AN ABSTRACT OF THE THESIS OF

Chen-chi Wu, for the degree of <u>Master of Science</u> in <u>Veterinary Science</u> presented on <u>September 11, 1996.</u> Title: <u>A Chromogenic *Limulus* Amebocyte Lysate Test for Determination of Endotoxin in the Chicken Plasma.</u>

Abstract approved: <u>*Redacted for Privacy*</u>

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Endotoxin is a part of the cell wall of gram-negative bacteria, consisting of serotype-specific polysaccharide, core oligosaccharide, and lipid A. Lipid A is responsible for an array of pathophysiologic reactions in animal hosts. Amebocyte lysate originated from *Limulus polyphemus* (horseshoe crab) has been used extensively in various assay systems to detect endotoxin. One of the assays, a chromogenic *Limulus* amebocyte lysate (CLAL) test was developed in 1978 and has been used extensively in human clinical fields for its high sensitivity and ease in quantitation. The use of the CLAL test in veterinary fields has been limited to dogs, horses and cattle. The objective

of the thesis research was to determine the level of endotoxin by the CLAL assay in broiler chickens. Since gram-negative septicemia is common in broiler chickens, the detection of endotoxemia would help in understanding the pathogenesis and in developing a new treatment or prophylactic mean.

By the use of a kinetic method, the CLAL assay detected the standard endotoxin (phenol-water extract from *Escherichia coli*, O55:B5 strain) in the range between 100 ng and 10 pg/ml. The intra-assay variation was 1.2% and interassay variation was 18.8% based on 1.0 ng standard. The substrate and lysate control showed spontaneous release of the chromophore starting around 40 min. after the start of the reaction. This spontaneous release was found not due to contamination of pyrogen-free water (PFW) or substrate by endotoxin. With chicken plasma, various non-specific reactions were detected. Plasma alone released the chromophore in a slow, steady manner, but this reaction was virtually eliminated by heating at 100 C. Chicken plasma contained both inhibitor(s) and enhancer(s) for the test. Endotoxin-free plasma samples were prepared by absorption and reconstituted with 1.0 ng/ml of endotoxin. After 1:10

dilution in PFW, heating (10 min.) at 100 C was found most adequate to inactivate these factors as compared with heating to 70 or 85 C. With plasma samples which had been diluted and heated at 100 C, however, still some nonspecific reaction was detected; the lysate, in the absence of substrate, caused some precipitates with chicken plasma in a nonspecific manner, making it difficult to interpret the OD readings. Because of these nonspecific reactions largely inherent to the state of lysate, sensitivity was judged only to 100 pg endotoxin/ml of chicken plasma. A commercial test kit also showed 100 pg/ml sensitivity with the end point method, but found unreliable since proper controls cannot be evaluated in a similar manner as the kinetic method.

Thirty chicken plasma were collected from 3 local broiler farms and was judged to contain less than 100 pg/ml in 29 birds, while one bird showed 12.0 ng/ml of endotoxin. Twenty-three per cent of the chickens showed gram-negative bacteremia without detectable endotoxemia, and the bird with endotoxemia did not have bacteremia. One microgram of the standard endotoxin was injected intravenously to 20 broiler chickens raised in the laboratory, and 5 were sacrificed at 2, 30, 60, and 90 minutes after the injection. The

endotoxin was found to be cleared from the blood at the rate of 152 pg/min.

To increase the sensitivity and to decrease the cost of the CLAL test, future efforts should be made; 1) to significantly decrease the nonspecific reaction between the lysate and substrate; and 2) to block the precipitation or clotting reaction between the lysate and chicken plasma. If these nonspecific reactions be controled, the CLAL test could be run in a simple end-point method and/or in an automated manner with chicken plasma.

A Chromogenic *Limulus* Amebocyte Lysate Test for

Determination of Endotoxin in the Chicken Plasma

by

Chen-Chi Wu

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Chapter 1
Introduction

The cell wall of gram-negative bacterium contains endotoxin that is responsible for a vast spectrum of biological activities; some of them are beneficiary and others are harmful for the host (Captain & Takada, 1990). Many activities of endotoxins are not necessarily side-effects of their toxic action as believed earlier but are induced independently by discrete structures in the lipid A. The pathophysiological activities of endotoxin are not direct lipopolysaccharide (LPS) effects, but are induced indirectly through the action of endogenous mediators that are formed after interaction of LPS with humoral and cellular targets (Natanson et al., 1994). Recent investigations have revealed that endotoxin or LPS binds to CD14 molecule on the surface of myeloid and nonmyeloid cells in association with the LPS-binding protein, leading to gene induction for synthesis of cytokines, adhesive proteins, and enzymes (Ulevitch & Tobias, 1995). The products of these inducible genes up regulate host defense

systems against the bacterial infection. More recent results suggest that this CD14-dependent cell activation also occurs with gram-positive bacteria as well (Pugin et al., 1994).

Various attempts have been made to detect or quantitate endotoxin in the blood (Hurley, 1995). The most sensitive test available at the moment is a *Limulus* amoebocyte lysate test, especially a chromogenic *Limulus* amoebocyte lysate (CLAL) test originally described by Iwanaga et al. (1978). The CLAL test is used routinely to detect endotoxemia in human patients with liver disease, hemodialysis, or adult respiratory distress syndrome. Such assessment of endotoxemia is understood to help prognosing septic shock and monitoring results of clinical treatments.

In veterinary fields, the CLAL test has been applied to dogs (Peterson et al, 1991; Bottoms et al., 1991), horses (King & Gerring, 1988; Henry & Moore, 1991), and cows (Motoi et al., 1993). This is a attempt to apply the CLAL test for the determination of endotoxin in the circulation of commercial broiler chickens as a part of investigation of bacteremia in broiler chickens.

Chapter 2
Literature Review

Chemistry and Biological Effects of Endotoxin

The injection of cell walls of gram-negative bacteria into experimental animals is followed by an array of toxic effects. These include fever, leukopenia, skin reactions, the Schwartzman phenomenon, shock and death, depending on the dose of material and the route of its administration. The term, endotoxin, indicating the active material, was introduced by Pfeiffer in 1904 to distinguish this heat-stable toxic principle from the heat-labile exotoxin that is released into the environment from live bacteria.

In 1930's, Boivin (Captain & Takada, 1990) introduced extraction of gram-negative cell walls with trichloroacetic acid which yielded a toxic macromolecular complex of protein, lipid and polysaccharide, with a molecular weight of the order of 1 to 10 million. Westphal and Lüderitz (1952) introduced the phenol/water extraction method, yielding protein-free lipopolysaccharide (LPS) which retained endotoxic activity. Lüderitz, Staub and Westphal (1966) also established that the polysaccharide component contained

the serologically active determinants in the form of defined oligosaccharides in the repeating units of the species-specific bacterial O-antigen.

Although Westphal and Lüderitz (1954) claimed that the Lipid A component is responsible for endotoxicity, it was not accepted for more than a decade. In some gram-negative species, LPS was purified from rough mutants and its structure was found devoid of complete O-antigenic polysaccharide (Lüderitz & Westphal, 1966). The most deficient strains, Re-mutants, produced only a trisaccharide composed of an unusual C_8 sugar acid, keto-deoxy octonic acid (KDO), bound to lipid A. Such low-molecular glycolipids exerted full endotoxicity. Free lipid A was produced from Re-glycolipids by mild acid treatment. After dispersion in water, lipid A was shown to exert practically all known endotoxic activities (Galanos et al., 1988).

Lipopolysaccharide is a part of an amphiphilic macromolecule embedded in the outer membrane of gram-negative bacteria and consists of a polysaccharide and a covalently bound lipid A (fig. 2.1). The polysaccharide component consists of O-specific chain and the core

oligosaccharide. A variety of non-enterobacterial wild type strains of pathogenic gram-negative bacteria including *Neisseria, Acinetobacter, Bordetella, Hemophilus, and Pasteurella* form LPS which consist only of the core and lipid A region, thus lacking the O-specific chain (Rietshcel, et al., 1988).

The core region of enterobacterial lipopoly-saccharides consists of a heterooligosaccharide which can be formally subdivided into the O-chain-proximal outer core and the lipid A-proximal inner core (fig. 2.2). The outer core contains the common sugars, D-glucose, D-galactose, and N-acetyl-D-glucosamine, whereas the inner core region is composed of the unusual sugars, heptose, mainly in the L-glycero-D-manno and the D-glycero-D-manno configuration, and 2-keto-3-deoxyoctonic acid. These residues are substituted by charged groups such as phosphate, pyrophosphate, phosphorylethanoamine and pyrophosphorylethanolamine, often in nonstoichiometric amounts. Therefore, the inner core region exhibits microheterogeneity and a considerable accumulation of charged residues.
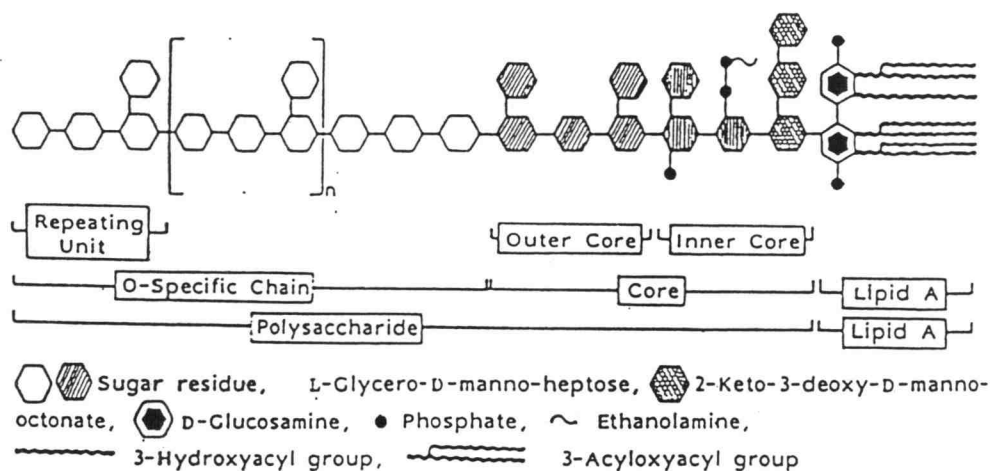
Fig. 2.1. Schematic structure of Salmonella LPS.
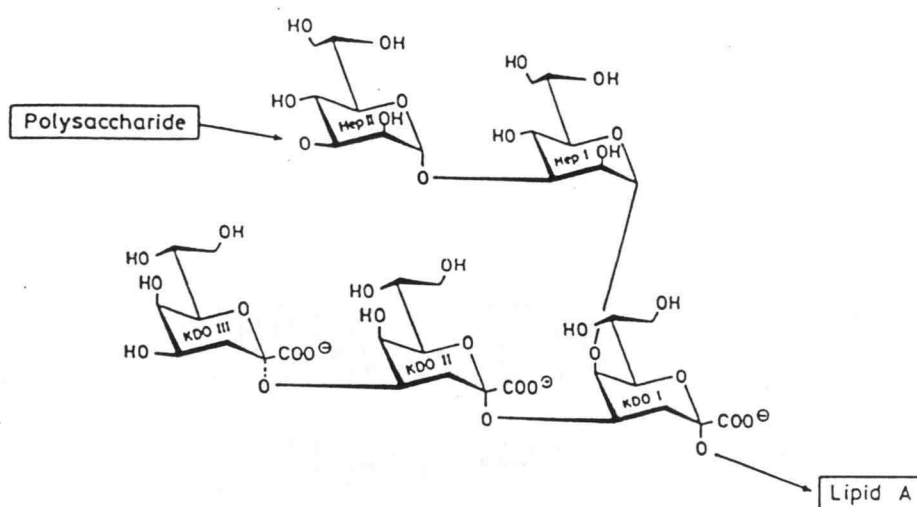
Fig. 2.2. Chemical structure of the core oligosaccharide
in the lipopolysaccharide of **S. Minnesota** chemotype Td1p
(strain R7).

