

AN ENZYME-LINKED IMMUNOSORBENT
ASSAY (ELISA) FOR ENDOTOXINS

by

MARK G. STEVENS

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Major Professor

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CHAPTER I

INTRODUCTION

Endotoxins are responsible for a variety of clinical entities in human and veterinary medicine. The biologic activity and toxic manifestations of endotoxin have been well documented. Extensive studies have been conducted on the alterations in blood pressure, body temperature, metabolism, immunity, circulating blood cells, and coagulation of blood caused by endotoxin. Information concerning the level of circulating endotoxin in these altered states is limited due to a lack of a specific and quantitative assay.

The procedure most commonly used to detect endotoxins is the *Limulus* amoebocyte lysate test. This assay is the most sensitive method available for measuring this substance. However the test is not absolutely specific for endotoxin, can be inhibited by interference factors present in various body fluids, and quantitative assays are difficult to perform.

Clinically the *Limulus* assay has been inconsistent in detecting endotoxin. Normal healthy individuals can yield a positive test due to the presence of endotoxin mimicking substances, and endotoxin inhibitors can produce a negative result in profoundly ill endotoxemic patients. Such discrepancies have made it difficult to interpret the test results.

A procedure specific and quantitative for endotoxin would be valuable both diagnostically and as a research tool. The diagnosis of

endotoxemia would no longer rely primarily on clinical signs, and an earlier and more effective treatment could be implemented. The ability to study the effects of circulating endotoxin and to ascertain its role in certain disease states could also be accomplished. In addition, possible inherent endotoxin levels present in healthy patients and what amount constitutes an abnormal condition could be determined.

A more consistent measurement of endotoxin was established when a radio-rocket immunoelectrophoresis and immunoradiometric assay using Lipid A antibody was developed. These methods exploited the fact that the core of the endotoxin molecule, which contains Lipid A, is similar for all endotoxins. By using antibody produced to Lipid A, a variety of different endotoxins could be detected and accurately quantitated.

Based on the concept that the core of all endotoxins is antigenically similar, this investigation was done to determine the feasibility of an enzyme-linked immunosorbent assay for endotoxin. Antisera to the endotoxin core has been achieved through use of bacteria defective in the synthesis of the outer cell wall. One of these organisms is the Escherichia coli strain 0111:B4 J5 mutant. Antibody produced to the exposed core region of endotoxin from this mutant was tested in the assay against a variety of different endotoxins in saline and serum. Several endotoxins from gram-negative bacteria could be detected. The sensitivity of the test varied among the endotoxins.

CHAPTER II

LITERATURE REVIEW

A. United States Pharmacopeial Pyrogen Test

The first test developed for detecting endotoxins was the United States Pharmacopeial Pyrogen (USP) test.⁹⁵ This procedure, conducted in rabbits, measures the pyrogenicity of intravenously administered endotoxin. Injected endotoxin has a direct pyrogenic effect and is capable of stimulating endogenous pyrogen release from phagocytic cells, mainly monocytes. Endotoxin and the liberated endogenous pyrogen, thought to be interleukin 1, influence the thermal regulatory areas in the brain resulting in the development of fever.¹⁰¹ The temperature rise following injection is monitored at 12.5 min intervals for at least 3 hr. The pyrogenic dose 50 (PD₅₀) is determined by the endotoxin concentration which gives an average febrile response of .6 C in 50% of a group of 4 or more rabbits tested.⁹⁵ A standard curve measuring temperature rise versus the concentration of a reference endotoxin is established and is used to determine endotoxin content of a test sample.

This procedure can detect pyrogenicity of endotoxin at a concentration of 1 ng/ml. This method is expensive, time consuming, and does not distinguish between endotoxin and other pyrogenic substances such as polynucleotides, dextran, and endogenous pyrogen produced by phagocytic cells. It is the only procedure approved by the Food and

Drug Administration (FDA) for use as a diagnostic test for determining endotoxemia.¹⁰¹

B. In vitro Endogenous Pyrogen Test

A modification of the above procedure has been used.²¹ An endotoxin sample is mixed with isolated rabbit blood monocytes. Endogenous pyrogen released from the cells is determined by injecting the supernatant into rabbits and monitoring the febrile response. This in vitro endogenous pyrogen test has been reported to have a greater sensitivity than injecting the suspect sample directly into rabbits. Endotoxin has been detected at .05-.1 ng/ml levels.

C. Limulus Amebocyte Lysate Test

(i) Biochemistry of the Reaction

A more widely used procedure for determining endotoxin in biological fluids, preparations, and biologics is the Limulus Amebocyte Lysate (LAL) test. This lysate is extracted from circulating blood cells (amebocytes) from the horseshoe crab Limulus polyphemus. Amebocytes, upon lysis and extraction, release a proclotting enzyme, thought to be a serine protease, of a M.W. between 84,000 and 150,000 daltons.^{92,93} The protease in the presence of endotoxin and divalent cations, like Mg and Ca, is activated to break down its substrate coagulogen. This heat stable 21,000 M.W. protein, upon enzymatic cleavage, has a small 7,000 M.W. fragment removed. The remaining larger fragment undergoes polymerization, believed to occur by oxidation of sulf-hydryl groups, to produce disulfide bonds.^{28,66} Polymerization of the coagulogen produces a gelatinous clot which is read visually as a positive test for endotoxin.

Gelation of the lysate, when mixed with endotoxin, measures the enzymatic effect of endotoxin and is not a direct measurement of endotoxin.

(ii) Current Status

Although the LAL test is not currently sanctioned by FDA for routine clinical use, FDA has granted limited approval for use of the test as a replacement for the USP rabbit test for detection of endotoxin in medical devices and qualified solutions.²⁵ Presently FDA will permit LAL testing final release of pharmaceuticals with the stipulation that parallel studies are conducted using the USP test. The results of the LAL assay must have an equal or greater sensitivity than the test conducted in rabbits.⁹⁷ Because the LAL test is cheaper and more sensitive, most manufacturers of parenteral products are using the procedure in conjunction with the USP test.⁷³

(iii) Sensitivity

The Limulus test appears to be the ideal method for detection of endotoxins clinically. It is relatively cheap, simple to perform, and can be completed within a days time.⁷³ However, important problems exist in its sensitivity and specificity for endotoxin.

One of the major problems with the sensitivity of the LAL assay is that some commercial preparations are more responsive to certain endotoxins than others.¹⁰¹ When 5 different commercial Limulus lysate products were tested for reactivity to different endotoxins, a range of detection between .003-.3 ng/ml for the Escherichia coli, Salmonella typhosa, and Serratia marcescens endotoxin was seen. However, response to Proteus mirabilis and Pseudomonas aeruginosa was much lower and varied between 10-500 ng/ml for the Pseudomonas and 30-700 ng/ml for the Proteus endotoxin.

A wide range of solubility, shelf life, and quality of the gel formed when reacting with endotoxin has been reported.¹⁰⁰ Criteria for interpreting LAL results have not been established.²⁷ These factors have contributed to reports that LAL test sensitivity varied for different endotoxins and for the same endotoxin tested by different commercial products.^{27,101}

The sensitivity of the LAL test is influenced by inhibitory factors present in serum and other body fluids. These substances can be partially or totally inhibitory. Inhibitors have been demonstrated in man, horse, dog, and most lab animals tested.^{12,13,26} Although some substances known to interfere with the assay have been identified, others have not.^{40,53,86} Their mechanism of action has not been identified.

Substances associated with inhibitory activity in serum include: penicillin;⁶⁷ polymyxin B;¹⁴ neomycin;⁵⁵ amidase inhibitors as anti-thrombin III;⁷² heat stable serum esterase;⁷⁴ two alpha-globulins;⁷⁴ a single serum protein called LPS-1.⁷⁴ They inactivate or cause disaggregation of endotoxin, resulting in a negative Limulus test. Heparin and ethylenediamino tetraacetic acid (EDTA) (presumably by chelating divalent cations) have reported to inhibit detection of endotoxin in plasma.^{77,84,91} High protein levels and high white blood cell counts caused interference when the test was conducted on synovial or cerebrospinal fluid.⁷⁷

Dimethyl sulfoxide (DMSO) inhibited the LAL test by preventing endotoxin activation of the proclotting enzyme.⁵⁴ Presumably DMSO rendered endotoxin ineffective as a catalyst for conversion of the proclotting enzyme to an active form by preventing its association

with the enzyme. As DMSO is used medically the inhibitory effects it may have on use of the LAL test may be a problem, but this remains to be determined.³⁹

The pH of the test sample is shown to effect sensitivity of the Limulus test. The optimum pH range for conducting this assay in any fluid is between 6.9 and 7.2.^{77,90} Solutions which are strongly acidic or basic need to be adjusted to the proper pH prior to assaying for endotoxins for the most accurate results.

