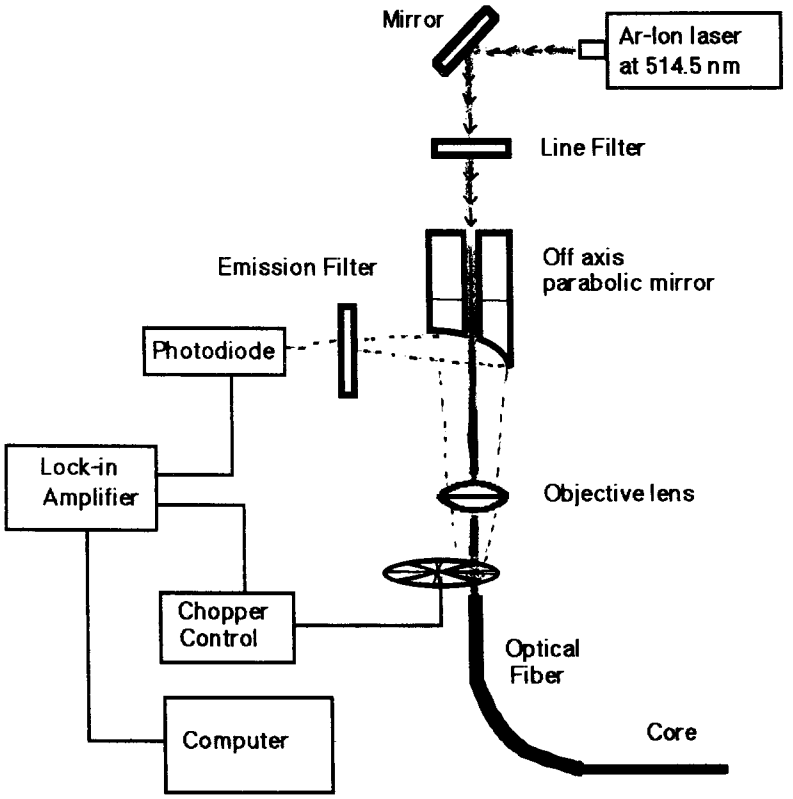

Fig. 1. The fiber optic biosensor consists of a 50 mW argon-ion laser as the excitation source. An off-axis parabolic mirror and a biconvex fused silica filter. A chopper is placed between the focusing lens and the fiber with a photodiode employed for signal conversion. A KV550 filter is positioned between the photodiode and the parabolic mirror. The chopper and the photodiode are interfaced with a lock-in amplifier and data is collected using a lap top computer.

Following tapering the fibers are rinsed and then immersed in boiling distilled water for 30 min. The fibers are removed and allowed to air dry. Once the fibers are dry they are immersed in a 2% solution of silane in toluene for 30 min. The silanized fibers are rinsed with toluene. The silanized fibers are immersed in a freshly prepared 2 m*M* solution of the heterobifunctional cross linker, γ-maleimidylbutyryl succinimide (GMBS, Fluka) ester (23) for 1 h. The fibers are then transferred to a cylinder containing a solution of PMB dissolved in PBS and incubated for 1 h. The fibers are then rinsed and stored in PBS. Various concentrations of PMB for the immobilization step were tested, 10 mg/mL proving to be optimal.

The fiber, with immobilized capture molecule, is mounted in a 200 μL capillary tube. Plastic T-fittings are attached to the ends of the capillary tube. The other end of the T is sealed using a hot glue gun. The remaining opening is attached to tubing led through a peristaltic pump. This allows uninterrupted sampling by the fiber. Within the tubing is a three-way

value that allows for the removal or addition of circulating sample without having to stop the flow. In this fashion, the assay may be performed in a sealed system.

Solutions

Plasma samples were prepared by extracting blood from the human donors into heparin tubes (LPS free). The blood was centrifuged for 15 min at 5000 rpm. The plasma was removed using a pipet and placed into LPS-free test tubes and stored at 4°C. For the experiments, the plasma was diluted into LPS-free phosphate buffered saline (PBS). The final plasma concentrations used were 20, 5, 1, and 0% in PBS + 2 mg/mL BSA.