The structural variability of the core within different bacterial species is limited (Tacken et al., 1986). In the genus *Salmonella* only one core type (Ra core) exists for all serotypes, and in *Escherichia coli* so far five core types have been described for more than a hundred different serotypes. The structural variability of core types relates primarily to the outer region, while the KDO-containing inner core appears to be structurally more conserved.

According to present knowledge, all lipopolysaccharides, independent of their bacterial origin, contain at least one pyranosidic or furanosidic KDO residue with a free carboxyl group occupying an internal position in the inner core region. KDO or a derivative represents a common and obligatory constituent of lipopolysaccharides. In all cases studied, this KDO group is A-ketosidically bound to the primary hydroxyl group of the distal glucosamine unit of the lipid A disaccharide backbone (fig. 2.3). It is this KDO residue which carries the polysaccharide chain and, thus, mediates the link between the polysaccharide and lipid A components in lipopolysaccharide.
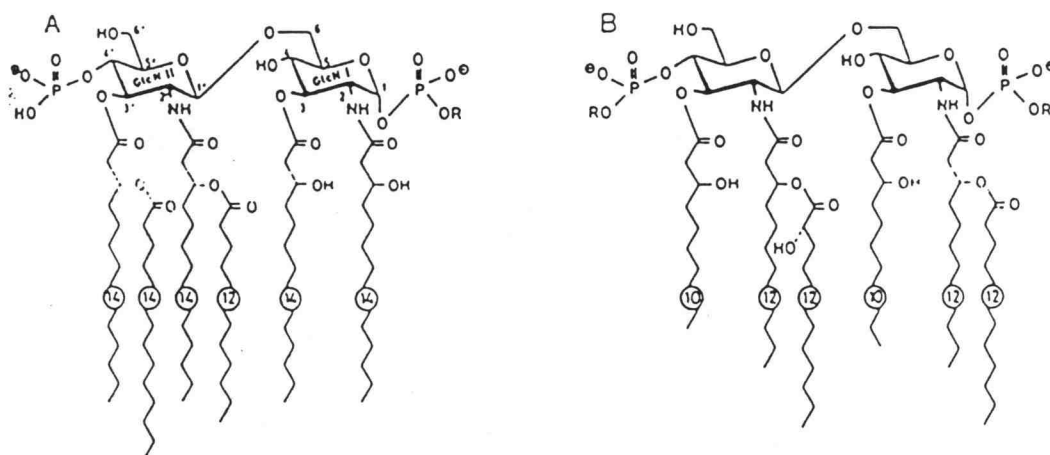
Fig. 2.3. Chemical structure of the lipid A component of (A) **Escherichia coli** and (B) **Chromobacterium violaceum**.

Lipid A represents the covalently linked lipid component of lipopolysaccharides. Polysaccharide-free lipid A does not exist in bacteria, a fact which is related to the biosynthesis of lipid A. Enzyme which cleaves the polysaccharide-lipid A bond are not known and hence, polysaccharide-derived free lipid A can only be prepared by acid catalyzed hydrolysis of lipopolysaccharide.

In fig. 2.3., lipid A structure of *Escherichia coli* is shown with molecular weight of 1796 Da. The lipid A is composed of a β-D-glucosaminyl-(1-6)-β-D-glucosamine

disaccharide which carries two phosphoryl groups: one in position 4' and one in position 1. This hydrophilic lipid A backbone is acylated by four residues of (R)-3-hydroxy fatty acids at positions 2, 3, 2', and 3'. As a further common feature lipid A contains two free hydroxyl groups at positions 4 and 6' (Rietschell et al., 1988).

Endotoxically active lipid A's of different bacterial origin are structurally related. Characteristic and common to them is the presence of a bisphosphorylated β(1-6)-linked D-glucosamine disaccharide. This structure has not been identified in other natural compounds and hence, is unique to lipid A. The lipid A backbone carries approximately four mole equivalence of (R)-3-hydroxy fatty acids (carbon number 10 to 18), two of which occupy amino functions and two of which are linked to backbone hydroxyl group. Both amide- and ester-bound 3-hydroxy-fatty acids are acylated at their 3-hydroxyl group.

The successful chemical synthesis of lipid A and partial structures established relationships of the chemical and physical structure to endotoxic activity at a molecular level. In summary endotoxic activity is determined by a molecule containing two D-glucosamine

residues, which are β(1-6)-inter linked, two phosphoryl groups and at least five, but not more than six, fatty acids including one or two 3-acyloxyacyl groups in a defined location as it is present in *E. coli* lipid A. Molecules lacking only one component, irrespective of its chemical nature, or molecules with a different distribution of components are less or not endotoxically active. Rietschell et al. (1990) hypothesized that for the expression of endotoxic activity, a particular supramolecular structure including at least partial melting of acyl chains at physiological temperature are prerequisites. A higher fluidity of hydrocarbon chains of lipid A should favor the interaction with the host cell membrane.

Kotani and Takada (1990), after their extensive studies with synthetic lipid A analogues, classified bioactivities of lipid A into three categories based on different structural requirements (Table 2.1)

Table 2.1. Classification of bioactivities of lipid A proposed on the basis of structural requirements

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Category I

  I-1 Lethal toxicity in chicken embryos
       Schwartzman reaction
  I-2 Pyrogenicity

Category II

  II-1 Lethal toxicity in galactosamine-treated mice
       IFN-α/β-inducing activity in *P. acnes*-primed mice
       TNF-inducing activity in *P. acnes*- or BCG-primed
       mice
       Immunoadjuvant activity
       Limulus activity (chromogenic method)
  II-2 Murine splenocyte stimulating effects;
       mitogenicity, polyclonal B cell stimulation (in
       vitro)
       Murine macrophage stimulating effects;
       enhancement of PGE_2 generation (in vitro)
       Guinea pig macrophage stimulating effects;
       enhancement of O_2^- generation and glucosamine
       incorporation (in vitro)
       Limulus activity (gelation method)

Category III

       Murine macrophage stimulating effects;
       enhancement of IL-1 generation (in vitro).
       Activation of complement cascade in human serum

Bioactivities in category I require an appropriate number (one or two) of 3-acyloxyacyl groups on the $\beta(1-6)$- linked D-glucosamine disaccharide bisphosphate backbone. Most of the "typical" endotoxic activities belong to this category. This category may further divided into two subcategories by degree of dependence on fine chemical structure. Subcategory I-1 type bioactivities show the greatest dependence on structure. Preparative activities for the Shwartzman reaction and the lethal toxicity in chick embryos and possible in mice belong to this category. Subcategory I-2 type bioactivities, such as pyrogenicity are exhibited by some monosaccharide lipid A analogues, although to a less extent than that of the disaccharide bisphosphate compounds.

Category II embraces most of the bioactivities other than endotoxicities listed in category I and includes the lethality in galactosamine-treated mice. A synthetic lipid A analogue having 3-hydroxyacyl groups but no double acyl groups (LA-14-PP) induced these activities at a level similar to that of disaccharide bisphosphate compounds having 3-acyloxyacyl groups. This category may be divided into two subcategories. The II-1 activities are scarcely

exhibited by acylated glucosamine phosphate compounds, while the II-2 type bioactivities are exhibited by some monosaccharide lipid A analogs. Some monosaccharide lipid A analogs are active in limited assays on bioactivities of this category, but they showed generally weaker activity than the disaccharide analogs. Similar dependency on phosphorylatiobn pattern is noted between bioactivities of categories I and II; generally, the strongest bioactivities of PP compounds were succeeded by PH compounds and then by HP compounds. Dephospho derivatives were practically inactive.

The ability to activate human complement cascade and to stimulate macrophages to release IL-1 is different from other bioactivities listed in category I and II, with respect to dependency on chemical structure (Category III). For instance, on one hand, the complement activation was caused by some monosaccharide analogs. On the other hand, LA-14-PP compound mentioned above scarcely caused complement activation, but minor substitutions in the bis pyrophosphate groups, namely, LA-14-PH and LA-14-HP compound, resulted in the activation.

Mediators and Mediator Activation

Endotoxins are endowed with a vast spectrum of biological activities that are both harmful and beneficiary for the host. Many of these activities, e. g. mitogenic and polyclonal stimulation of B cells, adjuvant activity or induction of prostaglandin and leucotriene synthesis in macrophages are not exclusively properties of endotoxin. They are also expressed by a number of other substances like zymosan (Aderem et al., 1986) or muramyl dipeptide (MDP; Tanaka et al., 1977) which are in some cases even more powerful inducers than endotoxin itself. Conversely, neither zymosan nor MDP elicit acute, hazardous effects that are in any way comparable to those seen in experimental endotoxin shock. Many activities of endotoxins are not necessarily side-effects of their toxic action as believed earlier but are induced independently by discrete structures in the lipid A as described above. Today it is generally agreed upon that the pathophysiological activities of endotoxin are not direct LPS effects, but are induced indirectly through the action of endogenous mediators that are formed after interaction of LPS with humoral and cellular targets.

The role of macrophages in endotoxin-induced lethality was studied in mice made hypersensitive to endotoxin by D-galactosamine. Treatment with D-galactosamine increases their sensitivity to endotoxin more than 100,000-fold (Galanos et al., 1988). D-galactosamine induces an early depletion of UTP in hepatocytes which leads to inhibition of RNA synthesis, and pretreatment with uridine prevents the depletion (Decker & Keppler, 1974). Induction of hypersensitivity to endotoxin by D-galactosamine was found to proceed in rabbits, rats, guinea pigs and in all endotoxin-responder mouse strains. Treatment of endotoxin-resistant mice (C3H/HeJ or C57B1/10 SccR) with D-galactosamine had no apparent effect on their high resistance to endotoxin (Freudenberg et al., 1986). Measurement of UTP levels in the liver of D-galactosamine-treated C3H/HeJ mice showed a strong UTP depletion identical to that seen in endotoxin-sensitive C3H/HeN mice. The sensitization of endotoxin-resistant mice by D-galactosamine became evident after adoptive transfer of endotoxin sensitive macrophages in these animals. Cultured macrophages from endotoxin-sensitive C3H/HeN mice injected into endotoxin-resistant C3H/HeJ rendered them sensitive to

the lethal activity of sub-microgram amounts of LPS after D-galactosamine treatment, showing that macrophages are the effector cells of the lethal toxicity of LPS.

Evidence as to be the possible nature of the relevant mediator involved in endotoxin lethality was obtained using human recombinant TNF in normal and D-galactosamine-sensitized mice (Lehman et al., 1987). TNF was found lethal in normal mice in a dose of 250-500 µg, and the toxic effect was enhanced by 5,000-fold by D-galactosamine. Thus, TNF is a major mediator for lethality in mice after LPS administration.

Pretreatment of mice either with minute amounts of LPS or with 10,000 times in excess of a lethal dose rendered them tolerant to a subsequent challenge with D-galactosamine and a second lethal dose of LPS, carried out 1 to 56 hours later (Freudenberg & Galanos, 1988). In the above-described adoptive transfer model, pretreatment of C3H/HeJ endotoxin resistant mice with LPS, before administration of D-galactosamine and macrophages, did not protect them of lethality. Complete protection from D-galactose and LPS toxicity, however, was observed, when at the time of LPS pretreatment, endotoxin-sensitive

macrophages were administered. The tolerance could not be broken by a second transfer of sensitive macrophages given at the time of challenge. Thus, the mice remained tolerant in the presence of macrophages that are capable of being triggered the LPS to cause lethality.