Sample contamination is also important in the sensitivity of the LAL test.⁷⁷ All glassware, reagents, and diluents which come in contact with the lysate must be rendered free of endotoxin. Glassware is depyrogenated by heating at 175 C for at least 4 hr, and commercially prepared sterile pyrogen free water is recommended as a diluent when performing the test. Precautions must also be observed when samples are obtained. The skin of the patient should be prepared as for surgery, and nonpyrogenic needles and syringes used for collection. Some procedures also advise that the test be conducted in a sterile glove box to reduce contamination by air currents.^{6,77}

(iv) Specificity

Endotoxin mimicking substances are most responsible for concern regarding specificity of the LAL test for endotoxin. Thrombin and thromboplastin, activators of the coagulation system, induces lysate gelation.²³ Bovine pancreatic ribonuclease, dextran derivatives, and certain polynucleotides exhibited a positive Limulus test.²³ The polynucleotides and dextran derivatives tested are not likely present in a normal biological system. However, thrombin and thromboplastin are present in biological fluids, and could influence the LAL test.

Endotoxin mimicking activity also occurs with peptidoglycan isolated from gram-positive organisms.¹⁰³ Intact gram-positive bacteria have not displayed a positive Limulus test. However peptidoglycan extracted from the cell wall of such organisms caused gelation of the lysate. Such reactions have occurred with peptidoglycan from group A Streptococcus, Staphylococcus aureus, and S. epidermidis.

(v) Extraction Methods

Various methods of treating and extracting serum and plasma samples prior to conducting the LAL assay have been utilized to rid samples of both endotoxin inhibitory and mimicking material.²⁰ These include perchloric acid precipitation of blood proteins,⁷² chloroform or heat extraction,^{6,20,53} pH shift,⁷⁶ gel filtration,³⁶ and dilution in distilled water.⁹⁹ The heat and perchloric acid methods have shown the most promise, although the results were variable, and there was some question about the accuracy and sensitivity of the test being altered using these procedures.^{6,20,53}

(vi) Clinical Trials

The use of the LAL test as a clinical diagnostic procedure has been criticized by clinicians because of a lack of consistent correlation between test results and the absence or presence of clinical signs of endotoxemia.^{19,23,61,74} Clinically normal patients have had a positive test, whereas profoundly ill individuals with clinical signs of endotoxemia and that were positive on bacterial blood cultures were negative. In some instances 0-5% of human patients with serious gram-negative infections and signs of septic shock were positive with the LAL test.^{15,61} When the test was conducted on normal individuals up to 33% gave a positive response.¹⁹

In patients with pyogenic arthritis 100% of the synovial fluids yielded a positive result in the Limulus test.²² However, 53% of those individuals with a nonseptic joint effusion also had a joint fluid specimen that produced lysate gelation.

In one study 46% of the serums from dogs had a positive LAL reaction.¹⁹ There was a significant correlation between lysate gelation and the presence of intestinal parasites. This lead to speculation that injury of the intestinal wall may have permitted endotoxin leakage to occur and produce a positive test in normal animals. Forty three percent of horses with clinical signs of gastrointestinal disease and accompanying hemostatic abnormalities, indicative of a possible endotoxemia, were positive with the Limulus test.⁶⁵

False negative results in the LAL assay have been attributed to endotoxin inhibitors and variability in the sensitivity of the commercial lysate used.^{61,74} False positive tests have been explained by contamination and the presence of endotoxin mimicking substances.^{19,61} False positive results may have occurred as the procedure has such a high sensitivity that very minute amounts of endotoxin were detected at a concentration where clinical signs are not obvious.¹⁹ Because of these results, FDA has recommended that the test not be used clinically for detection of endotoxemia until the problems of inhibitor removal and endotoxin mimicking substances are better understood, and a more uniform test can be developed.⁷⁴ FDA has also proposed guidelines to standardize sensitivity of the LAL assay by controlling its production process.²⁵

(vii) Quantitating Ability

The use of the Limulus test clinically is also in question because of its limited quantitating capacity, important in determining disease severity and a prognosis.⁷⁴ The LAL test as originally designed is difficult to perform quantitatively.¹⁰¹ Results are read as either positive or negative for the presence of endotoxin at a set level according to the manufacturers recommendation, with the reference endotoxin provided. Any small change in lysate viscosity or opacity is difficult to assess visually, and excessive manipulation must be avoided to prevent disruption of the gel.¹⁰¹ The quality of the gel formed tends to vary among commercial lysates. Some preparations form a firm clot while others exhibit only a soft gel when reacting with endotoxin, making the endpoint hard to determine.^{74,101}

A spectrophotometric method measuring light absorbance, and a nephelometric procedure measuring light scattering of the clot formation have been implemented to aid in increasing the quantitating ability of the test.^{74,91} A modification of the LAL test using a chromogenic substrate, and monitoring the change in optical density after its addition has also been used.^{65,74} These methods may assist in making the test more quantitative but are still hindered by endotoxin inhibitors and mimicking substances. The condition of the serum or plasma sample is also important as to whether an inherent opacity exists due to hemolysis or lipemia, when using these procedures.^{76,91}

D. Immunologic Measurement of Endotoxin

An immunoradiometric assay has been developed to better quantitate endotoxins.⁶⁹ This method utilized antibody (Ab) made to the

Lipid A moiety of endotoxin, which is similar if not identical for all endotoxin producing bacteria. Lipid A Ab produced to the 595 mutant strain of Salmonella minnesota has been shown to cross react with Lipid A from S. minnesota, Shigella flexneri, and 3 serotypes of Escherichia coli. Lipid A Ab reacted only to free Lipid A and not to the intact endotoxin molecule. The endotoxin was acid hydrolyzed to release Lipid A prior to conducting the assay. The determination of free Lipid A was then a measure of the endotoxin from which the Lipid A was derived. Measurement of Lipid A hydrolyzed from these bacterial endotoxins in saline could detect endotoxin at 10-25 ng/ml.

Quantitation of endotoxins has also been achieved by radio-rocket immunoelectrophoresis which used S. minnesota 595 Lipid A Ab.⁸⁹ The procedure could measure endotoxin from E. coli, S. minnesota, and Klebsiella pneumoniae at 50 ng/ml in saline.

E. Enzyme-Linked Immunosorbent Assay

An alternative to radiolabeled Ab for detecting endotoxin is enzyme labeled Ab. Many quantitative procedures using Ab tagged with radioisotopes are being replaced by the enzyme-linked immunosorbent assay (ELISA).¹⁰⁶ This procedure has repeatedly demonstrated an equal or greater sensitivity than methods using radiolabeled Ab and avoids the problems of elaborate equipment, radiation hazards, and short reagent shelf life inherent to the use of radioisotopes.

In principal the ELISA can be used to measure any thing to which an Ab can be made. There are three basic procedures employed using this test to quantitate antigen (Ag) in a test sample.¹⁰⁶ The sandwich

ELISA uses a capture Ab to immobilize the Ag being measured to which a second Ab labeled with enzyme, from the same or a different species, is then reacted. The test Ag is thus "sandwiched" between the capture and labeled Ab.

A modification of this procedure is the double sandwich (Indirect) ELISA which utilized a third Ab. This Ab is labeled with enzyme and reacts with the unlabeled second Ab already bound to the captured Ag. This procedure is more sensitive than the sandwich ELISA but requires Ag specific Ab from two different species. Both of these techniques demand that the Ag in question have at least two Ab binding sites for the sandwich to form.

In the competitive ELISA, labeled Ag competes with unlabeled Ag in the test sample for binding to a limited quantity of Ab. The Ab bound Ag is separated from the free Ag by washing and enzyme activity is determined in the bound fraction. This activity is then related to the concentration of the unlabeled Ag in the test sample. This procedure is similar to that of the classical radioimmunoassay (RIA) and is the most sensitive of the three methods.