Assays

Because of known problems with aggregation of LPS, extensive precautions were taken to insure that LPS was dispersed throughout the solution in the actual experiment. All samples were vortexed for at least 1 min and sonicated for 1 min immediately prior to testing. Two different solutions of *E. coli* LPS O128:B12 were prepared; one unlabeled, and one labeled with TRITC. The final concentrations of TRITC-LPS tested range from 1–500 ng/mL.

In the competition assays, the final concentration of TRITC-LPS was kept constant at 200 ng/mL. Unlabeled LPS (at various concentrations) was added with or without plasma.

All experiments were done at room temperature. The samples were injected and fluorescence measured for 2 min at 30 s intervals. The laser light was blocked between readings in order minimize photobleaching.

Background was determined by injecting the plasma solution to be tested (containing no LPS) and monitoring the change in fluorescence, if any. In practice, none of the plasma samples generated a fluorescent signal. The solution was removed and a sample containing TRITC-LPS injected in increasing concentrations. A standard binding curve was performed for each concentration of plasma in PBS + 2 mg/mL BSA, using a minimum of three fibers per test. The average percent change in signal per assay was calculated using a minimum of three fiber-optic probes and a standard deviation calculated and graphed.

RESULTS

Optimization of PMB Concentration Immobilized on a Microtiter Plate or Fiber-Optic Probe

The effect of the PMB concentration used for immobilization on binding TRITC-LPS was examined in both an ELISA and the fiber-optic system. Microtiter plates or fiber-optic probes were coated with PMB using 0, 0.1, 1.0, 10, and 100 mg/mL solutions of PMB and assayed for the ability of the immobilized PMB to bind TRITC-LPS.

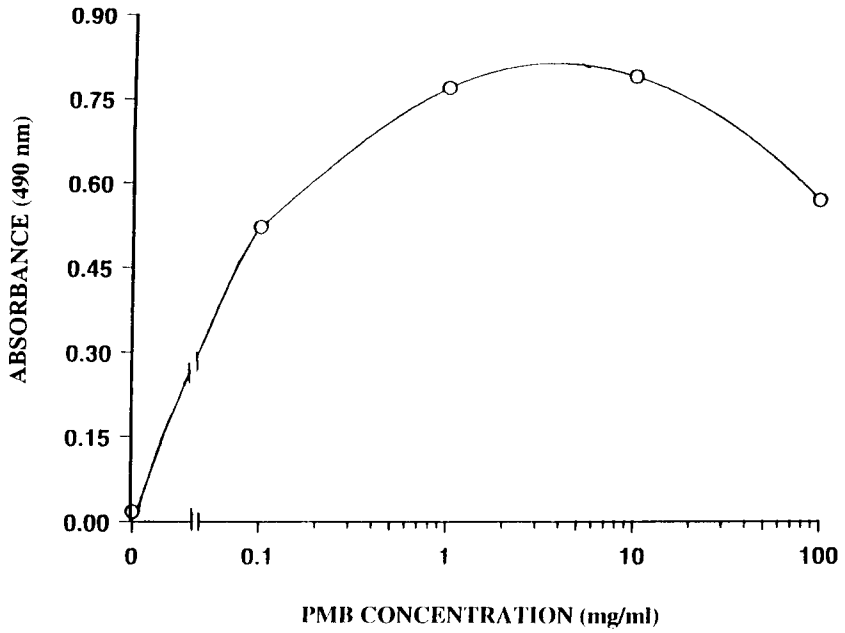

Fig. 2. Optimization of PMB concentration for immobilization in an ELISA assay. PMB was adsorbed to the surface of microtiter plates at a solution concentration of 0, 0.1, 1, 10, or 10 mg/mL. TRITC-LPS was detected using anti *E. coli* antibody.

Figure 2 shows the effect of PMB concentration in an ELISA. It is interesting to note that there are three distinct parts to this binding profile. From 0 to 1 mg/mL PMB, the binding sensitivity increases. At 0.1 mg/mL PMB, the sensitivity of detection is approximately 64.8% of that at the optimal PMB concentration. There is a plateau between 1.0 mg/mL and 10 mg/mL where changing the concentration of PMB appears to have little effect. This is followed by a region on the binding curve where increasing the concentration of PMB appears to be inhibitory. At 100 mg/mL of PMB, there is a significant decrease or inhibition in binding to 44% of the level using 1–10 mg/mL.