Endotoxin-responder mice made tolerant to LPS/D-galactosamine by pretreatment with LPS are also found to be tolerant to the lethal activity of TNF (Galanos & Freundenberg, 1988). Conversely, pretreatment of the animals with TNF renders them tolerant to both TNF and LPS. The tolerance-inducing property of TNF may be demonstrated directly also in D-galactosamine-treated C3H/HeJ mice. Pretreatment of these animals with TNF makes them tolerant to a subsequent challenge with D-galactosamine and lethal amounts of TNF.

Molecular Mechanisms of LPS-Cell Interaction

Recognition of LPS triggers gene induction by myeloid and nonmyeloid lineage cells. These inducible genes encode proteins that include cytokines, adhesive proteins, and enzymes that produce low molecular weight proinflammatory mediators. Together the products of these inducible genes up regulate host defense systems that participate in

eliminating the bacterial infection. In 1986, a plasma protein termed LPS-binding protein (LBP) was discovered (Tobias et al., 1986), which led to intensive investigation into molecular mechanisms involved in the cell activation.

LBP was identified in experiments that compared LPS binding to high-density lipoproteins (HDL) in normal and acute phase serum (Tobias et al., 1986). Isopycnic equilibrium density gradient centrifugation in CsCl of mixtures of ^{3}H-Re595 LPS, isolated from R-form *Salmonella minnesota* Re595 strain, and serum revealed that the rate of binding of LPS to HDL was markedly slowed in acute phase serum because of the formation of a stable complex between LPS and proteins present in acute phase serum. Fractionation of serum revealed that a 60-kDa glycoprotein was responsible for complexing with the LPS; this protein was named LBP. The complete primary structure of human and lapine LBP was deduced from cDNA cloning (Schumann et al., 1990). LBP is synthesized in hepatocytes as a single polypeptide, glycosylated, and released into blood as a 60-kD glycoprotein (Ramadori et al., 1990). LBP synthesis is under the control of cytokines and steroid hormones (Grube et al., 1994).

An ELISA-based binding assay was used to show that LBP binds to LPS from rough or smooth form gram-negative bacteria via lipid A. The presence of core and/or O-specific polysaccharide did not significantly influence LBP-lipid A binding (Tobias et al., 1989). LPS-LBP binding has a Kd in the nM range and a stoichiometry of 1:1. Tobias et al. (1988) first reported that LBP shared primary amino acid sequence with another LPS/lipid A binding protein, namely, bactericidal/permeability-increasing protein (BPI). BPI is a 50 kD protein localized in a granule of neutrophils; it is an antibacterial protein specific for gram-negative bacteria (Elsbach & Weiss, 1993). Thus LBP is a member of a family of proteins that bind amphipathic molecules and transport such molecules in aqueous environments.

A major function of LBP is to enable LPS binding to either membrane or soluble CD14 (Kirkland et al., 1993). LBP appears to have two functional domains, one for LPS binding and another that fosters LPS-CD14 interactions. The LPS binding domain of BPI has been localized to an amino terminal 25 kDa fragment of the molecule. Two laboratories about the same time prepared exactly the same

fragment of human LBP, comprising residues 1-197, and showed that it was capable of binding LPS with affinity similar to that of intact LBP.

Measurements of induction of TNF with a series of LPS preparations as well as synthetic lipid A showed that the presence of LBP lowered the threshold stimulatory concentration of LPS and markedly enhanced the rate of TNF production (Mathison et al., 1992). LBP also enhances the effects of LPS on the induction of other cytokines as well as on NO release (Corradin et al., 1992). LBP also enhances LPS-induced upregulation of adhesive protein function and priming of arachidonic acid metabolism in neutrophils. Immunodepletion of LBP from plasma markedly reduces LPS-induced cell activation (Schumann et al., 1990). Using D-galactosamine-treated mice, Gallay et al. (1993) showed that depletion of LBP with anti-murine LBP antibody prevents LPS-induced lethality.

CD14 is present in two forms: In myeloid lineage cells CD14 is expressed as a glycosylphosphatidylinositol (GPI)-anchored membrane glycoproein and serum contains a soluble form of CD14 lacking the GPI tail (Ziegler-Heitbrock & Ulevitch, 1993). LBP functions as an opsonin for LPS-

bearing particles including whole gram-negative bacteria or red blood cells coated with LPS (E-LPS), facilitating attachment of these particles to myeloid cells. When E-LPS are mixed with LBP (E-LPS-LBP) the particles form rosettes with monocytes/macrophages (Wright et al., 1989). Anti-CD14 monoclonal antibodies or pretreatment of cells with phosphtidylinositol-specific phospholipase C blocks rosetting of E-LPS-LBP.

How a GPI-anchored protein like CD14, which does not directly communicate with the cell interior, mediates ligand-specific cell activation ? The most current view suggests that GPI-anchored proteins and src-like protein tyrosine kinases associate with each other (Brown, 1993). Weinstein et al. (1992) first showed that LPS treatment of macrophages increased protein tyrosine phosphorylation and identified MAP kinase isoforms as well as other proteins as targets.

Myeloid lineage cells have an important role during ·the initial host responses to microbial pathogens. Recognition of these pathogens by myeloid cells leads to induction of nonspecific host-defense mechanisms with production of various cytokines and enzymes that produce

small molecule immune/inflammatory mediators. Cells participating in these innate, nonadaptive immune responses are regulated by receptors that are nonclonal and not coded for by rearranging gene families; such receptors detect common or structurally related components of microbial pathogens. CD14 is a prototypic example of such a receptor. Polyuronic acid polymers (Espevik et al., 1993), lipoarabinomannan (Zhang et al., 1994), and cell wall preparations from gram-positive bacteria (Pugin et al., 1994) were shown to activate myeloid lineage cells via CD14-dependent mechanisms.

Limulus Amebocyte Lysate Test for Endotoxin

Limulus amoebocyte lysate (LAL) assay , used to detect the endotoxin *in vitro*, was first developed by Bang (Bang, 1956) who discovered that the endotoxin of a *Vibrio* species from seawater, pathogenic for the horseshoe crab (*Limulus polyphemus*), caused fatal intravascular coagulation triggered by an endotoxin-initiated reaction causing the enzymatic conversion of a clottable protein derived from the circulating blood cell (amebocyte) of the crab (Levin & Bang, 1968). The hemocytes circulating in horseshoe crab (*Limulus)* hemolymph contain a coagulation system which

participates both in hemostasis and in defense against

invading microorganisms (Young et al., 1972). The

coagulation system of *L. polyphemus*, considered homologous

to the Japanese horseshoe crab, *Tachypleus tridentatus* that

has been studied extensively (Iwanaga et al., 1985),

consists of several enzymes that are arranged in three

pathways in a fashion which resembles the classic,

alternate, and common mammalian coagulation cascade

pathways. The components of cascade of *Tachypleus*

tridentatus are Factor B, Factor C, Factor G, proclotting

enzyme, and coagulogen (fig. 2.4.).
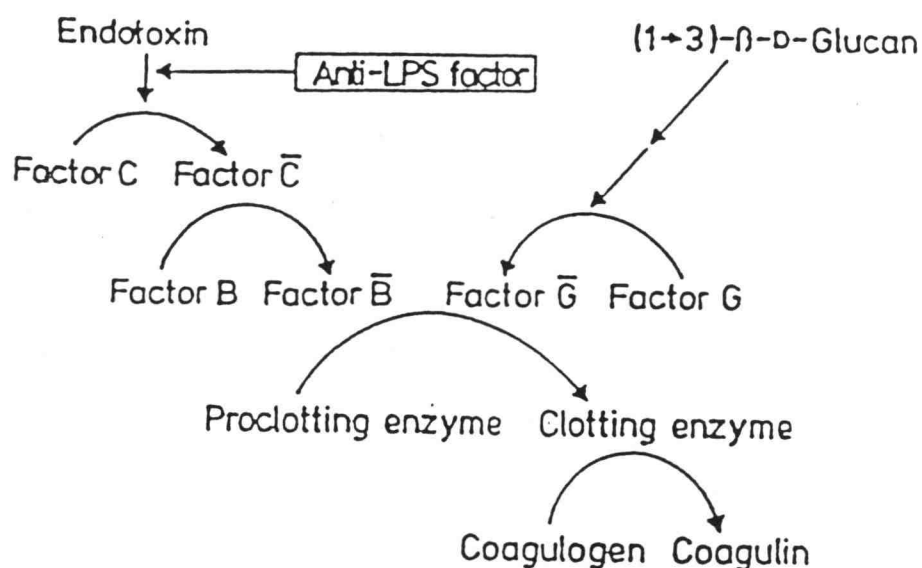
Fig. 2.4. Schematic representation of coagulation system found in horseshoe crab hemocytes. A (1→3)-β-D-glucan-mediated pathway linked with the activation of proclotting enzyme is also shown.

Coagulogen is an invertebrate fibrinogen-like substance, consists a single basic polypeptide chain and has a molecular weight of 19,000, which is converted from a soluble form to an insoluble gel, named coagulin, after the addition of endotoxins to the lysate. The conversion to a gel is mediated by a clotting enzyme in the lysate. Proclotting enzyme, a factor Xa-like serine protease, is a single-chain glycoprotein with the molecular weight of 54,000 and insensitive to endotoxins; however, in the

presence of activated factor B, a proclotting enzyme is fully activated and result in transformation of clotting enzyme consisting of two-chain polypeptides with light (molecular weight, 27,000) and heavy(molecular weight 31,000) chains which contains a diisopropyl phosphofluoridate (DFP)-sensitive active site (Morita et al., 1983). The clotting enzyme may transform coagulogen to coagulin gel and hydrolyze the chromogenic substrate *t*-butyloxycarbonyl (BOC)-Leu-Gly-Arg-ρ-nitroanilide, liberating ρ-nitroaniline (Nakamura et al., 1977). Factor B, a molecular weight of 64,000 and consisting of two chains held by disulfide linkages, is able to transform proclotting enzyme to the active form by limited proteolysis. Factor C , a activator of factor B, consists of two-chain polypeptides, with a heavy (molecular weight 80,000) and a light (molecular weight 40,000) chains. Factor C is highly sensitive to endotoxin and is activated autocatalytically to activated factor without any decrease of the molecular weight of 130,000. The pathway of factor G triggered by (*1*→3)-β-D-Glucan links with the activation of proclotting enzyme. This cascade sequence results in an amplification of the original stimulus which accounts for

the sensitivity of the *Limulus* coagulation system to endotoxin at picogram-per-milliliter concentrations, and an additional component of *Limulus* amebocytes is an anti-LPS factor which has high molecular weight, heat labile (Young et al., 1972), and anti-endotoxin properties (Warren et al., 1992).

The endotoxin-activated clotting enzyme cleaves the coagulogen to form a gel clot, a positive indicator. To perform this gel clot test, a small amount of LAL solution is added to an equal volume of a standard dilution or a sample in a small tubes. If a firm gel forms and remains solid in the bottom of the reaction tube when the tube is inverted, the test is scored positive. Methods to improve the visualization of clot formation in microtiter volume have been described (Gardi & Arpagaus, 1980). With all gel clot-based techniques, a semiquantitative result can be obtained through serial dilution of samples and standards.

In the chromogenic LAL assay method (Iwanaga et al., 1978), the coagulogen is completely or partially removed to be replaced by a chromogenic substrate (Scully et al., 1980), a synthesized n-Benzoyl-Val-Gly-Arg-ρ-nitroanilide (synthetic peptide linked to a chromophore) containing an

amino acid sequence similar to that present at the site in the clotting protein cleaved by the clotting enzyme. The chromogenic LAL assay usually has two stages: a LAL activation stage and a chromophore release stage. In the presence of endotoxin, factors in LAL are activated in a proteolytic cascade that results in the cleavage of a colorless artificial peptide substrate, n-Benzoyl-Val-Gly-Arg-ρ-nitroanilide. Proteolytic cleavage of the substrate liberates ρ-nitroanilide (ρNA), a chromophore, which is yellow and absorbs at 405 nm (fig. 2.5.).