Although an ELISA for measuring a spectrum of endotoxins has not been developed, this procedure has been used for quantitating many different toxins. The sandwich ELISA has been used to measure type A, B, and E exotoxin of Clostridium botulinum,^{70,48,71} Staphylococcus aureus enterotoxin A,⁸⁰ Pseudomonas aeruginosa exotoxin A,⁸³ and the enterotoxin and endotoxin of Vibrio cholera.³⁷ Mycotoxins such as Aflatoxin B₁ and B₂ of Aspergillus flavis⁷⁵ and various toxins present in snake venoms have been measured with this procedure.⁹⁴

The double sandwich ELISA has been used to quantitate S. aureus A, B, and C enterotoxin,⁷³ Escherichia coli heat labile enterotoxin,⁵ C. perfringens type E toxin,⁶⁰ and the toxin A of C. difficile.⁶⁰ The sensitivity obtained for these toxins was variable depending on the ELISA methodology used. Sensitivity ranged from .3 ng/ml for the P. aeruginosa exotoxin A⁸³ and S. aureus enterotoxin A,⁸⁰ to 1.3 ug/ml for the endotoxin of V. cholera.³⁷

A competitive ELISA has been used to detect levels less than .5 ng/ml. This method has been used for the S. aureus type A, B, and C enterotoxin, enabling measurement of .1 ng/ml.⁹⁰

ELISA procedures for measuring drugs, antibiotics, hormones, and various regulatory proteins are also in use in many diagnostic laboratories. Drugs with potential toxicity have been accurately monitored in blood using this test, and have proven valuable in evaluating the effectiveness of the treatment protocol. Methotrexate, prescribed for treatment of human lymphoblastic leukemia,¹ digoxin for congestive heart failure,¹⁷ and the anticonvulsants phenytoin,⁹⁶ primidone,⁹⁶ phenobarbital,⁹⁶ and diazepam¹⁰² have all been accurately quantitated in blood using a sandwich ELISA. A competitive ELISA has been used to measure lidocaine⁵⁰ and theophylline.⁹⁶

Detection limits for these compounds with the ELISA are between 28 ug/ml for phenobarbital and diazepam, to 1.5 ng/ml for digoxin and phenytoin. These results have been beneficial in quantitating toxic and therapeutic blood levels for these drugs. It has also enabled production of commercial ELISA kits to monitor serum levels of diazepam, phenobarbital, primidone, and digoxin in man.⁹⁶

Gentamycin has been measured by an ELISA and levels of .001-.4 ng/ml of serum were detected using a competitive procedure.^{62,88} The method proved to be more rapid and sensitive in determining nephrotoxic and ototoxic blood levels in man than the original RIA.

Radioimmunoassays for hormonal imbalances are being replaced by the ELISA which is more rapid and has an equal or greater sensitivity. By far the most widely used procedure is the competitive ELISA. This method has been used to determine serum levels of the adrenal gland hormones cortisol,⁴⁵ epinephrine,⁸⁷ and norepinephrine.⁸⁷ It was employed for serum determination of thyroxine (T_4),⁸² insulin,⁴⁴ testosterone,⁴¹ progesterone,⁴² estriol,⁴⁷ follicle stimulating hormone,⁵⁶ and dopamine.⁸⁷ This assay measured cortisol at 10 ng/ml, T_4 at 20 ng/ml, .05 ng/ml for testosterone, and 5 milli-int units/l for insulin. Because the test gives rapid results a commercial ELISA is available to measure serum insulin in man.¹⁰⁸

Serum regulatory proteins like alphafetoprotein and ferritin can be measured by an ELISA. Alphafetoprotein, an important marker in assessing the prognosis after therapy for primary liver carcinoma, and in detecting possible congenital abnormalities in man, has been measured at levels 3-50 ng/ml.^{2,3} Serum ferritin has been quantitated at 10 ng/ml, a level lower than the RIA and Farr technique originally used to measure this protein.⁴

Use of an ELISA has also permitted quantitation of microbial Ags and enzymes in various body fluids. The polysaccharide capsular Ag of Hemophilus influenzae type B in human serum has been detected at 1 ng/ml.¹⁸ This procedure was 25 times more sensitive than counter-

immuno-electrophoresis first used to determine blood levels of this antigen. Streptococcus pneumoniae type 3 polysaccharide Ag has been measured at 2 ng/ml in serum and cerebrospinal fluid.³³ The Candida albicans polysaccharide Ag (25 ng/ml) and the Candida proteinase (.1 ng/ml) in serum from immunosuppressed cancer patients has been measured with the ELISA.^{85,79} Viral Ags like Hepatitis B surface Ag were detected at 5 ng/ml in plasma.⁹⁸ Clinical measurement of these Ags by the ELISA has been used to confirm an early diagnosis of these infections in man prior to the appearance of antibodies.

The ELISA has been used to quantitate Abs and was a sensitive method for demonstrating titer increases during infection and evaluating the degree of immunity. Such is the case with the measurement of Rubella I_gG Ab where 1 ug/ml has been detected.⁵¹

Other uses of the ELISA have been to measure serum proteins and enzymes in establishing a diagnosis or as a method of study for some pathologic conditions. Low density apolipoproteins (C-I, C-II, C-III, and E) have been quantitated at 100-200 ng/ml in serum.³⁸ Human serum prostatic antigen (prostatic acid phosphatase) has been measured at 1 ng/ml.⁴⁹ This permitted an early diagnosis of prostatic cancer prior to metastasis and was of value during treatment evaluation. Serum fibronectin released from malignant fibroblasts has been detected at 10 ng/ml to study the role of this substance in initiating malignancy.¹⁰⁰

F. Immunochemistry and Structure of Endotoxin

Development of an endotoxin ELISA requires an Ab specific for a wide range of bacterial endotoxins. All endotoxins contain 3 main

antigenic components.⁹ They are Lipid A, the core polysaccharide, and the O antigenic side chains. The core polysaccharide and O Ags are good immunogens while Lipid A is poorly immunogenic due to its small size and insolubility in water.

Lipid A is a phospholipid composed of glucosamine-4-phosphate, long chain fatty acids and ethanolamine.⁵⁷ The core polysaccharide is linked to Lipid A by 2-keto-3-deoxyoctonate (KDO) and contains heptose, ethanolamine, and 3 hexoses consisting of galactose, glucose, and N-Acetyl glucosamine. The O Ags are long chains of repeating short polysaccharide units of 3-5 sugars each. These Ags form the outermost region of the endotoxin molecule and vary in structure between species and serotypes of bacteria. Lipid A and the core polysaccharide together form the inner and outer core of the molecule and are antigenically similar if not identical for all endotoxin producing bacteria.⁵⁸

Production of antiserum to this common core of endotoxin has been achieved by use of rough bacteria defective in their synthesis of the outer cell wall containing the O Ags.^{11,68} One such defective mutant is Echerichia coli strain 0111:B4 J5. This mutant lacks the enzyme uridine diphosphate-galactose epimerase and cannot incorporate galactose into the core polysaccharide.⁶⁸ This results in the inability to either synthesize the O Ags or attach them to the core of the endotoxin. The J5 endotoxin contains only Lipid A, KDO, two heptoses, and glucose.^{9,34} Antibody to this endotoxin is made to the common core and has been shown to cross react with other bacterial endotoxins.

G. Immunotherapy with Antiserum to J5 Endotoxin

In laboratory animals actively immunized or given J5 antiserum the toxic effects of endotoxin were prevented or reduced in: (1) death from challenge from intravenous endotoxin, (2) the local (dermal) Schwartzman reaction, (3) the generalized Schwartzman reaction (disseminated intravascular coagulation).⁹ This protection was provided by the I_{gG} and I_{gM} fractions of the antiserum. Normally I_{gM} is the main immunoglobulin made to endotoxin but with repeated immunizations an amamnestic response can be elicited with I_{gG} being the major Ab produced.^{8,11,69}

Bacteremia and endotoxic shock from Eschericha coli, Pseudomonas aeruginosa, and Klebsiella pneumoniae in laboratory animals has been consistently reduced by administration of Ab to the J5 core endotoxin.^{10,11,34,109} These three species account for 65% of the bacterial endotoxemias seen in man and are common causes in animals.¹⁰ This has been exploited in immunotherapy in man for endotoxemia resulting from primary infections and secondary infections associated with immunosuppressive diseases or therapy.

Treatment with the J5 antiserum in humans in profound gram-negative shock increased the rate of recovery from 29 to 82%.¹¹ The incidence of a febrile attack and bacteremia in patients with leukemia and lymphoma was reduced by use of the J5 antiserum. Vaccination with the J5 mutant in man prior to immunosuppressive therapy assisted in preventing subsequent bacteremia and endotoxemia.