Figure 3 shows the effect of PMB concentration when PMB was immobilized onto the fiber-optic probe. The highest signal was obtained using a 10 mg/mL solution of PMB. As with the ELISA, there is a significant decrease in binding capacity when a solution of >10 mg/mL PMB was used for immobilization. Since using 10 mg/mL PMB for immobilization produced more signal than either 1 mg/mL or 100 mg/mL, 10 mg/mL was chosen as the standard concentration at which PMB was immobilized.

Kinetics of LPS Binding

The kinetics and stability of LPS binding to the polymyxin B immobilized on the fiber-optic probe is shown in Fig. 4. The binding was observed

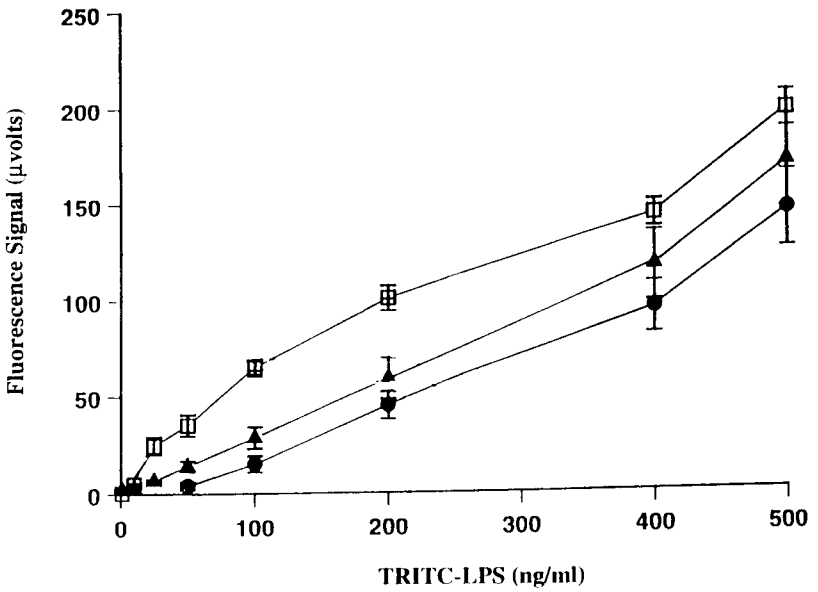

Fig. 3. The effect of PMB concentration during immobilization onto the fiber-optic probe. PMB was dissolved in PBS at concentrations of 1 mg/mL (▲), 10 mg/mL (□), or 100 mg/mL (●), and reacted with the surface of a fiber-optic probe coated with γ-maleimidylbutyryl succinimide (GMBS). Excess or unreacted PMB was washed off using PBS prior to sample addition. TRITC-LPS was added at concentration of 0–500 ng/mL and the increase in fluorescence measured after 2 min. Values represent the mean ± S.E. from three fibers.

at 22°C and is essentially complete within 30 s. There is no change in fluorescence signal following the initial binding, indicating that there is no detectable nonspecific adsorption that would result in an increase in signal as a function of time. There is no loss in signal observed even after extensive washing, verifying that there is little photobleaching and that the LPS is tightly bound.

Detection of TRITC-LPS in Buffer and Plasma

Quantitative detection of TRITC-LPS was observed in buffer and increasing plasma concentrations in a direct binding format. Figure 5 shows the effect of increasing TRITC-LPS concentration, (0 to 500 ng/mL) vs change in emission signal as a function of binding to the surface of the fiber optic probe. Under all solution conditions tested, the increasing concentration of LPS was reflected in a linear change in fluorescence signal. The largest net change in signal was observed when the LPS was dissolved in PBS + 2 mg/mL BSA. Galanos et al. (*10*) have shown that BSA solubilizes LPS in aqueous solutions and appears to reduce aggregation. This reduced aggregation has been shown by Takayama et al. (*23a*) to increase the activity of LPS in terms of binding to various receptors. However, the data using 1% plasma solution is very similar to that without plasma. At