Boc-Val-Leu-Gly-Arg-CONH-⟨ ⟩-NO$_2$

H$_2$O | Clotting enzyme

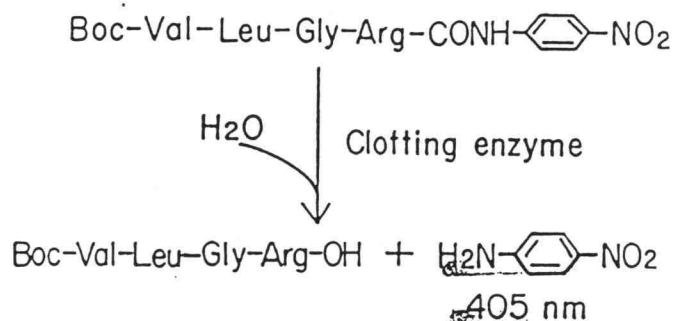Boc-Val-Leu-Gly-Arg-OH + H$_2$N-⟨ ⟩-NO$_2$
405 nm

fig. 2.5. A typical chromogenic substrate for *Limulus* clotting enzyme. ρ-nitroanilide (ρNA) liberated is measured spectrophotometrically at 405 nm.

The strength of the yellow color, measured by optical density (OD) at 405 nm in a spectrophotometer is a function

of the amount of active clotting enzyme, correlated to the amount of endotoxin present in the test. Both stages of the chromogenic reaction are critically time and temperature dependent, but within these limitations, the chromogenic assay is sensitive to 10 pg/ml (Thomas et al., 1981). A new kinetic single-stage limulus amebocyte lysate method, based on components (LAL and, chromogenic substrate) originally intended for a two-stage end-point method, has been described: The components can be pooled and subsequently used in a single-stage kinetic procedure adapted to microplate (Dunner, 1993), with a help of a kinetic software.

There are two pathways leading to the coagulation of LAL: one activated by endotoxin triggered by factor C; the other is activated by β-glucans triggered by a glucan-reactive factor G, specifically blocked by polymyxin and laminarin, respectively (Zhang et al., 1994), and two pathways joint at the step of activation of proclotting enzyme. therefore, the use of polymyxin and laminarin can improve specificity of LAL test to endotoxin.

Generally only LPS can produce a positive LAL assay at concentrations as low as picograms per milliliter. When

reactions with other microbial products were described, for example, peptidoglycan derived from the cell walls of gram-positive organisms (Kimura, 1976; Wildfeuer et al., 1975)or (1→3)-β-D-glucans (Roslansky, 1991; Zhang et al., 1988), the concentrations required were 1,000 to 400,000 times higher than the required concentration of endotoxin. In other words, contamination of peptidoglycan with 0.00025% endotoxin could account for a positive LAL assay and is difficult to exclude. However, there are other reports describing no LAL reactivity for the same compounds(Yin, 1975).

The use of LAL test for detecting endotoxin in human plasma, however, is still controversial (Urbaschek et al., 1985). One of the difficulties can be explained by the techniques used in the assay as well as the different treatments of the plasma used to eliminate plasma-related inhibitors of the LAL-endotoxin reaction first described by Levin (Levin et al., 1970). Enhancer can enhance a LAL reactivity after dilution-heating procedure (Ditter et al., 1982). To overcome this difficulties, a new test system was developed by Urbaschek. The slope of OD over different endotoxin concentration can be described by the

mathematic model $\Delta O.D.$ max/min $= A + F_{10} \times \log (X + C)$, where C represents the unknown endotoxin concentration of the sample, X is the endotoxin spike added, and F_{10} is the climb of the curve indicating the extent of the interference. This factor relative to water (F_{10r}) is >1 when the LAL-endotoxin reaction is enhanced and <1 when it is inhibited. A deviation of endotoxin concentration in the intra-indivisual has been described. (Urbaschek et al., 1985).

There are three characteristics of the progress of the LAL gelation reaction when monitored as a change in OD. These three characteristics need to be concerned in the design of a quantitative assay for endotoxin (fig. 2.6) (Hurley et al., 1991; Hurley, 1995). (I) The progress of the reaction follows a sigmoid curve, with an initial plateau, a phase of rapid rise, and a terminal plateau. (ii) The absolute increase in OD is determined by the concentration of LAL clottable protein. (iii) The rate of increase in OD is determined by the concentration of endotoxin.
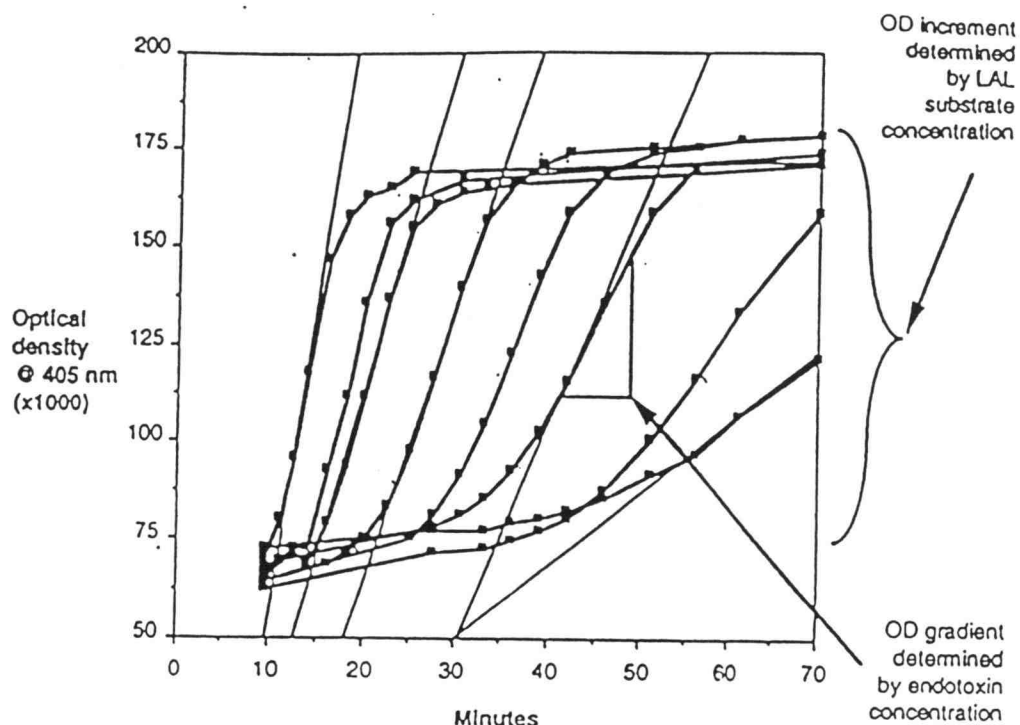
Fig 2.6. LAL reaction curves showing the progress of the endotoxin-activated gelation of LAL at eight concentrations of endotoxin (from right to left, respectively: 0.033, 0.1, 1, 3.3, 1., 33.3, and 100 ng/ml) as change in OD at 405 nm in a microtiter tray (Hurley et al., 1991).

The progress of the LAL assay can be monitored in endpoint and kinetic methods. In general, a kinetics assay has several advantages over an endpoint assay. For example, a kinetic assay is able to quantify the concentration of endotoxin over a wider range (Hurley, 1995). However, with the microtiter format, some loss of precision will occur because of timing errors in the addition of reagent to

multiple wells of a 96-well plate, and , with the repeated readings, inability to control the incubation temperature in the microtiter plate readers as commonly available in clinical laboratories.

To solve the problem of interference from the color or turbidity of the specimens, an enzyme-linked immunosorbent assay against synthetic *Limulus* peptide C, a 28-amino-acid fragment of coagulogen formed by the reaction of endotoxin with *Limulus* amebocyte lysate, was developed. Besides, the outcome was more correlated well with endotoxin concentrations in the plasma samples and with raw lysate, which was much more stable in solution than chloroform-treated lysate, the assay was still highly sensitive to endotoxin but was totally unresponsive to natural glucans which include curdlan, laminarin, and LAL-reactive materials(Zhang et al., 1994). However, the assay is time-consuming, usually 5 to 6 hours for performance of a test.

Clinical Application of LAL Test

The amount of endotoxin associated with a single gram-negative organism is 3 to 4 x 10^{-14} g. Assuming an origin from bacteria within the intravascular compartment, it would be expected that a positive LAL assay at a detection

limit of 10 pg/ml would represent the detection of an amount of endotoxin equivalent to that within 300 organisms/ml (Hurley, 1995). In patients with sepsis, endotoxemia is typically at levels as high as 400 pg/ml or higher, although much greater levels are seen in patients with meningococcemia (Brandtzaeg et al., 1989). Hence, it would be difficult to account for the positive results in patients with gram-negative bacteremia on the basis of the numbers of bacteria present, typically less than 10 CFU/ml in adults.

The significance of endotoxemia in patients with liver disease is unclear. Some studies have found an association between endotoxemia and abnormalities in routine biochemical liver function tests (Bigatello et al., 1987), whereas others have not (Fukui et al., 1991), although these tests are relatively insensitive indicators of liver dysfunction in comparison to histological evidence.

There is a poor concordance between gram-negative bacteremia and endotoxemia. Endotoxemia is detected in approximately half or less of those with gram-negative bacteremia, and similarly, gram-negative bacteremia is detected in approximately half of those with endotoxemia

(Hurley, 1994). While gram-negative bacteremia is itself a relatively weak predictor of clinical outcome, endotoxemia is somewhat more predictive of clinical outcome. In a study of 473 patients of whom 31 were found to have endotoxemia, 53 were found to have gram-negative bacteremia and 17 were found to have both. The positive predictive value for the subsequent development of clinical septicemia was higher for the detection of endotoxemia (positive predictive value = 48%) than for the detection of gram-negative bacteremia (positive predictive value = 28%)(van Deventer et al., 1988). The co-presence of gram-negative bacteremia with endotoxemia appears to be an important determinant of prognosis.

The detection of endotoxemia has been shown to have some value for prognosis of the following conditions; neutropenia (Yoshida et al., 1994), neonates and children (Scheifele et al., 1985), burn injuries (Endo et al., 1992), or ARDS-related sepsis (Parsons et al., 1989).

Only limited attempts have been made to use the *Limulus* amoebocyte lysate test for the detection of endotoxemia in veterinary species. The LAL test was applied to determine the levels of endotoxin in the blood

of dogs with porto systemic shunts before and after the corrective surgery (Peterson et al., 1991). There was no significant differences in the level of endotoxin between the surgically corrected and the control group. The level of endotoxin in normal healthy dogs is below 25 pg/ml, which was the limit of sensitivity of the chromogenic LAL assay (Bottoms et al., 1991). In horses, 4 to 70 pg/ml of endotoxin was detected in plasma of colic horses compared with less than 2 pg/ml in plasma of healthy controls (Henry and Moore, 1991). The authors also noted that horses with endotoxin levels higher than 10 pg/ml did not survive. Using a turbidometric assay, Motoi et al. (1993) found that the serum levels of dairy cows varied from 1 to 4 pg/ml.

Chapter 3
MATERIALS and METHODS

Chickens

Commercial broiler chickens at ages between 4 and 8 weeks-old were obtained from local farms. For the endotoxin clearance experiment, one day-old broiler chicks were raised in battery cages in isolation for 3 weeks under conventional management.

Glassware

All the glasswares were soaked in detergent solution (E-Toxa-Clean; Sigma Chemical Co., St. Louis, MO) overnight and rinsed well with distilled and pyrogen-free water (PFW: BioWhittaker, Walkersville, MD). They were covered by metal caps and aluminum foil and autoclaved at 121 C for 1 hr followed by baking at 230 C for 5 hrs.