CHAPTER III

MATERIAL AND METHODS

A. Isolation of Endotoxins

The Escherichia coli strain 0111:B4 J5 endotoxin was isolated by the phenol-chloroform-petroleum ether method of Galanos.²⁹ The Pseudomonas aeruginosa, Klebsiella pneumoniae, Pasteurella haemolytica, and P. multocida endotoxins were extracted by a modification of the Westphal method according to Carter.¹⁶ After extraction the endotoxins were lyophilized and stored at 0 C. The J5 endotoxin analyzed spectrophotometrically was free of nucleic acid and protein contamination.¹⁰⁴ A protein analysis also revealed the absence of protein.^a The presence of Lipid A in the isolate was confirmed by the method of Westphal and Luderitz.⁴³ The Serratia marcescens,^b Salmonella typhimurium,^b S. enteritidis,^b S. abortus equi,^b S. minnesota,^b S. minnesota strain 595,^c and the various E. coli serotypes^b were commercially prepared.

B. Production of J5 Endotoxin Antiserum

Antibody to the J5 endotoxin was prepared in 6 rabbits by 4 injections at weekly intervals using Freund's incomplete adjuvant.^b To render the J5 endotoxin soluble it was necessary to saponify it by adding .25 N NaOH and incubating in a 37 C water bath for 10 min.

a. Bio-Rad Laboratories, Richmond, CA 94804

b. Sigma Chemical Co. St. Louis, MO 63178

c. Cal Biochem-Behring Corp. La Jolla, CA 92037

The mixture was neutralized with .25 N HCL and an emulsion prepared by adding neutralized endotoxin to the adjuvant (1:2) and homogenizing. One ml of the emulsion was used for each weekly injection and initially contained .025 mg/ml followed by increasing dosages of .125, .25, and .50 mg/ml. Injections were given in multiple sites both subcutaneously and intramuscularly.

Animals were bled by the marginal ear vein one week after the last injection. Blood was allowed to clot at 37 C for 30 min and the serum then collected by centrifugation after an additional 2 hr at 4 C.

C. J5 Antiserum Titer Determination by an Indirect ELISA

(i) Equipment

All washing steps were done with .15 M phosphate buffered saline (PBS) pH 7.4 using an automated lymphocyte cell harvester.^a This machine produced a continuous flow of .6 ml of fluid into and out each well per sec. The addition of reagents to the wells and serum dilutions were performed using a 12 channel microtiter pipet dispenser.^b

(ii) Microplate Preparation

Flat bottom polystyrene microtiter plates^c were coated with 100 ul per well of a 15 ug/ml solution of purified J5 endotoxin, in a .06 M carbonate-bicarbonate buffer pH 9.6 containing .3% sodium azide. Preliminary trials established that this concentration of endotoxin was the minimum amount that could be used to saturate the

a. Cambridge Technology, Inc. Cambridge, MA 02139

b. Titertek, Flow Laboratories. Finland

c. Dynatech, Laboratories, Inc. Alexandria, VA 22314

coating surface. Plates were covered tightly and incubated in a 60 C water bath for 12 hr. Plates not used immediately were stored at 4 C with the coating buffer in the wells.

(iii) Test Procedure

The Indirect ELISA for determination of the antibody titer was performed as follows:

Step 1. The prepared plates were emptied by inverting, and the wells washed for 3 sec using the lymphocyte cell harvester.

Step 2. The wells were emptied and blocked by adding 200 ul of a blocking buffer consisting of .15 M PBS pH 7.4 containing .25 M glycine (Aminoacetic acid)^a and .5% bovine serum albumin (BSA) fraction V.^b Plates were incubated for 30 min at 37 C.

Step 3. The fluid was shaken out of the plates and 100 ul of blocking buffer added to each well. One hundred ul of heat inactivated (56 C for 30 min) pre-immune and immune serum sample from each rabbit was added, and serial two fold dilutions performed in the wells. All samples were run in duplicate. Plates were covered and incubated at 37 C for 2 hr.

Step 4. The fluid was removed from the wells by inverting the plate and two separate 5 sec washes performed, in which the plate was inverted and emptied between the first and second wash.

Step 5. Step 2 was repeated.

Step 6. The wells were emptied of blocking buffer, and to each was added 100 ul of a 1:5000 dilution of goat antirabbit I_g (heavy

a. Sigma Chemical Co. St. Louis, MO 63178

b. United States Biochem Corp. Cleveland, OH 44126

and light chain specific) peroxidase conjugate^b in blocking buffer. Prior conjugate titrations determined that this dilution produced the least amount of background. Plates were covered and incubated 1 hr at 37 C.

Step 7. Step 4 was repeated.

Step 8. The plate was emptied and 100 ul of the substrate solution was added to each well. This solution contained 40 mg o-phenylenediamine^a and .4 ml of 3% hydrogen peroxide per 100 ml of .1 M phosphate-citrate buffer pH 5.0. The reaction was allowed to proceed in the dark for 15 min at room temperature.

Step 9. The reaction was stopped by adding 50 ul of 2 M H₂SO₄. The optical density (OD) was then read at 490 nm with an enzyme immunoassay (EIA) reader equipped with a readout thermal printer.^c The machine was blanked on an endotoxin coated well containing only substrate and acid.

The endpoint titer was determined in the following manner. The average OD reading was found for the 1:32 dilution of the 6 pre-immune rabbit serums run in duplicate. Three standard deviations from this value was used to establish an endpoint OD reading. The highest dilution of the immune serum which gave an average OD value greater than this reading was taken as the endpoint titer. The titer for the immunized animals varied between 512 to 4096 and measured both I_gG and I_gM Ab to endotoxin, because the conjugate used was both I_gG light and heavy chain specific.

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- a. Sigma Chemical Co. St. Louis, MO 63178
 - b. United States Biochem Corp. Cleveland, OH 44126
 - c. BIO-TEK Instruments, Inc. Burlington, VM 05401

D. Isolation of J5 Gammaglobulins

Animals with a titer of 2048 and 4096 were rebled and the serum collected as described above. Serum from each animal was kept separate, and the globulin fraction isolated by ammonium sulfate precipitation.³⁵ Residual sulfate was removed by dialysis and the globulins were lyophilized and stored at -70 C. The Abs from the serum having a titer of 4096 were used in preparing the conjugate and as the coating Ab in the endotoxin ELISA.

E. Preparation of the J5 Ab Conjugate

The J5 Ab conjugate was prepared using horseradish peroxidase type VI^a according to the sodium periodate^a method of conjugation by Wilson and Nakane¹⁰⁴ with the following modifications: (1) After reacting the enzyme with periodate the reaction was quenched, to prevent excess oxidation, by adding 1.0 ml of .16 M ethylene glycol in distilled water. (2) The conjugate was separated from free peroxidase by ammonium sulfate precipitation.⁷⁸ (3) BSA was not added to the purified conjugate prior to storage. After purification the conjugate was dissolved in .15 M PBS pH 7.4 and 50 ul aliquots were dispensed into sterile glass vials, quick frozen at -70 C and stored at 0 C.

F. ELISA for Endotoxins in Saline

(i) Equipment preparation

All glassware, pipets, dilution tubes, and dispensing trays were rendered free of endotoxin by the conventional method of rinsing

a. Sigma Chemical Co. St. Louis, MO 63178

in distilled water, autoclaving at 121 C for 30 min, and baking at 175 C for 4 hr. The dispensing of fluids into the plates and their washing was performed with a 12 channel microtiter pipet dispenser.^a The pipet tips for the dispenser, the distilled water for washing, and the PBS used for washing, reagent preparation, and as a diluent for endotoxin were all sterile.

(ii) Microplate Preparation

Flat bottom polystyrene microtiter plates^b were used as the test vehicle. To assure firm attachment of the J5 coating antibody, plates were pre-treated with BSA as outlined by Saunders and Clinard.⁸¹ The wells were rinsed 3 times in distilled water and each received 50 ul of a .2 mg/ml aqueous solution of BSA fraction V.^c After the BSA was allowed to air dry at room temperature, it was fixed to the plate by adding 50 ul of .25% glutaraldehyde in .15 M PBS, pH adjusted to 7.0 with .1 M K_2HPO_4 . Fixation was performed at room temperature for 30 min, after which the wells were washed 4 times in distilled water. Fifty ul of PBS containing 15 ug/ml of the J5 globulins was added to each well and allowed to air dry in a 30 C incubator. Other concentrations of coating antibody were used in initial trials, but this amount of coating material produced the optimum sensitivity. Prepared plates retained their activity for 3 weeks when covered and stored dry at room temperature.