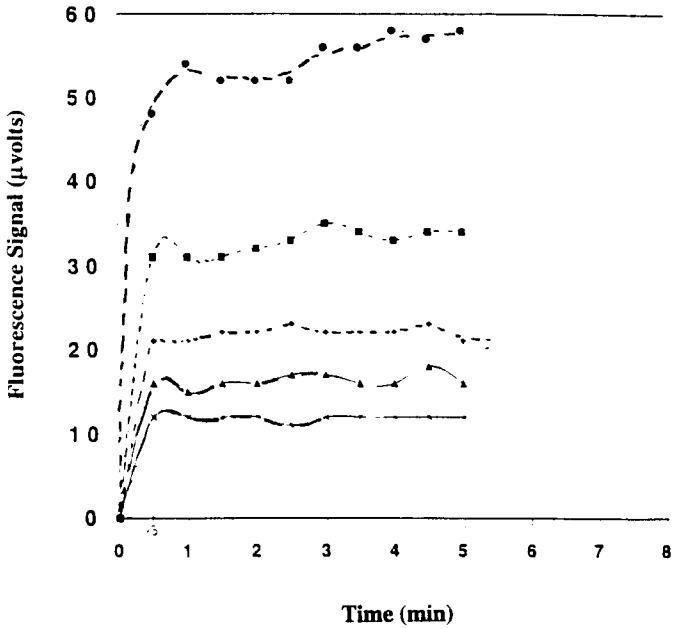

Fig. 4. The kinetics and stability of binding LPS to the polymyxin B immobilized onto the fiber-optic probe. The change in fluorescence signal over time at TRITC-LPS concentrations 10 ng/mL (*), 25 ng/mL (▲), 50 ng/mL (◆), 100 ng/mL (■), and 200 ng/mL (●). TRITC-LPS was allowed to react with a fiber-optic probe to which 10 mg/mL PMB had been covalently immobilized. Changes in fluorescence signals were observed every 30 s. Between measurements the excitation light shutter was closed to prevent photobleaching.

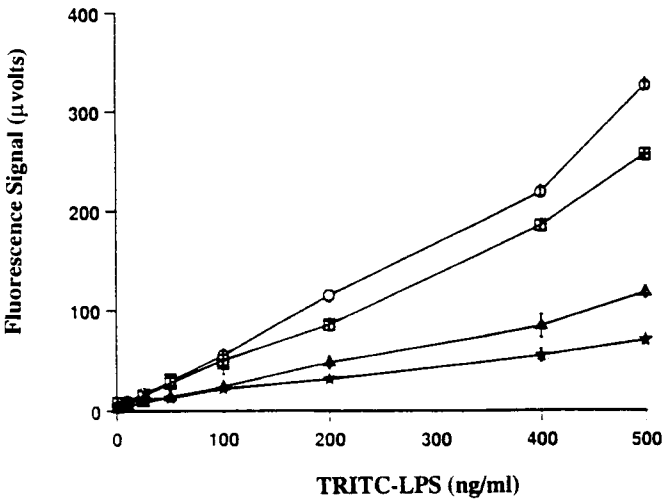

Fig. 5. Effect of solution on the binding of TRITC-LPS to PMB. TRITC-LPS (0–500 ng/mL) was dissolved in PBS + 2 mg/mL BSA 0% (○) or 1% (□), 5% (△) or 20% (☆) plasma and allowed to bind to immobilized PMB on the fiber-optic probe. The change in fluorescence emission as a function of each solution condition is shown. In all solutions the binding of LPS to the fiber optic is linear. Values represent the means ± S.E. of data from 3 different fibers.