Lysate

Lyophilized powder of *Limulus polyphemus* amebocyte lysate (E-Toxate; Sigma catalogue number 210-50) was reconstituted in 5 ml of PFW water as instructed.

Immediately prior to the use in the test, the lysate was
mixed with 10 mM MgCl$_2$ and 50 mM tris-HCl buffer, pH 7.3
(BioWhitaker) at the ratio of 5:2:2.

Substrate

A N-benzoyl-val-gly-arg ρ-nitroanilide (Sigma) was
dissolved in PFW to 2 mM immediately prior to each test.

Endotoxin Standard

Lipopolysaccharide (LPS) purified by the phenol-water
method from Escherichia coli, strain O55:B5 (Difco
Laboratories, Detroit, MI) was dissolved in PFW at the
concentration varying from 100 to 0.1 ng/ml except in some
experiments where other concentrations were examined.
Immediately prior to dispensing, the LPS suspension was
mixed well by a Vortex mixer.

Plasma

Heads of the chickens were disarticulated at the
atlanto-occipital joints, and dipped in an aseptic solution
contain detergent. Transverse incisions were made with
sterile scissors through the pectoral muscles on each side
of the keel and over costochondral junctions, and the
coracoid and clavicle bones were cut. The ventral

abdominal wall and breast were removed, and 5 ml blood samples were taken directly from the heart into syringes containing 50 units of sterile sodium heparin. After 0.1 ml was plated onto a MacConkey agar plate, the blood was immediately placed on ice and centrifuged at 1,000 x *g* for 10 min at 4 C (Obayashi, 1984). Plasma was separated and stored at -70 C. The bacteriological cultures were examined at 24 and 48 hrs to enumerate colonies.

Heat Inactivation

Plasma samples were diluted to 1:10 in PFW and immersed in boiling water for 10 min. The endotoxin standard in various concentrations was also diluted in 1:10 before testing. In an experiment where various inactivation temperatures were examined, a plasma sample was absorbed by the procedure described below to remove any endotoxin, quadreplicated, added 1 ng of the standard endotoxin to each tube, diluted in 1:10 in PFW, and heated in water kept at 70, 85 or 100 C.

Chromogenic Limulus Amebocyte Lysate Test

Samples and control standards were delivered in 100 µl into 13 x 100 mm glass tubes, and 900 µl of pyrogen-free

water was added. The tubes were immersed in boiling water for 10 min. followed by transferring 100 µl of each sample or standards into wells of the microtiter plate in duplicates or triplicates (Microwell; Nunc, Roskilde, Denmark). In an experiment, in which different inactivating temperature was examined, the diluted standards were heated at 70 or 85°C in addition to 100°C. The substrate solution was added in 50 µl into each well followed immediately by the addition of the lysate solution in 50 µl, and the plate was incubated at 30 C for additional 10 min. Optical density at 405 nm was read with an automatic microplate reader (Multiskan MK II; Flow Laboratories, McLean, VA). The results were plotted with the O. D. values on Y-axis and the reaction time in min. on X-axis by the use of the computer program (SigmaPlot, Jandel Scientific Software, San Rafel, CA).

Absorption of Endotoxin

One milliliter of plasma, substrate or lysate was added into the endotoxin removal affinity resin supplied in a tube (END-X B15; Cape Cod Associates, Woodshole, MA) and mixed with a rotator for 8 hours at 4 C. The mixture was

centrifuged at 1,200 x *g* for 2 minutes, and supernatant was transferred to receiver tubes supplied with the resin.

Commercial Test Kit

Pyrochrome LAL chromogenic test kits were purchased (Cape Cod Associates), and diluted, heat-treated samples/standards were tested according to the end-point method described by the manufacturer.

Endotoxin Clearance

One microgram of purified LPS was injected intravenously into 20 chickens and endotoxin levels were determined with blood samples taken within 90 minutes.

Statistics

Intra-assay precision (coefficient of variation) was determined by four repeated measurements of 1 ng standard endotoxin in PFW within one assay. Interassay precision was determined by four repeated measurements of 1 ng standard endotoxin in PFW on four different days (Bottoms et al., 1991). Student's t-test was used to analyze other data.

Chapter 4
Results

Standards

The standard endotoxin in different amounts were added to PFW, and CLAL test was run for 140 min. (Fig 4.1a). Although not shown in the figure, the substrate and lysate control were tested for each endotoxin concentration, and these control values were subtracted from each O. D. value at a given time. Since the rate of enzymatic reactions corresponds to the concentration of endotoxin (Hurley et al., 1991), a straight line was drawn manually passing the midpoint of the rapid O. D. increase phases described previously (Fig 4.1b; Hurley et al., 1991). The slopes of each straight line was determined and standards in PFW after subtraction of lysate and substrate controls with straight lines representing the rate of rapid OD increases plotted against endotoxin concentrations in $\log_{10}$ (Fig 4.1c); $r^2 = 0.966$. When the concentration of endotoxin was lower than 100 pg, reproducibility of the results was poor; in some tests, there was no measurable increase in the rate of O. D. increase beyond spontaneous ρ-nitroanilide release

by the lysate. Intra-assay precision based on 1 ng

endotoxin standard was 1.2%, while interassay precision was

18.8%.
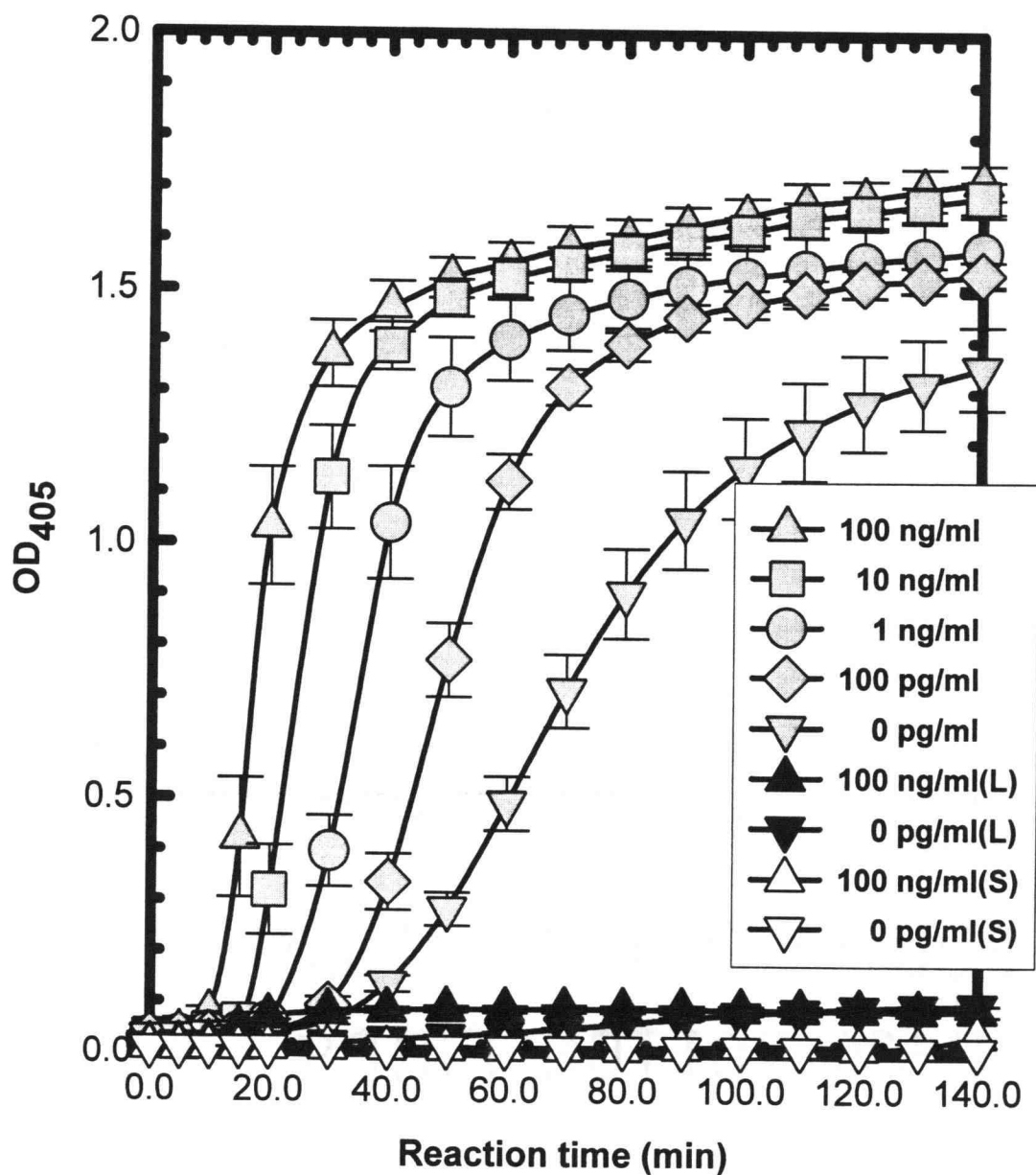
Fig 4.1a. CLAL reaction curves with endotoxin standards in PFW (mean ± s. d. with 3 samples). L: lysate controls, S: Substrate controls.
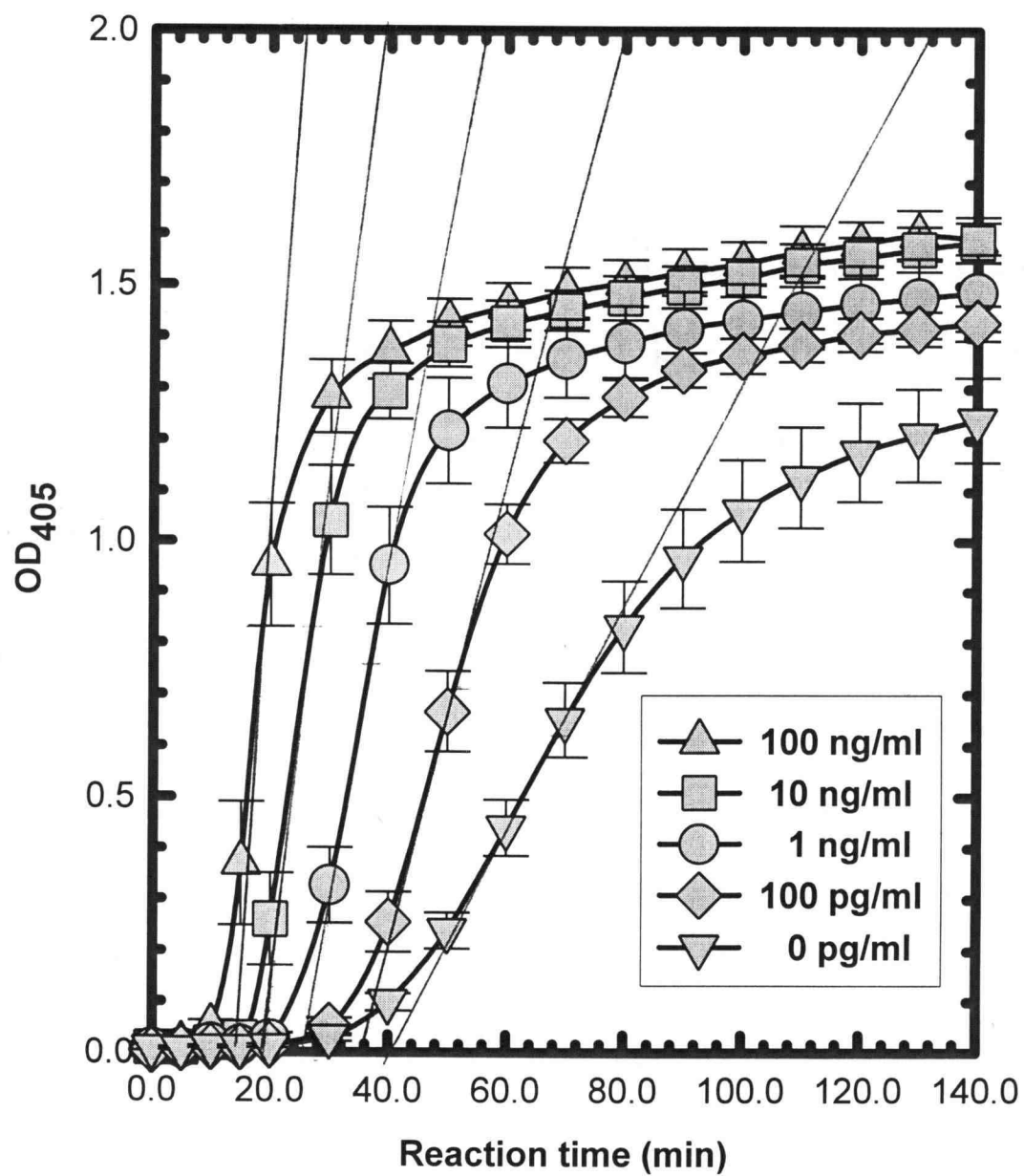
Fig 4.1b. CLAL reaction curves of the endotoxin standards in PFW after subtraction of the lysate and substrate controls. Straight lines represent the maximum of rapid OD increases.
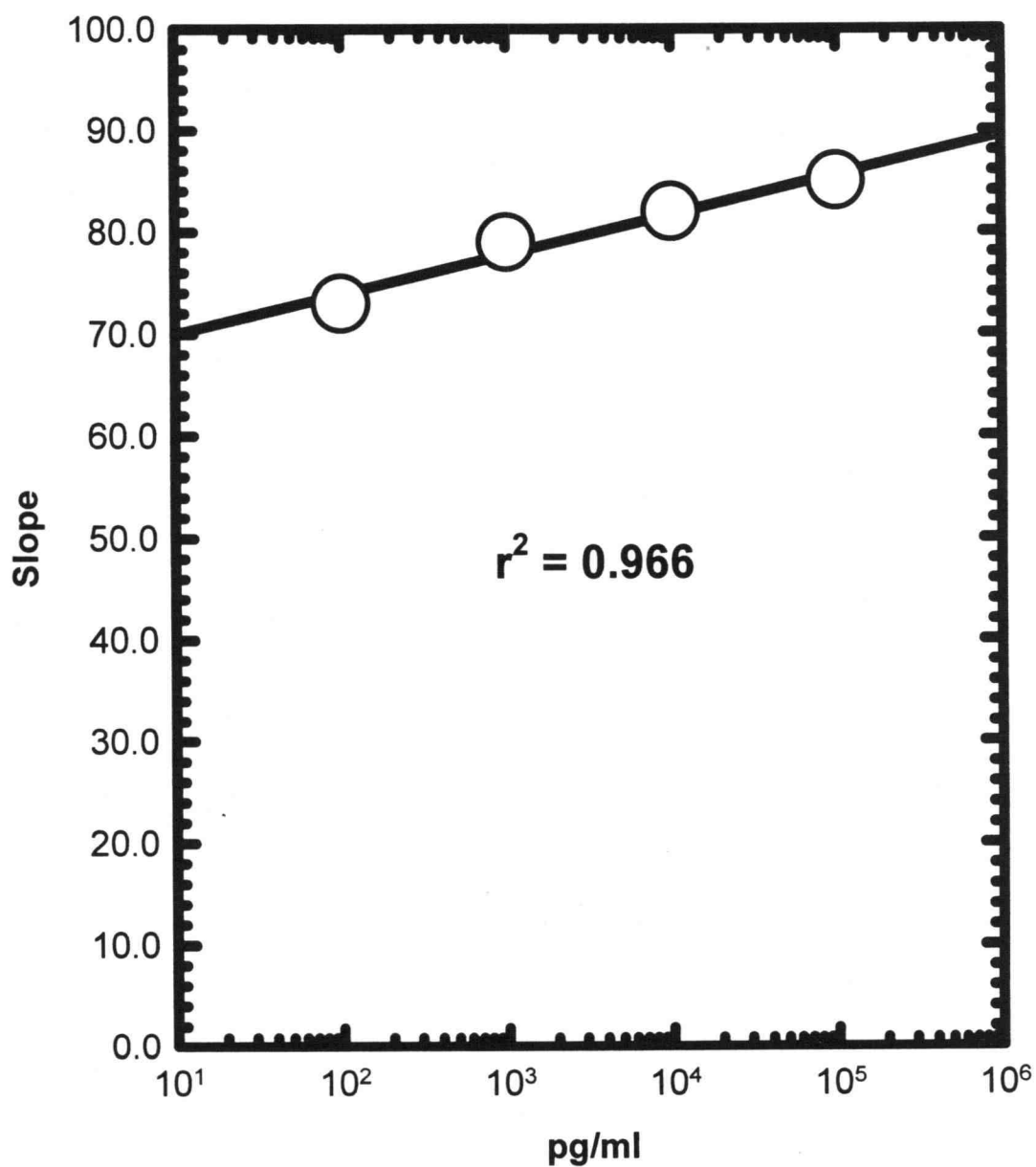
Fig 4.1c.   The slope of the straight lines plotted against $\log_{10}$ concentrations of endotoxin and their linear regression line.