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- a. Titertek, Flow Laboratories. Finland
 - b. Dynatech Laboratories, Inc. Alexandria, VA 22314
 - c. United States Biochem Corp. Cleveland, OH 44126

(iii) Endotoxin Dilutions

Each endotoxin was weighed out in a 1 or 2 mg sample and dissolved in .15 M PBS pH 7.4 with the exception of the J5 and Salmonella minnesota strain 595 endotoxin. These endotoxins were made soluble by saponification as previously described. After neutralization they were added to PBS. For each endotoxin a stock solution of 100 ng/ml was prepared and stored at 4 C. Stock solutions were used within 1 week. Serial two-fold dilutions from 100 to .8 ng/ml were made immediately prior to conducting the assay.

(iv) Test Procedure

The procedure for the ELISA measuring endotoxins in saline was as follows:

Step 1. Prepared plates were washed 3 times in PBS .15 M pH 7.4 to remove any unadhered J5 antibody.

Step 2. A 100 ul sample of each dilution of the endotoxin was added to a replicate of 6 wells. For each plate 12 wells received only PBS which served as the negative control. The perimeter wells of the plate were not used, as they consistently produce OD readings different from the remaining 5 in the replicate. These wells also gave elevated background readings for the negative control. A similar observation of the variability of the outer wells of microtiter plates has been reported.⁶⁰

Step 3. Plates were sealed by an adhesive plastic cover and incubated at room temperature for 2 hr. During this time samples were rotated on a horizontal clinical rotator^a at 1 rev/sec for 1 min, after 30, 60, and 90 min of incubation.

a. Eberbach Corp. Ann Arbor, MI

Step 4. The wells were emptied, washed 4 times in PBS, and blocked by adding 50 ul of .15 M PBS pH 7.4 containing .25 M glycine, .5% BSA fraction V, and 20 ug/ml of normal rabbit globulins. Plates were sealed and incubated at room temperature for 30 min.

Step 5. The fluid was removed from the wells which were then rinsed once with PBS. Each well received 50 ul of a 1:200 dilution of the J5 antibody conjugate in PBS containing .25 M glycine and .5% BSA fraction V. Plates were resealed and incubated for 1 hr at room temperature. After 30 min, samples were rotated for 1 min.

Step 6. Plates were emptied and washed 8 times in PBS. One hundred ul of the substrate solution containing 40 mg of O-phenylenediamine and .4 ml of 3% hydrogen peroxide, per 100 ml of .1 M phosphate-citrate buffer was added to the wells.

Step 7. Samples were incubated in the dark for 30 min at room temperature and the reaction stopped by adding 50 ul of 2 M H_2SO_4 .

Step 8. The OD was read at 490 nm with an EIA reader. The instrument was blanked on an antibody coated well containing substrate and acid.

G. ELISA for Endotoxin in Serum

(i) Serum Preparation

For serum samples spiked with endotoxin, fetal calf serum (FCS) was chosen to minimize the presence of immunoglobulins which may bind to endotoxin and render it ineffective in the assay. Some endotoxins bound to serum proteins and could not be detected. The endotoxins could be freed by treating serum in the following manner. Tubes

containing .8 ml of sterile FCS^a were spiked with .1 ml of various concentrations of endotoxin in PBS. The samples were mixed and incubated at 37 C for 30 min to allow for endotoxin-protein binding to occur. One tenth ml of PBS containing .5 mg/ml of protease Type XIV^b was added to each tube and the samples were incubated for 4 hr at 37 C.

Protease activity was stopped and undigested proteins were precipitated by adding 1 ml of 5% phosphotungstic acid (PTA). The precipitate was gently stirred and pelleted by centrifugation at 1,500 g for 30 min. One ml of the supernatant was removed and brought to a pH of 7.4 by adding .1 ml of .3 M NaOH, followed by .9 ml of PBS .15 M pH 7.4. After neutralization the initial concentration of endotoxin added to the FCS was diluted 4 fold.

(ii) Test Procedure

The ELISA method was identical to that used for endotoxin in saline. Endotoxin free serum that was digested, precipitated, and neutralized, was used as the negative control. Neutralized supernatants were spiked with varying concentrations of endotoxin and served as positive controls. Each concentration of endotoxin in the test sample and positive control were added to a replicate of 6 wells. Each plate received 12 samples of the unspiked serum (negative control).

H. Statistical Methods

For each dilution of endotoxin in saline and serum the highest and lowest OD reading within the replicate of 6 was removed. The two

a. KC Biological Inc. Lenexa, KS

b. Sigma Chemical Co. St. Louis, MO 63178

highest and lowest OD values for the 12 negative controls per plate were also disregarded. The sensitivity of the test was established by the concentration of endotoxin which gave an average OD reading greater than the average OD of the background plus 2 standard deviations.

A coefficient of variation within and between-assays for the J5 and S. minnesota 595 endotoxin in saline was used to determine the precision of the test. Ten PBS samples containing 100, 25, 6, and 3 ng/ml were analyzed to estimate the within-assay variation. To estimate between-assay variation the same 4 concentrations were divided into 5 aliquots and frozen, and on 5 different days the aliquots were analyzed.

CHAPTER IV

RESULTS

A. Assay Sensitivity and Precision for Endotoxin in Saline

Antibody to the J5 endotoxin reacted with all endotoxins tested. The lowest concentration of Salmonella endotoxins detected by the ELISA ranged from .8 to 3.1 ng/ml (Figure 1). S. abortus equi endotoxin could be detected at .8 ng/ml, S. enteritidis and S. minnesota at 1.6 ng/ml, and 3.1 ng/ml for the endotoxins of S. minnesota 595 and S. typhimurium. Lipid A from the S. minnesota 595 endotoxin could not be measured by the ELISA. Endotoxin from the E. coli serotypes were measured at 1.6 to 3.1 ng/ml except for 055:B5 in which 6.25 ng/ml could be detected (Figure 2). The OD of the reaction was linear over a 50-100 fold concentration range for both the Salmonella and E. coli endotoxins (Figures 1 & 2). Other endotoxins tested also demonstrated the same type of linearity.

For the 16 endotoxins examined the sensitivity varied 30 fold and ranged between .8-25.0 ng/ml (Table 1). Most of the time the assay gave a measurement of 1.6-3.1 ng/ml, while 80% of the endotoxins could be detected at less than 12.5 ng/ml. The ELISA had a greater sensitivity for Salmonella endotoxins than for endotoxins from E. coli. The J5 antiserum cross-reacted best with the Salmonella endotoxins, followed next by those of E. coli, and were least reactive to the P. aeruginosa and Pasteurella endotoxins. The assay detected the two

rough endotoxins, J5 and S. minnesota 595, equally well measuring both at 3.1 ng/ml.

Tables 2 and 3 contain precision data on the assay for the J5 and S. minnesota 595 endotoxin. Between-assay and within-assay coefficient of variation over the 3-100 ng/ml range were between 8.3-18.0% and 8.3-16.6% for the S. minnesota 595 and J5 endotoxin, respectively. The best precision occurred for concentrations in the mid-range of the standard curve for both endotoxins. Overall a higher precision resulted for between-assays than for within the assay.

B. Assay Sensitivity and Accuracy for Endotoxin in Serum

Techniques used to remove serum inhibitors in the Limulus procedure such as heat and chloroform extraction were not successful in the ELISA. The results of ELISA tests for endotoxins in FCS appear in Figure 3. The actual concentration in the spiked serum samples is 4 fold higher than the concentration assayed as serum is diluted when proteins are precipitated and samples neutralized. When the concentrations were corrected to those originally present in the spiked FCS the assay measured endotoxin between 3.1-25.0 ng/ml (Table 4). The S. abortus equi endotoxin in serum, as in saline, was detected at the lowest level for all endotoxins assayed, being measured at 3.1 ng/ml. The S. typhimurium and P. aeruginosa endotoxin were detected at 12.5 ng/ml and those of the E. coli and K. pneumoniae at 25 ng/ml. The reactions were linear over the range of 1-100 ng/ml for all endotoxins assayed in serum (Figure 3).

The protease and PTA enabled a good recovery of endotoxin from FCS. Some endotoxins could be recovered 100% from serum samples (Table 4). The ELISA test for endotoxins treated with protease and PTA had higher OD readings than the same concentration in positive controls which had not been exposed to either of these reagents.