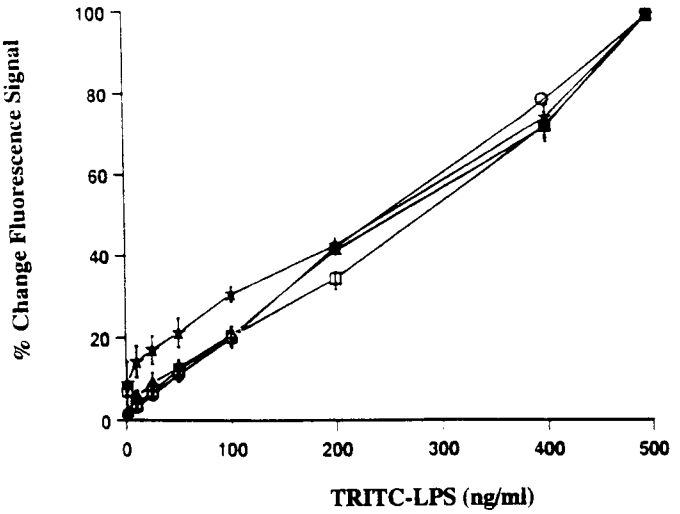

Fig. 6. The effect of solution on the binding of TRITC-LPS to immobilized PMB as a function of percent signal change. Data is analyzed and plotted by taking the maximum observed change in signal at 500 ng/mL TRITC-LPS in each solution as a 100% and dividing the observed signal changes of the other samples by this value. TRITC-LPS was dissolved in PBS + 2 mg/mL BSA 0% (○), 5% (△), or 20% (☆)plasma. Values represent the means ± S.E. of data from 3 different fibers.

5% plasma, there is a significant decrease in maximum signal observed (relative to PBS + 2 mg/mL BSA) with further reduction in emission signal at 20% plasma. However, the standard deviations are small and indicate that, even in 20% plasma, the observed change in signal is useful in terms of determining TRITC-LPS concentration.

When the percent change in signal is plotted as a function of TRITC-LPS concentration, the effect of plasma on detection is negligible (Fig. 6). The slopes of the samples in solution for the 1, 5, and 20% plasma are 0.186, 0.184, and 0.176 respectively. The effect of plasma is likely to be both a viscosity effect (diffusion limitation) as well as a sequestering of the LPS by its association with plasma proteins. The slope information can be applied in the data analysis for the determination of the concentration of unlabeled LPS in each concentration of plasma.

Competition Assay, for the Detection of Unlabeled LPS

The percent inhibition of TRITC-LPS binding vs. increasing concentrations of unlabeled LPS as a function of plasma concentration is shown in Fig. 7. With increasing unlabeled LPS there is a concomitant increase in inhibition. When the TRITC-LPS concentration equals that of the unlabeled LPS, (200 ng/mL) the signal is decreased or inhibited by 50%. The lowest concentration, 12.5 ng/mL unlabeled LPS, results in a measurable change in signal for all concentrations of plasma tested. The slopes of the

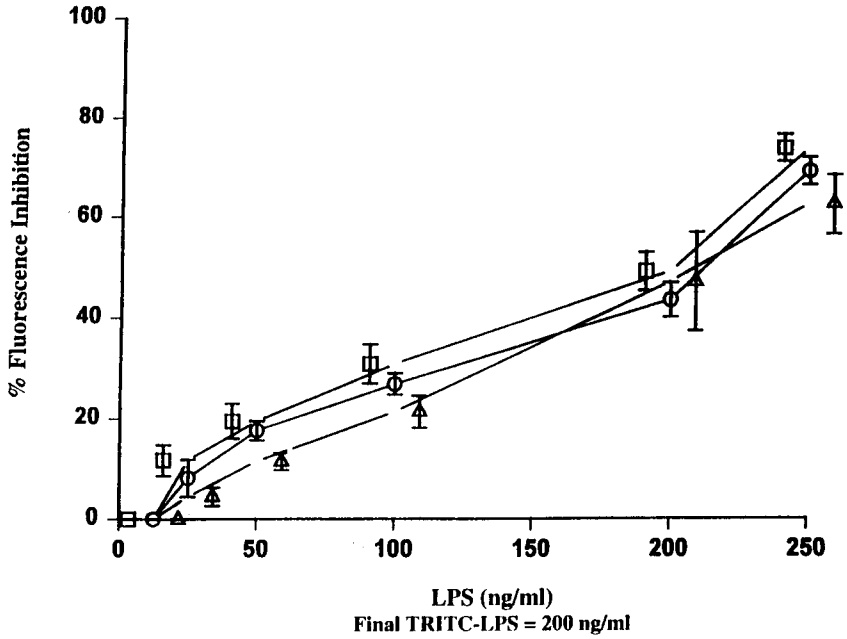

Fig. 7. Percent inhibition of TRITC-LPS binding as a function of increasing unlabeled LPS and as a function of plasma concentration. Using a constant final concentration of TRITC-LPS of 200 ng/mL, 0–250 ng/mL of unlabeled LPS was added. The effect of plasma concentration was tested for each sample 1% plasma (□), 5% plasma, (○), and 20% plasma (△). The decrease in observed signal was calculated as a percent of the expected fluorescence signal for 200 ng/mL TRITC-LPS and plotted as percent inhibition versus concentration of unlabeled LPS. Values represent the means ± S.E. of data from 3 different fibers.