Heat Inactivation

Non-treated plasma samples caused the release of the dye from the substrate in the absence of the lysate, and this reaction was reduced to a negligible level after the heat treatment (100 C, 10 min.; Fig. 4.2). To remove any inhibitory or enhancing factors in plasma, various inactivation temperatures were tested in a subsequent experiment, and the results were summarized in Table 4.1. There was no significant ($P<0.05$) difference between the PFW control and 100 C treatment group. No treatment or 85 C treatment showed significantly ($P<0.05$) lower concentrations, while the 75 C treatment group showed higher concentration.
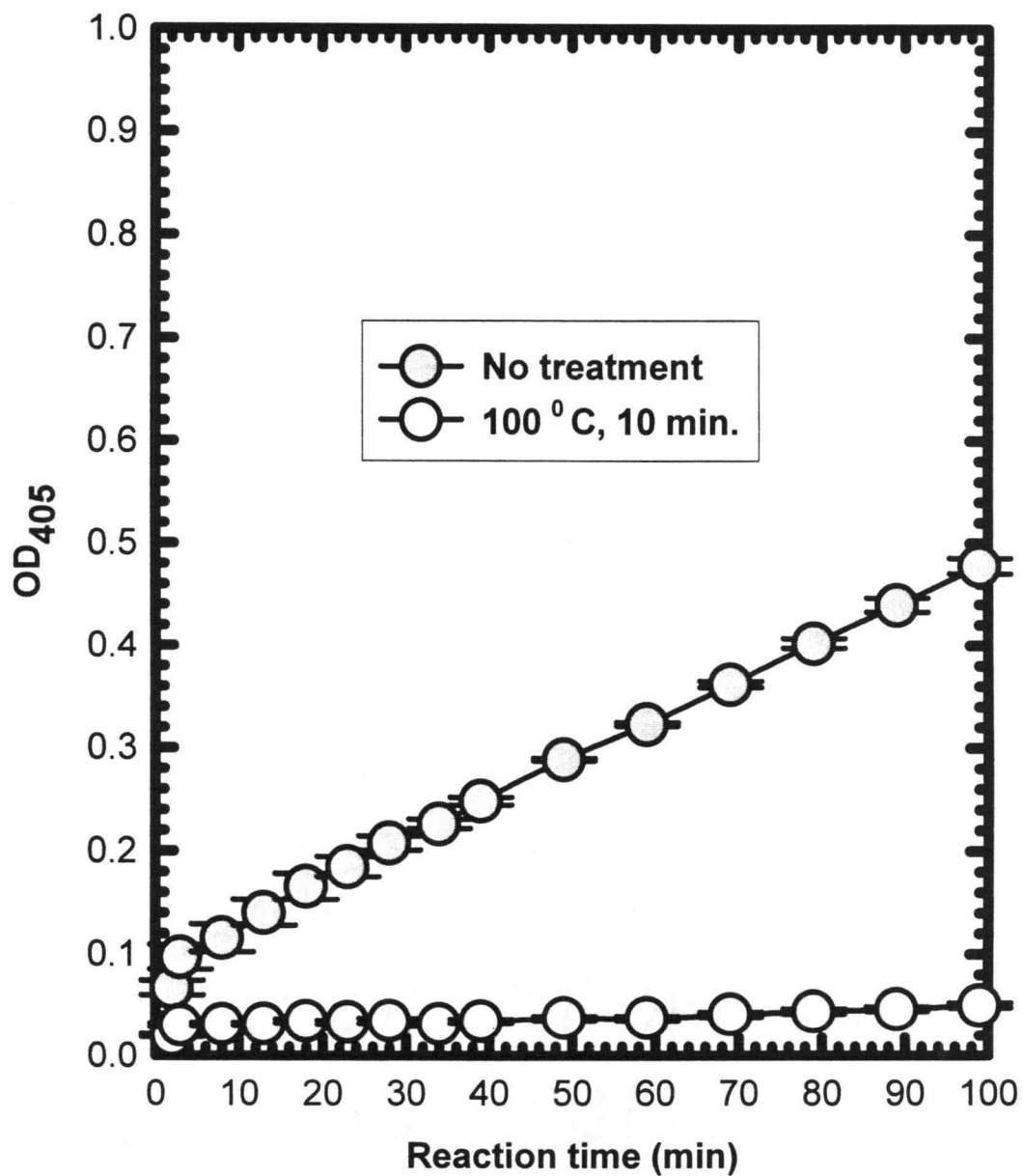
Fig 4.2. Nonspecific reaction between non-treated or heat (100 ^{0}C, 10 min.)-treated chicken plasma previously absorbed for endotoxin.

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Table 4.1. The effect of treatment at various temperatures on the absorbed chicken plasma subsequently reconstituted with endotoxin in comparison with the PFW control.

Sample	Endotoxin added ng/ml	Slope (degree) Mean±S.D.	%
PFW (no treatment)	1.0	77.63±0.48 [a]	100.0
Plasma (no treatment)	1.0	74.88±0.48 [c]	94.5
Plasma-70°C	1.0	79.63±0.63 [d]	102.6
Plasma-85°C	1.0	73.63±1.25 [c]	94.9
Plasma-100°C	1.0	76.50±0.91 [a]	98.6

Non-specific Reaction with the Lysate and Substrate

As shown in fig 3.1a, the PFW control containing the lysate and substrate resulted in releasing the dye in a delayed and slow manner. To make sure that this is not due to a minute amount of endotoxin, PFW was treated with the endotoxin absorption procedure to remove any endotoxin present in the water or substrate solution, but repeated attempts failed to show any notable change in O. D. values compared with nontreated PFW (data not shown).