When comparing ELISA sensitivity for endotoxin in saline to that obtained for endotoxin spiked FCS, which had been treated with protease and PTA, similar values were found (Table 4). The same sensitivity could be achieved in saline and treated serum for the S. abortus equi, S. typhimurium, and K. pneumoniae endotoxin. The E. coli and P. aeruginosa endotoxins were measured at a 2 fold higher and 4 fold lower concentration, respectively, in the treated serum. This indicates the assay performed equally well in saline and deproteinated serum, and the 4 fold dilution which occurred during serum treatment made the sensitivity in serum lower.

C. Procedures Affecting the Assay Sensitivity

Several steps in the assay were essential to obtain the reported sensitivity for endotoxin. Pretreating the plates with BSA was an effective method of attaching J5 antibody and it was virtually impossible to remove them by normal washing procedures. In initial trials use of the conventional carbonate buffer did not produce firm attachment of the J5 antibody. The sensitivity was decreased 10 fold and a higher background was produced when the BSA coating step was omitted. The slope of the standard curve was reduced when the carbonate buffer was used indicating antibody detachment had occurred during incubation and washing, or there was less adherence during the coating process.

The second important procedure was to use 50 ul of coating antibody and conjugate, while adding 100 ul of the test sample and substrate solution. By adding a larger sample volume than was used for antibody coating more endotoxin became immobilized per surface area of the capture antibody than if using an equal volume of coating material and test sample. The OD difference between samples and the background was greater using this procedure. The addition of a greater volume of substrate solution than was used for the conjugate enabled a longer enzymatic reaction time and precluded having to dilute the samples prior to reading.

Lastly, detergents such as sodium dodecyl sulfate and Tween 20 and 80, which are used to reduce nonspecific attachment of the conjugate, are not recommended for use in this assay. These substances have been used successfully with protein^{60,83} and polysaccharide antigens,^{18,33} however caution must be observed when such agents are used with any lipid containing antigen. Disaggregation of the endotoxin molecule has been shown to occur upon reacting with Tween and sodium dodecyl sulfate.^{12,63,107} When Tween 20 (0.5%) was incorporated in the ELISA it totally eliminated the ability to detect endotoxin.

Figure 1. ELISA Optical Density for Salmonella
Endotoxins in Saline, Standard Curves
for Endotoxin Dilutions.

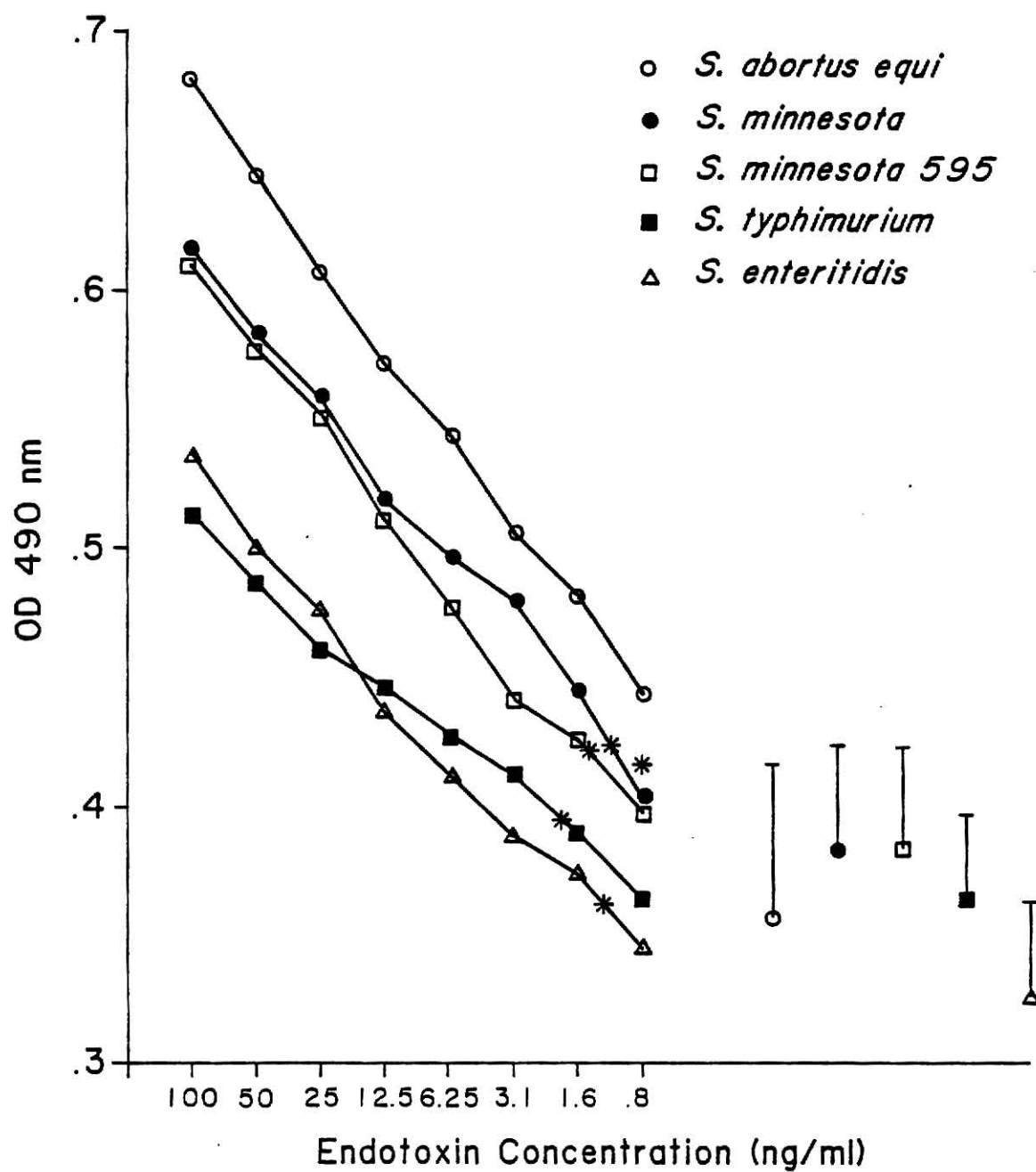


Figure 2. ELISA Optical Density for E. Coli Endotoxins
in Saline, Standard Curves for Endotoxin
Dilutions.