competition assay in 1, 5, and 20% plasma are 0.183, 0.184, and 0.176. These slopes are in good agreement with those determined in the direct binding assay, indicating that the presence of unlabeled LPS does not interfere with the binding of TRITC-LPS in either a constructive or destructive manner. Thus the competition observed is a standard competition binding assay, where two similar molecules are competing for the same or similar binding sites with the equivalent affinities.

Discussion

The detection of LPS is of significant medical, environmental, and economic importance. Assays for LPS should be specific for all Gram-negative bacteria, sensitive, rapid, easy to perform, quantitative, and resistant to interfering substances found in biological or environmental samples. No assay currently available meets all of these requirements. The detection of LPS using the fiber-optic biosensor does meet these requirements and has the added benefit of being able to perform the assays for LPS on site whether in a clinic or a waste treatment plant. The immobilized PMB is capable of binding most Gram-negative LPS. Alternatively,

one can immobilize a capture molecule specific for a given serotype or species of Gram-negative bacteria, such as *E. coli, Salmonella,* etc. in order to detect and serotype the LPS simultaneously.

Both the fiber-optic and ELISA data show that the binding of LPS is affected by the concentration of PMB immobilized on the capture surface. The change in concentration of the PMB in solution used to coat the microtiter plate shows the ELISA to be biphasic in response to LPS detection. Increasing the concentration above 10 mg/mL resulted in a decrease in detection sensitivity rather than the expected plateau which typically is a result of microtiter plate saturation (*24*).

A similar effect is observed when comparing the fiber-optic probes using increasing concentrations of PMB in solution in the immobilization step. In both cases the 10 mg/mL concentration is optimal in terms of overall detection. However, at higher concentrations of LPS, the difference between PMB surface immobilized concentration diminishes (Fig. 3). The greatest difference in detection sensitivities caused by immobilizing different densities of PMB can be seen between the 50 ng/mL and 200 ng/mL LPS.

The similarity between the ELISA and the fiber-optic probe in terms of PMB immobilized in surprising. In the case of the ELISA, the PMB is absorbed to the microtiter plate wells, while on the fiber-optic surface the PMB is covalently immobilized via primary amine groups. The different methods of applying PMB to the surface could result in a change in net charge between that of PMB covalently immobilized the fiber-optic probe, versus that of PMB absorbed to the plastic microtiter well. The binding of PMB to LPS is based primarily due to electrostatic interactions between the two molecules. One might predict that the change in charge would effect the binding characteristics (*25*), but this does not seem to be the case.

The inhibitory effect observed with PMB concentrations greater than 10 mg/mL can be understood in terms of PMB structure. The structure of PMB allows significant ring stacking and charge interactions to occur. Indeed repulsive forces may be contributing to the observed effects. As the concentration of the PMB on a surface increases, there will be increased ring stacking, charge interaction, and loss of rotational freedom (increase in steric hindrance). PMB in solution does not show a concentration inhibition effect (*15*), indicating that the molecules free in solution are less susceptible to charge and stacking effects. The binding kinetics, (Figs. 2, 3) are best described as a "step" function rather than a "logarithmic curve," which is characteristic of LPS binding to an immobilized antibody. This suggests that there is tight binding of LPS to PMB with little or no detectable reorientation *(26)*. The antibody-LPS binding curve in contrast indicates that the association and binding is more complex, than that of LPS to PMB.

The binding kinetics of LPS to polymyxin have been studied solution. Bader et al. (*15*) reported an apparent Ka = $4.3 \times 10^4 M^{-1}$. Bader also demonstrated that the ratio of PMB binding to the LPS monomer was approx

1:1. Association constants as high as $4 \times 10^{8} M^{-1}$ have also been reported (*14*). Matsumae et al. (*27*) have shown that the K_d of *E. coli* O128:B12 for covalently immobilized PMB is $2.46 \times 10^{-9} M$, this is reflected in the stability of the fluorescent signal seen in the competition assay. The rapid binding observed on the fiber-optic probe supports the theory (*15*) that PMB:LPS interaction is primarily ionic and hydrophobic in nature and that no covalent bonds are made or broken.