Nonspecific Reactions with Plasma

When chicken plasma samples were tested in a CLAL test, it became clear that the lysate enzyme systems react with components of plasma, resulting in the formation of white precipitate in the absence of substrate (Fig 4.3). The reaction, however, appeared to be a single step reaction, and produced only a small increase in the O. D. within 40 min. Samples containing heat-treated plasma with the substrate or plasma only caused negligible change in the O. D. values (Fig 3.2). Based on these results, plasma+lysate and plasma+substrate controls were concurrently run for each sample in further tests, and O. D. values were corrected for these controls at each

measurement points. The reasons for not subtracting PFW
control values will be discussed below.
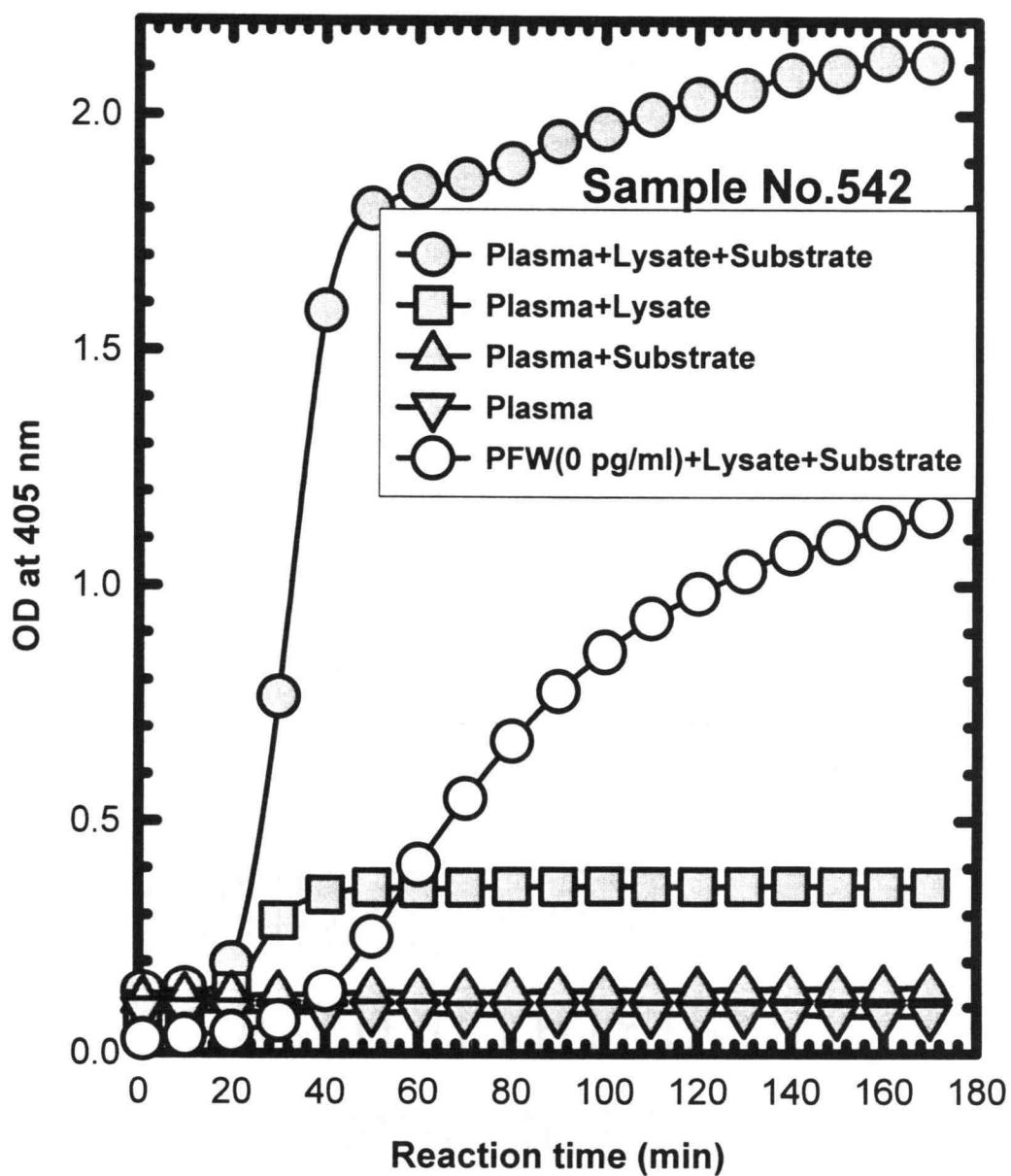
Fig 4.3. CLAL reaction curves of a chicken plasma sample with various controls.

Field Samples

Ten blood samples were obtained from each of the three different commercial broiler farms. Only one bird showed a plasma endotoxin level at 12 ng/ml, while other 29 birds showed less than 100 pg/ml of endotoxin in plasma including 7 bacteremic birds (Table 4.2). No bacteria was detected on MaConkey medium with the blood of the endotoxemic bird.

Table 4.2. The results of endotoxin determination by the CLAL test in correlation with bacterial isolations on MacConkey agar plate with 30 broiler chicken samples.

Location	Sample No.	Bacteria (CFU/ml)	Endotoxin (pg/ml)
Farm A	540	< 10	< 100
(60 days	541	10 (lac⁻)[a]	< 100
old)	542	< 10	12,000
	543	10 (lac⁻)	< 100
	544	< 10	< 100
	545	< 10	< 100
	546	< 10	< 100
	547	70 (lac⁺)	< 100
	548	< 10	< 100
	549	< 10	< 100

Table 4.2 (continued)

Location	Sample No.	Bacteria (CFU/ml)	Endotoxin (pg/ml)
Farm B (38 days old)	732	< 10	< 100
	733	< 10	< 100
	734	< 10	< 100
	735	< 10	< 100
	736	20 (lac$^+$)	< 100
	737	< 10	< 100
	738	20 (lac$^+$)	< 100
	739	20 (lac$^+$)	< 100
	740	< 10	< 100
	741	170 (lac$^-$)	< 100
Farm C (32 days old)	881	< 10	< 100
	882	< 10	< 100
	883	< 10	< 100
	884	< 10	< 100
	885	< 10	< 100
	886	< 10	< 100
	887	< 10	< 100
	888	< 10	< 100
	889	< 10	< 100
	890	< 10	< 100

[a] lac$^-$: lactose-negative; lac$^+$: lactose-positive colonies on MacConkey agar medium.

CLAL Test Kit

A commercially available test kit claims its sensitivity as low as 10 pg. With the endotoxin standard in PFW, the kit demonstrated a linear correlation between O. D. values and endotoxin concentrations down to 1 pg level (data not shown). However, when chicken plasma, which had been preabsorbed for endotoxin and subsequently reconstituted with standard amounts of endotoxin was used, 1 and 10 pg levels failed to show significant (P<0.05) absorbance increase over the blank plasma (Fig 4.4).
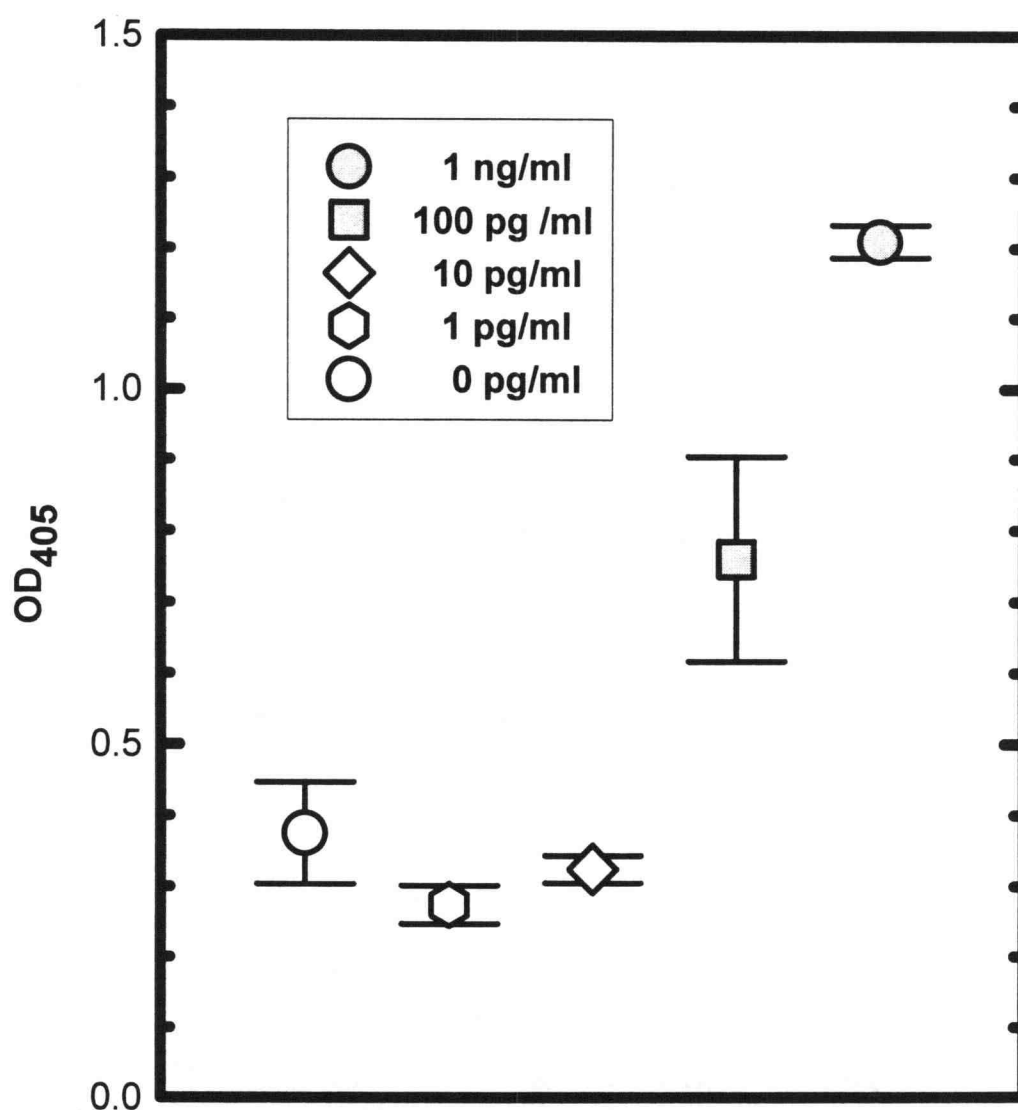
Fig 4.4. Concentrations (mean + s. d. ; n=3) detected by the commercial test kit with various amounts of endotoxin standards reconstituted in chicken plasma previously absorbed for endotoxin. The endpoint method (t=45 min.) was used. Treatment groups without common alphabets (a, b, c) on significantly (p < 0.05) different.

Endotoxin Clearance

One microgram of purified LPS was injected intra-
venously into 20 chickens and endotoxin levels were
determined with blood samples taken within 90 minutes.
The endotoxin level decreased slowly in a linear fashion
although considerable individual variations were observed;
the mean level decreased from 64.0 ng at 2 min. to 50.6 ng
at 90 min.; the rate of clearance was 152 pg/min. (Fig
4.5).

Fig 4.5. Plasma concentration of endotoxin in chickens bled at various times after the intravenous injection of 1.0 ug/ml endotoxin (mean ± s. d.).