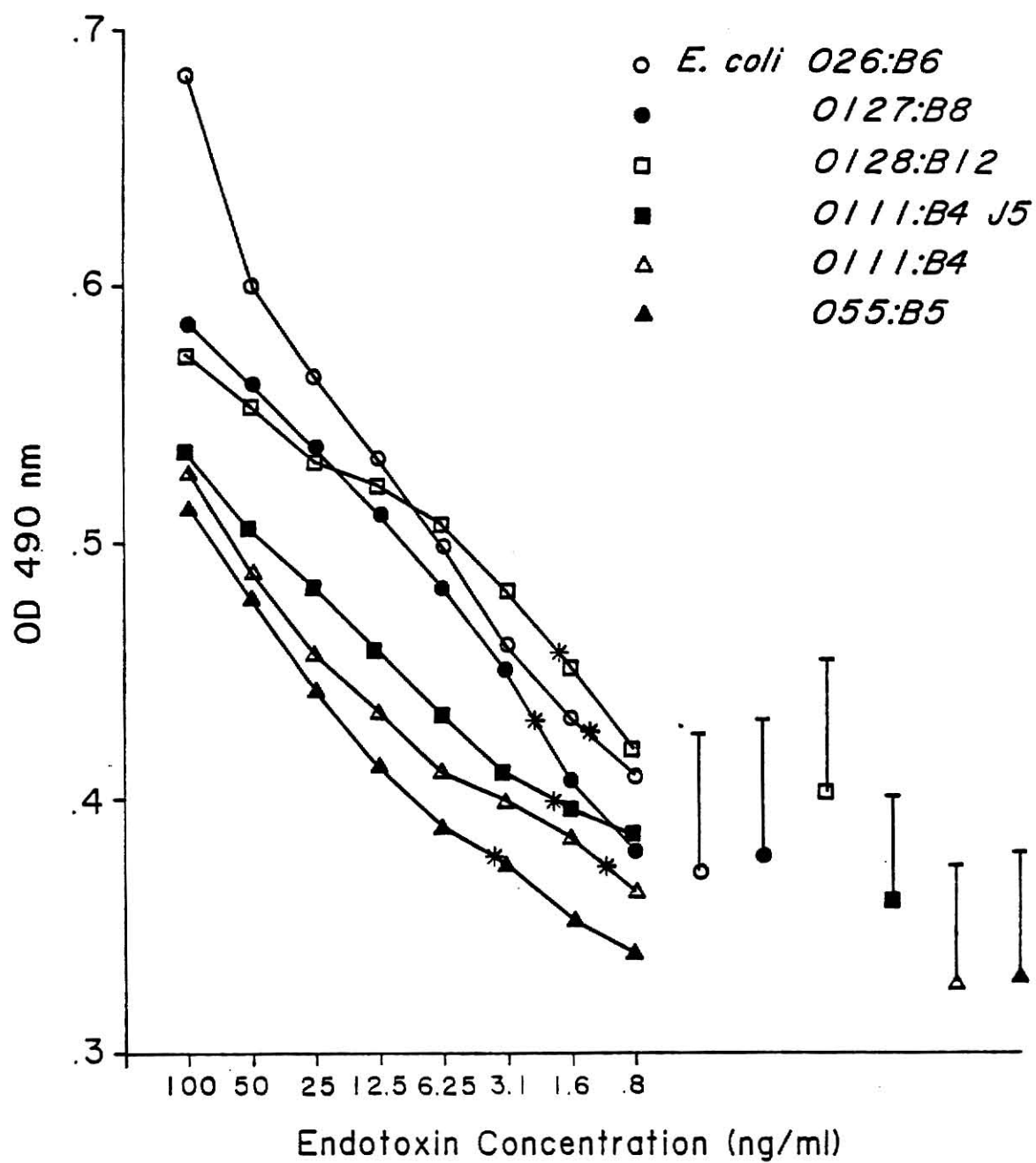


Table 1
ELISA Sensitivity for Endotoxins
In Saline

Endotoxin	Concentration (ng/ml)	Endotoxin	Concentration (ng/ml)
<i>S. abortus equi</i>	0.8	<i>E. coli</i> 0111:B4	1.6
<i>S. minnesota</i>	1.6	<i>E. coli</i> 0111:B4 J5	3.1
<i>S. enteritidis</i>	1.6	<i>E. coli</i> 026:B6	1.6
<i>S. minnesota</i> 595	3.1	<i>E. coli</i> 0127:B8	3.1
<i>S. typhimurium</i>	3.1	<i>E. coli</i> 0128:B12	3.1
<i>S. marcescens</i>	3.1	<i>E. coli</i> 055:B5	6.25
<i>K. pneumoniae</i>	6.25	<i>P. haemolytica</i>	12.5
<i>P. aeruginosa</i>	12.50	<i>P. multocida</i>	25.0

Table 2
Precision of Assay for E. coli J5 Endotoxin

Within-assay	n	$\bar{x}$ ng/ml	SD ng/ml	CV%
Rep 1	10	100	15.00	15.0
Rep 2	10	25	2.00	8.0
Rep 3	10	6	0.75	12.5
Rep 4	10	3	0.50	16.6
Between-assay				
Rep 1	10	100	12.00	12.0
Rep 2	10	25	4.00	16.0
Rep 3	10	6	0.50	8.3
Rep 4	10	3	0.25	8.3

Table 3

Precision of Assay for S. minnesota 595 Endotoxin

Within-assay	n	x ng/ml	SD ng/ml	CV%
Rep 1	10	100	18.00	18.0
Rep 2	10	25	2.50	10.0
Rep 3	10	6	0.50	8.3
Rep 4	10	3	0.45	15.0
Between-assay				
Rep 1	10	100	14.00	14.0
Rep 2	10	25	3.00	12.0
Rep 3	10	6	0.75	12.5
Rep 4	10	3	0.25	8.3

Figure 3. ELISA Optical Density for Endotoxins
in Serum, Standard Curves for Endotoxin
Dilutions.

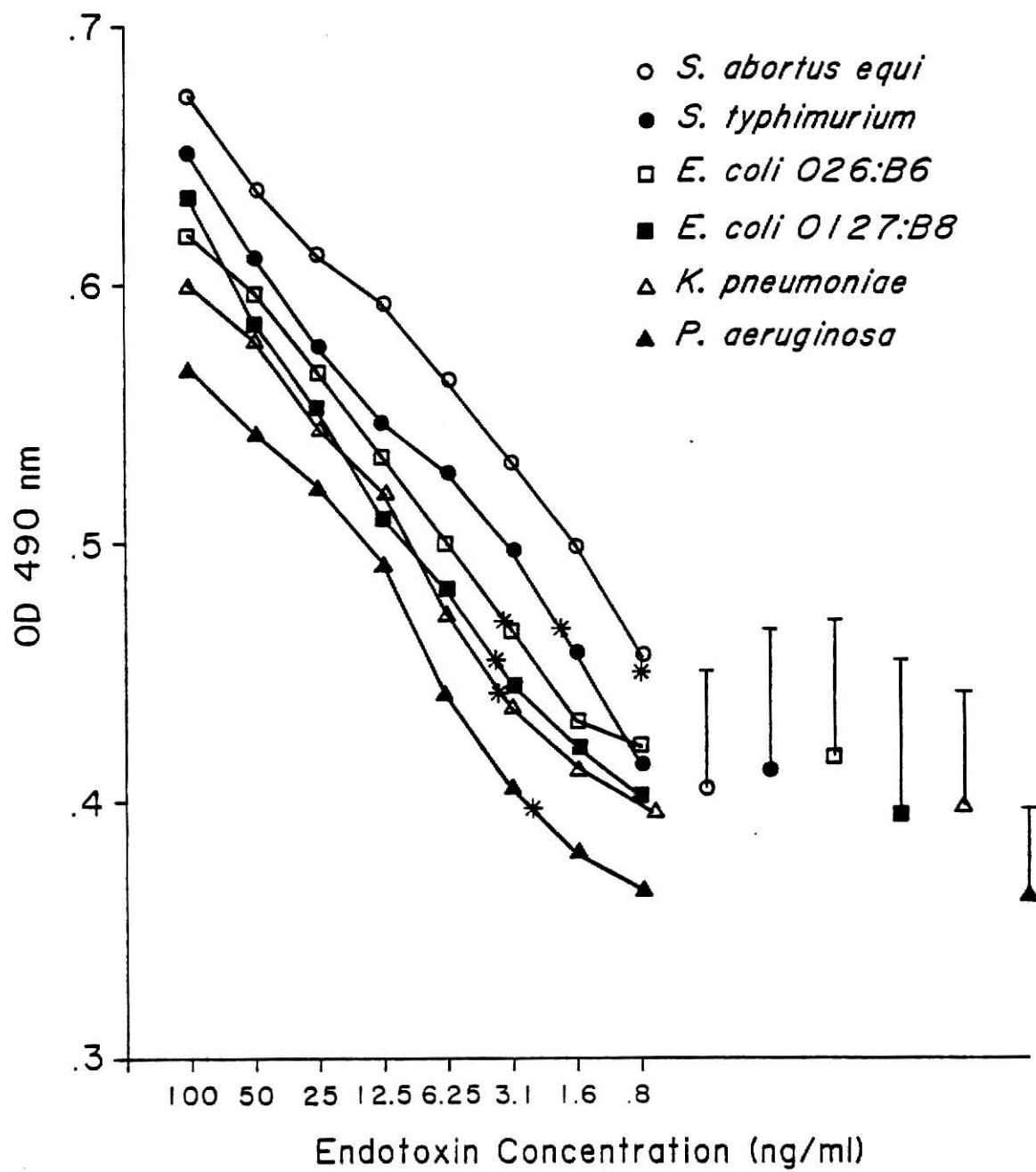


Table 4

Comparison of ELISA Sensitivity for Endotoxins
in Saline, Serum and Serum Equivalent (NG/ml)

Endotoxin	Saline	Serum	Serum Equivalent
<i>S. abortus equi</i>	0.80	0.80	3.12
<i>S. typhimurium</i>	3.12	3.12	12.50
<i>E. coli</i> 026:B6	3.12	6.25	25.00
<i>E. coli</i> 0127:B8	3.12	6.25	25.00
<i>K. pneumoniae</i>	6.25	6.25	25.00
<i>P. aeruginosa</i>	12.50	3.12	12.50

CHAPTER V

DISCUSSION

This ELISA had a 10-20 fold greater sensitivity for measuring endotoxin in saline than the immunoradiometric assay (IRMA) and radio-rocket immunoelectrophoresis (RIE) which used Lipid A antibody.^{69,89} The ELISA was capable of measuring the P. aeruginosa endotoxin while the RIE did not. The ELISA in saline was a quicker procedure being completed in 4 hours while the RIE required 3 days. Both the RIE and IRMA performed poorly in serum as they detected 0-65% of the endotoxin from spiked serum samples. This ELISA was capable of 100% recovery when serum proteins were digested and precipitated.