The decrease in signal as a function of increasing plasma concentration cannot be attributed to a decrease in diffusion rate alone. The greatest relative change is observed between 1 and 5% plasma, which is a 5-fold increase in plasma. At 500 ng/mL the signal maximum is 280 μvolts and 120 μvolts respectively. The change in signal when increasing the plasma concentration 4-fold from 5–20% are 120 μvolts and 50 μvolts. The overall effect of plasma on detection in this system is most likely attributable to several factors. One of the contributing factors is clearly diffusion. However, another factor likely to interfere with the observed binding is the presence of circulating LPS-binding proteins within plasma.

The competition assay was able to detect the presence of 12.5 ng/mL LPS in PBS and 25 ng/mL LPS in all concentrations of plasma tested. The entire assay was complete within 30 s, which is the time required for the sample to be injected and for the LPS to diffuse to the surface of the fiber optic probe. While the absolute signal in plasma changed as a function of plasma concentration, the detection limits were not significantly affected. Even the presence of LPS-binding proteins can be addressed either by measuring the relative or percent change, or by analyzing the change in slopes of competition assay vs. the direct binding values. Immobilizing a capture molecule with a very high binding constant will further help overcome the competition between plasma binding proteins and the immobilized molecule. Such a very high binding affinity exists with the LPS-binding molecule known as LPS Neutralizing Protein (ENP).

Serotype determination is also possible using an antibody specific for LPS from one bacterial strain as the capture molecule. In addition to detection and serotyping the LPS, it has become important to determine the degree to which a given bacteria binds antibiotics (*28*). Using several different antibiotics immobilized onto different fiber-optic probes and observing the pattern of binding, it may be possible to address the bacterial sensitivity to a given antibiotic. David et al. (*29*) has developed several cationic amphiphilic drugs that interact with lipid A and act as antagonist in the Limulus gelation assay. Phenothiazines, aminoquinooine, biguanides, and aromatic diamidines and others could be rapidly screened for LPS binding using this technique. Standard clinical assays for antibiotic sensitivity may take as long as a week to complete.

This study demonstrates the speed, ease, and efficacy of a competitive assay for LPS performed using the evanescent wave fiber-optic biosensor. Many other techniques exist for detecting LPS, but most require several hours or even days to complete the assay. The more rapid and

sensitive techniques such as the LAL still require hours to perform and are difficult to standardize in complex solutions such as plasma or contaminated waters due to the many inhibitory substances found in these solutions, and the immuno-dye reagent assays are not quantitative (*30*). One of the significant advantages of the method described here is that it requires no enzymes to visualize the presence of LPS, is relatively insensitive to proteases that are typically present in plasma of bacteremic individuals, and provides excellent quantitation of LPS in the ng/mL range.