Chapter 5
Discussion and Conclusions

Discussion

The chromogenic test is superior to other forms of
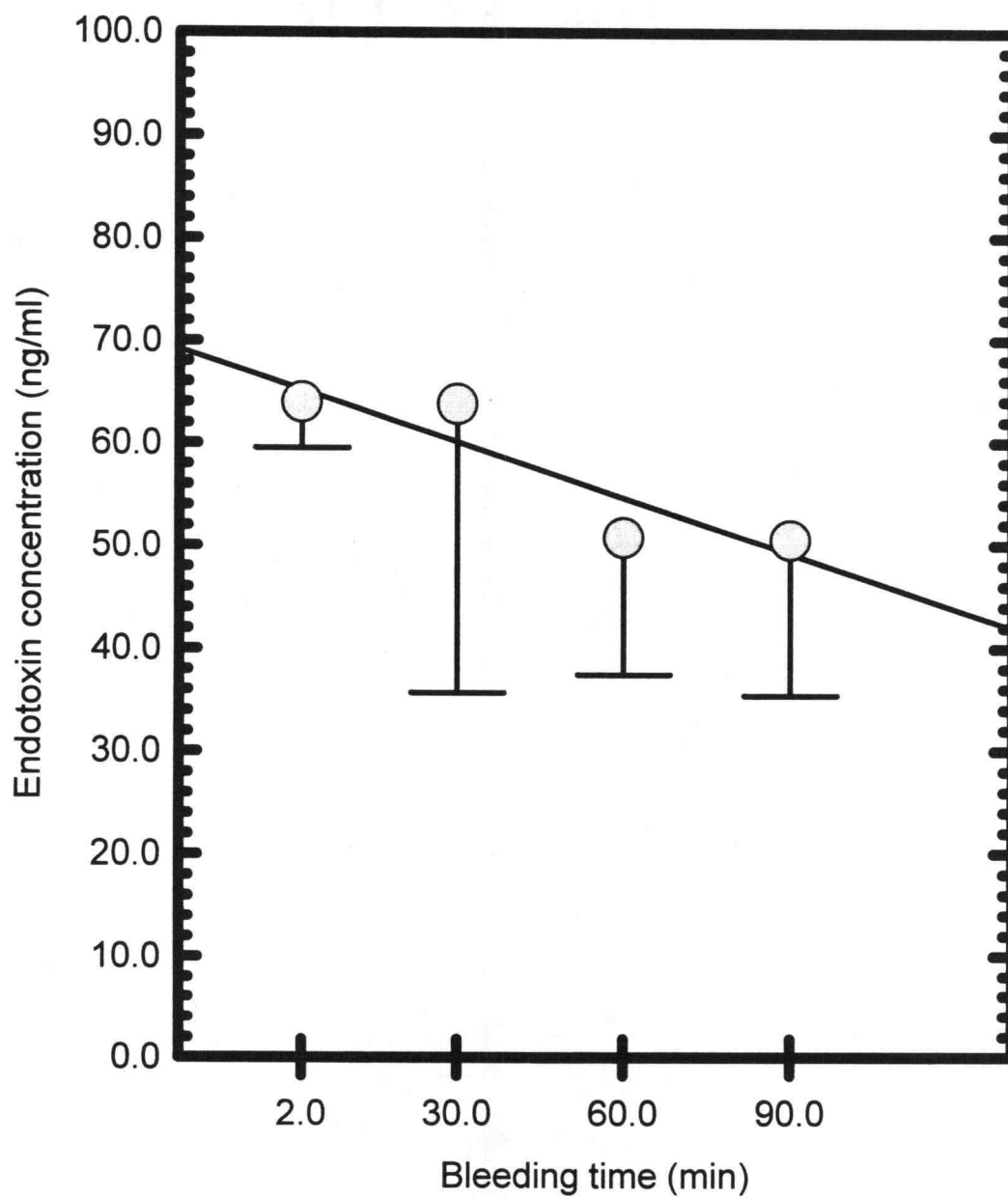LAL test for its high sensitivity and ease in quantitative
measurement (Hurley, 1995). It is indicated in the
present study, however, that concurrent running of proper
controls are essential as interference by nonspecific
factors occurs routinely. The lysate and chicken plasma
were found to form colorless to white precipitate, causing
non-specific increase in OD values. This may be due to
coagulin formation converted from residual coagulogen by
the clotting enzyme (Iwanaga et al., 1985). The
coagulogen is supposed to be blocked for the use in the
CLAL test (Scully et al., 1980). No further attempts,
however, were made to remove coaglulogen in the present
study. The substrate and chicken plasma was initially
found to release the chromophore, but the reaction was
greatly reduced after the heat treatment (Fig 3.2). These
two nonspecific reactions, even though they result in
relatively minor increase in OD values and occurs in a
predictable manner, caused a significant problem in

determining low concentrations of endotoxin in plasma. This is the major reason for limiting sensitivity of the CLAL test in the current form to 100 pg/ml (or 10 pg/ml for 1:10 diluted plasma sample) as these nonspecific reactions are virtually absent in the endotoxin controls in PFW.

Another interfering phenomenon, spontaneous ρ-aniline dye release from the substrate by the lysate in the absence of plasma sample (PFW+lysate+substrate control), was found not due to endotoxin contamination of PFW or substrate. Subtracting this PFW control value from the OD reading of plasma samples seemed reasonable, but, in practice, several factors must be considered. First, as explained above, plasma+lysate and plasma+substrate control consistently cause low degrees of OD increase in comparison to negligible OD increase in correspondent controls in PFW. Second, the onset timing of the rapid OD increase phase are somewhat variable from a plasma sample to another. When the concentration of endotoxin is higher than 100 pg/ml in plasma, the effect of these factors are relatively minor; however, they become major sources of error, when the concentration is below this level.

The spontaneous release of the chromophore by the lysate was not reported in the absence of endotoxin by the authors who first developed the CLAL (Harada et al., 1979). It may be due to some contamination of impure materials in the substrate. However, the commercially available test kit, which supposedly use a different source of the substrate, showed similar degrees of non-specific reactions with PFW (data not shown). The exact cause of this reaction should be investigated in the future.

The progress of the LAL assay can be monitored in two ways, using the endpoint or kinetic methodology. With the endpoint method, the detection range is limited, but measurements and calculations are simple and straight forward; it usually takes a short period of time. The plasma+lysate and plasma+substrate controls should be concurrently run even though these control are included in each test, when the onset timing for the rapid OD increase, or, more generally, when the kinetic pattern of plasma sample differs from that of PFW and two plasma controls, subtracting these control values from the plasma OD value at a fixed reaction time leads to erroneous

results. This is a serious shortcoming of the endpoint method. Hence, one must be careful in interpreting some results run by the endpoint method. The kinetic method, on the other hand, uses the rate of reaction for calculating the endotoxin concentration; it is free of the onset timing variations. In addition, one can obtain exact kinetic patterns for controls.

A drawback of the kinetic method is to find an accurate method of the rate determinations. In the present study, efforts were made to evaluate different procedures to calculate the exact slope of the OD increase in the sigmoidal curve. Conversion of the sigmoidal curve to the log-log or log-logit scale produced straight lines (Bottoms et al., 1991). Such conversion, however, was found to be another source of error, especially when one tries to convert some plotted results that deviate from an atypical sigmoidal curve; a typical example is in a result where 0 and 100% does not form a straight line. Subtracting controls also frequently resulted in producing an atypical shape of sigmoidal curve. Therefore, at this developing stage, it is concluded that manual drawing of a straight line at the phase of the rapid OD increase

prevents gross errors in the calculations. Future efforts, however, should be made to find a reasonable calculation program.

Some commercial determination kits including one tested here are inadequate for the use, at least, with the chicken plasma. As explained above, the use of endpoint method may lead to erroneous results. More seriously, the lysate and substrate are premixed, and, hence, it is impossible to run the lysate or substrate control separately. As the plasma causes nonspecific reactions, which are different from nonspecific reactions with PFW, the mere presence of the PFW control is not sufficient. One must examine absorbance values generated by the plasma+lysate and plasma+substrate control, or, alternatively, run a control containing a plasma preabsorbed for endotoxin for each sample. It should be mentioned, however, that these potential error factors are important only in the low range (<100 pg/ml) of endotoxin.

. Absorption of endotoxin by the commercially available resin appeared to be very efficient. The manufacturer specifies that it can absorb as much as 1 µg of endotoxin. While it was not systematically investigated, absorbed

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plasma or PFW did not show any detectable endotoxin in the present study. The enzymatic activity of the lysate, however, was greatly reduced after the absorption procedure.

Published papers indicated the inhibitory effect of heparin, plastics or storage without inactivation process as reviewed by Hurley (1995). No systematic investigations were made in the present study to make sure such inhibition does not occur. However, circumstantial evidences indicated that an overt difference was not noticed between sterile heparin and "endotoxin-free" heparin, and that plasma endotoxin remained stable at - 70 C for at least 3 months polystyrene microtubes.

The results of this study indicate that blood endotoxin level is lower than 100 pg/ml in normal broiler chicken. The level seems comparable with other mammalian species such as human (<10 pg/ml; Hurley, 1995), dogs (<25 pg/ml; Bottoms, et al., 1991), and horses (<2 pg/ml; Henry & Moore, 1991). Endotoxemia was detected only in one chicken with a high endotoxin level (12 ng/ml). In human patients with systemic meningococcal infection, the blood endotoxin level as high as 170 ng/ml was reported

(Brandtzaeg et al., 1989). The chicken with endotoxemia did not have gram-negative bacteremia. Hurley (1994) indicated that endotoxemia is detected in approximately half or less of those with gram-negative bacteremia, and similarly, gram-negative bacteremia is detected in approximately half of those with endotoxemia in human patients.

While this is the attempt to determine endotoxin levels in chickens, more efforts are definitely needed in performing the CLAL test to decrease nonspecific reactions and to increase sensitivity and reproducibility.

Conclusions

1. Attempts were made to determine endotoxin levels in broiler chickens by the chromogenic test with amebocyte lysate orginated from *Limuls polyphemus*.

2. The kinetic method was found superior to the end-point method, because; 1) free of the onset timing of the reaction; 2) wider range of detection; 3) higher reliability by examining kinetic patterns of samples with those of controls for a period of time instead of measuring OD values at a single point of time in the end-

point method. The kinetic method, however, is time-consuming for both measurements and calculation.

3. Various nonspecific reactions were found in the test. There was a spontaneous release of the chromophore from the substrate by the lysate itself. The reaction was delayed with the average onset time of approximately 30 minutes, slowly reaching to the peak OD values around at 120 minutes. The chicken plasma released the chromophore from the substrate in the absence of the lysate, but heating at 100 C greatly reduced this reaction. The plasma and lysate without the substrate formed colorless precipitates by a single step reaction manner.

4. Heating at 100 C for 10 min. was more efficient than 70 or 85 C to inactivate plasma substances that inhibit or enhance the endotoxin determination.

5. The sensitivity of the test was defined as 100 pg/ml of chicken plasma, considering nonspecific reactions mentioned above. Intra-assay variation was 1.2%, while interassay variation was 18.8% with 1 ng endotoxin standard.

6. The level of endotoxin was determined with 30 plasma samples from broiler chickens obtained from local farms.

Twenty-nine samples had lower than 100 pg/ml, while one sample showed 12 ng/ml endotoxemia. No relationship was observed between bacteremia and endotoxemia with 100 pg/ml sensitivity of the test.

7. One microgram endotoxin was intravenously injected into broiler chickens raised in the isolation. The endotoxin clearance rate in the blood was 152 pg/min.

8. Absorption of endotoxin by the commercially available resin appeared to be very efficient. The manufacturer specifies that it can absorb as much as 1 μg of endotoxin. While it was not systematically investigated, absorbed plasma or PFW did not show any detectable endotoxin in the present study. The enzymatic activity of the lysate, however, was greatly reduced after the absorption procedure.

9. The results of this study indicate that blood endotoxin level is lower than 100 pg/ml in normal broiler chicken. The level seems comparable with other mammalian species such as human (<10 pg/ml; Hurley, 1995), dogs (<25 pg/ml; Bottoms, et al., 1991), and horses (<2 pg/ml; Henry & Moore, 1991). Endotoxemia was detected only in one chicken with a high endotoxin level (12 ng/ml). In human

patients with systemic meningococcal infection, the blood endotoxin level as high as 170 ng/ml was reported (Brandtzaeg et al., 1989). The chicken with endotoxemia did not have gram-negative bacteremia. Hurley (1994) indicated that endotoxemia is detected in approximately half or less of those with gram-negative bacteremia, and similarly, gram-negative bacteremia is detected in approximately half of those with endotoxemia in human patients. While this is a attempt to determine endotoxin levels in chickens, more efforts are definitely needed in performing the CLAL test to decrease nonspecific reactions and to increase sensitivity and reproducibility.

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