The ELISA was 10-100 times less sensitive than the Limulus assay, however, it measured endotoxin from different sources more uniformly. When comparing the Limulus sensitivity for the endotoxin of E. coli to that for Pseudomonas a 100-8000 fold difference exists.¹⁰¹ This assay had only a 30 fold difference in sensitivity between all endotoxins tested including that from P. aeruginosa.

The time required for the Limulus assay in serum is 2-24 hours, depending on how the test is read and what extraction method is used.^{6,22,52} The ELISA in serum can be completed in 10 hours. Although the ELISA requires more time than some Limulus procedures, it is a quantifiable test which is a lacking feature of the Limulus assay.

The ability of this assay to detect S. minnesota 595 endotoxin, while not able to measure S. minnesota 595 Lipid A, indicates that antiserum to J5 endotoxin contains some antibodies to KDO. The S. minnesota 595 endotoxin contains only Lipid A and KDO. In order for J5 antiserum to cross react with this endotoxin, antibody to J5 KDO must have been present.

KDO is the dominant antigenic determinant of endotoxins from the various S. minnesota rough mutants.⁴⁶ Because of the structural similarity of these endotoxins to that of the J5 it is likely that KDO has a similar dominance in the J5 endotoxin. Further supporting evidence of KDO being the major cross reacting antigen comes from the fact that the major immunodeterminant group of Lipid A is Beta-hydroxy myristic acid,^{57,59} which is absent from the Lipid A of P. aeruginosa.²⁴ In previous studies when Lipid A antibody was used to measure this endotoxin it could not be detected.⁸⁹ The ability to measure the P. aeruginosa endotoxin in this assay suggests that the cross-reactive antibody is to the J5 KDO rather than Lipid A.

A final consideration is that the immunogenicity of Lipid A is suppressed when Lipid A is present as an integral part of the endotoxin molecule, possibly because it is masked by KDO.⁵⁹ Because of this suppression Lipid A antibody cannot be adequately produced from the intact molecule, nor can it cross react to Lipid A of other bacteria when it is a structural part of the endotoxin.^{30,59,89} Because the intact J5 endotoxin was used as the immunogen the presence of cross-reactive Lipid A antibody in the antiserum is doubtful. It therefore

is most likely that the cross-reactive antibodies are those made to the KDO component.

The difference in the ELISA sensitivity for the tested endotoxins may be explained in several ways. The variation could have been due to the fact that some endotoxins are more antigenically similar to J5 endotoxin than others. However, this does not explain why some Salmonella endotoxins were measured at a lower level than endotoxin from E. coli 0111:B4 J5 or its parent strain 0111:B4. One would expect that the J5 antibody should react best to the immunogen used to produce the antiserum.

Endotoxin isolates vary in lipopolysaccharide and protein content. A wide degree of heterogeneity both in purity and chemical composition, of supposedly identical commercial endotoxin preparations has been reported.³² The protein content between different batches of the same endotoxin serotype can vary 2 fold. The KDO composition remains similar for the same serotype but changes for different types of endotoxin isolated. The difference in purity and chemical composition of the various endotoxins is a more probable explanation for the variable sensitivity observed.

An alternative possibility is the occurrence of a preexisting titer in the J5 immunized animals to some bacterial O antigens or their endotoxin. Because non-purified immunoglobulins were used for coating and preparing the conjugate, the presence of any O antigen or endotoxin specific antibody would complement the J5 antibody in measuring that particular endotoxin.

The precision of the ELISA with a CV% of 8.3-18 for within and between-assays indicates that the procedure is reproducible on a day to day basis. As might be expected the precision was best for concentrations occurring in the mid-range of the standard curve, and a higher precision existed for the J5 than for the S. minnesota 595 endotoxin. Of the several factors that can influence the precision, plate preparation is probably one of the most important. Precoating and fixing BSA to the plates produced a secure antibody binding and lowered nonspecific conjugate binding. The lowered background can be attributed to the fact that immunoglobulins have a lower affinity for BSA than for plastic.¹⁵ The firm adherence of the coating antibody greatly reduced the amount of detachment occurring during incubation and washing, and also probably reduced unequal antibody binding.

The inability to measure endotoxin in FCS was not totally unexpected. Similar inhibition in serum has occurred in the RIE⁸⁹ and Limulus assays.^{12,26,74} Precipitation of serum proteins did permit some endotoxins to be measured, however others like those of E. coli and P. aeruginosa were apparently protein-bound and were removed from the samples. By digesting the proteins prior to precipitation the bound endotoxin was freed or enough of the attached proteins removed so that measurement could be accomplished.

It was of interest to note that endotoxin exposed to PTA gave higher OD readings than the same concentration added to precipitated and neutralized serum. Acid treatment of endotoxin has been shown to alter the KDO component.³¹ Such an alteration may have exposed previously concealed antigens to which the J5 antibody reacted. A

change in the KDO structure by PTA may explain why P. aeruginosa endotoxin could be measured at a lower level in serum than in saline.

Exactly what substance(s) bound to endotoxin in FCS remains speculative. Endotoxin has been shown to bind to albumin,³¹ lipoproteins,²⁶ alpha,⁶⁴ beta,⁶⁴ and gammaglobulins,⁷⁴ complement,^{7,8} platelets,⁶⁴ enzymes (esterases),⁷⁴ and proteins which have not yet been well characterized.⁶⁴ Whether these and other substances present in different animal serums will also inhibit the ELISA, and how successful the protease and PTA will be in removing them remains to be determined.

Endotoxin contamination by equipment and reagents has been minimized but not totally eliminated from this assay. It is impossible to conduct the ELISA free of endogenous endotoxin as plates and many of the reagents cannot be depyrogenated. However, because the test and control samples are treated in the same manner, contamination is uniform throughout the assay and becomes part of the background reading.

CHAPTER VI

CONCLUSIONS

Antibody produced to the E. coli 0111:B4 J5 endotoxin cross-reacts with a wide variety of bacterial endotoxins. Especially important are those of the Enterobacteriaceae which are responsible for the majority of clinical problems seen medically. The dominant antigenic determinant of the J5 endotoxin appears to be KDO. Antibody made to this determinant probably is most responsible for the ability of J5 antiserum to bind to heterologous endotoxins.

The ELISA can detect endotoxin at .8-25.0 ng/ml in saline and 3.1-25.0 ng/ml in serum, while capable of accurate quantitation over a 1-100 ng/ml range. The procedure has a 10-20 fold greater sensitivity than other immunologic methods to measure endotoxin and requires less time and sophisticated equipment.

The assay is 10-100 times less sensitive than the Limulus test, however it has the advantage of being a quantifiable procedure, and it possesses a more uniform sensitivity for different endotoxins. The sensitivity of the assay can probably be improved by purification of the J5 antiserum, developing a competitive assay, or production of a monoclonal antibody. The ELISA should have potential both as a diagnostic test and as a method of study of endotoxemia as the assay sensitivity becomes improved.

CHAPTER VII

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AN ENZYME-LINKED IMMUNOSORBENT
ASSAY (ELISA) FOR ENDOTOXINS

by

MARK G. STEVENS

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ABSTRACT

Tests now used to detect endotoxin are semiquantitative assays. The most widely utilized is the *Limulus* amebocyte lysate test. This procedure is the most sensitive method available for measuring endotoxin. However the test is not absolutely specific for endotoxin, can be inhibited by interference factors present in various body fluids, and is difficult to perform quantitatively.

The objectives of this study were to develop and determine the feasibility of an enzyme-linked immunosorbent assay for detection and quantitation of endotoxin, irrespective of its bacterial origin.

The assay was a sandwich technique that utilized rabbit antibody produced to endotoxin of the *Escherichia coli* strain 0111:B4 J5 mutant. Horseradish peroxidase was used to prepare the conjugate. Hydrogen peroxide served as the substrate and o-phenylenediamine as the chromogen.

The test had a sensitivity of .8-25 ng/ml in saline for the 16 endotoxins examined. A sensitivity of 3.1-25 ng/ml in serum was achieved after freeing protein bound endotoxin by digestion and precipitation of serum proteins. The assay was more rapid and had a greater sensitivity than other reported immunologic methods for measuring endotoxin. The procedure was less sensitive than reports from the *Limulus* assay. However the test was quantitative over a 50-100 fold concentration range, and had a rather uniform sensitivity for different endotoxins. Both of these are lacking features of the *Limulus* method.