REFERENCES

1. Dipiro, J. T., Hamilton, R. G., Howdieshell, T. R., Adkinson, N. F., and Mansberger, A. R. (1994), *Clin. Diagn. Lab. Immunol.* **1,** 295–298.
2. Munster, A. M., Smithmeek, M., Dickerson, C., and Winchurch, R. A. (1993), *Ann. Surg.* **218,** 321–327.
3. Mertsola, J., Cope, L. D., Munford, R. S., McCracken, G. H., and Hansen, E. J. (1991), *J. Infect. Dis* **164,** 353–358.
4. Jackson, B. M., Becksague, C. M., Bland, L. A., Arduino, M. J., Meyer, L., and Jarvis, W. R. (1994), *Amer. J. Nephrol.* **14,** 85–89.
5. Oppenheim, B. A., Barclay, G. R., Morris, J., Knox, F., Barson, A., Drucker, D. B., Crawley, B. A., and Morris, J. A. (1994), *Arch. Dis. Child* **70,** 95–98.
6. Jacobs, R. R. (1994), Sampling environments containing organic dust. *Amer. J. Ind. Med.* **25,** 3–11.
7. Smid, T., Heederik, D., Houba, R., and Quanjer, P. H. (1994), *Amer. J. Ind. Med.* **25,** 877–888.
8. Teeuw, K. B., Vandenbrouckegrauls, C. M. J. E., and Verhoef, J. (1994), *Arch. Intern. Med.* **154,** 2339–2345.
9. Takahashi, K., Fukada, M., Kawai, M., and Yokochi, T. (1992), *J. Immunol. Meth.* **153,** 67–71.
10. Galanos, C., Rietschel, O., Luderitz, O., and Westphal, O. (1972), *Eur. J. Biochem.* **31,** 230–233.
11. Appelmelk, B. J., Verweij-van Vught, A. M. J. J., Maclaren, D. M., and Thijs, L. G. (1985), *J. Immunol. Methods* **82,** 199–207.
12. Lichtman, S. N., Wang, J., Schwab, J. H., and Lemasters, J. J. (1994), *Hepatology* **19,** 1013–1022.
13. Appelmelk, B. J., Su, D., Maarian, A., Verweij-van Vught, J. J., Thijs, B. G., and MacLaren, D. M. (1992), *Ana. Biochem.* **207,** 311–316.
14. Umeda, M., Niwa, M., Yamagami, S., Kishimot, T., Maekawa, M., and Sawada, Y. (1990), *Biomat. Art. Cells Art. Orig.* **18(4),** 4913497.
15. Bader, J. and Teuber, M. (1973), *Z. Naturforsch.* **28c,** 422–430.
16. Finney, D. J. (1978b), in *Statistical Methods in Biological Assays,* Griffin, London, pp. 69–76.
17. Satoshi, M., Masahiro, N., Taizo, W., Tadashi, S., and Tetsuya, T. (1983), *Anal. Biochem.* **198,** 292–299.
18. Pearson, F. C., Dubczak, J., Weary, M., Bruszer, G., and Donohue, G. (1985), *J. Clin. Microbiol* **21,** 865–868.
19. Westphal, O. and Jaun K. (1965), *Meth. Carbohyd. Chem.* **5,** 83–91.
20. Skelly, R., Munkenbeck, P., and Morrison, D. C. (1979), *Infect. Immun.* **23,** 287–293.
21. Golden, J. P., Anderson, G. P., Ogert, R. A., Breslin, K. A., and Ligler, F. S. (1992), *Proc. SPIE Int. Soc. Opt. Eng.* **1796,** 2–8.

22. Ligler, F. S., Golden, J. P., Shriver-Lake, L. C., Ogert, R. A., Wijesuria, D., and Anderson, G. P. (1993), *Immunomethods* **3,** 122–127.
23. Bhatia, S. K., Thompson, R. B., Shriver-Lake, L. C., Levine, M., and Ligler, F. S. (1989), *SPIE* **1054,** 184–190.
23*a*. Takayama, K. and Oureshi, N. (1991), *PCT Int. Appl.* WO9101134 A1 910207, 65 pp.
24. Chalifour, R. J., Michon, F., Jennings, H. J., and Lacroix, M. (1992), *J. Immunol. Meth.* **154,** 69–76.
25. Brintzinger, H. (1961), *Helvetica Chimica Acta.* **86,** 744–753.
26. Laethem, M. E., Belpaire, F. M., Wijnant, P., Rosseel, M. T., and Bogaert, M. G. (1994), *Chirality* **6,** 405–410.
27. Matsumae, H., Minobe, S., Kindan, K., Watanabe, T., Sato, T., and Tosa, T. (1990), *Biotech. Appl. Biochem.* **12,** 129–140.
28. Boll, M., Radziejewskalebrecht, J., Warth, C., Krajewskapietrasik, D., and Mayer, H. (1994), *Fems. Immunol. Med. Microbiol.* **8,** 329–341.
29. David, S. A., Bechtel, B., Annaiah, C., Mathan, V. I., and Balaram, P. (1994), *BBA-Lipid Metab.* **1212,** 167–175.
30. Williams, T. J., Lee, C.-H., and Johnson, A. O. (1992), Immuno-dye reagent and assay for detection of endotoxin. U.S. Patent 5,093,235.