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Triglyceride-Rich Lipoproteins Can Inhibit the Detection
of Bacterial Endotoxin *In Vitro*

by

Eldan B. Eichbaum

A Thesis

Submitted in partial satisfaction of the
requirements for the M.D. with Thesis Program

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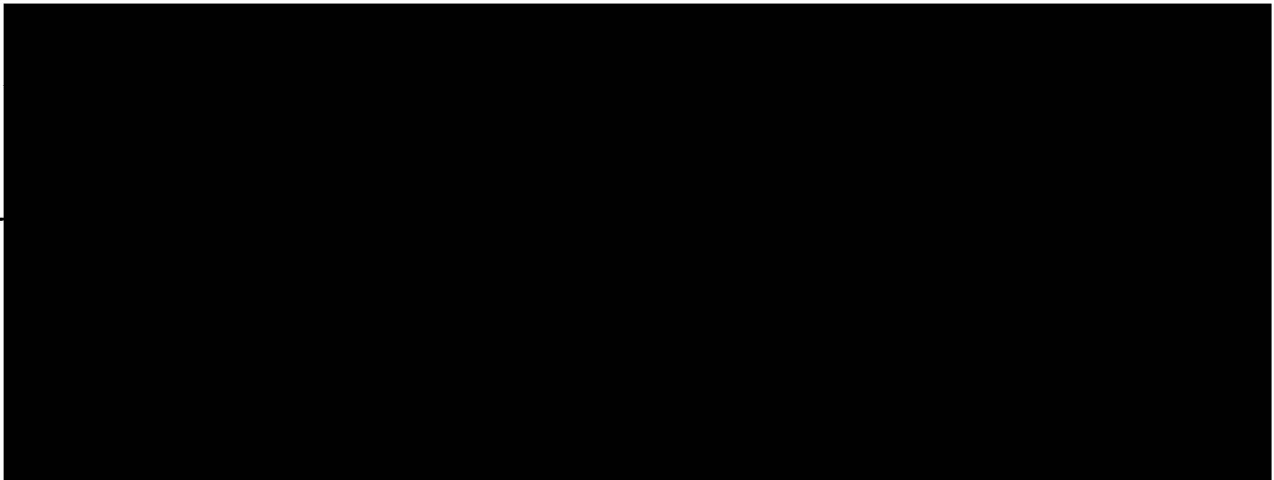

Triglyceride-Rich Lipoproteins Can Inhibit the Detection of Bacterial Endotoxin *In Vitro*

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Endotoxemia causes a variety of pathophysiologic responses including fever, leukocytosis, and disturbances in lipid metabolism. The most dramatic of these disturbances in lipid metabolism is an increase in circulating plasma triglyceride-rich lipoproteins. This hypertriglyceridemia may be a defensive mechanism against endotoxemia, as triglyceride-rich lipoproteins may bind endotoxin and facilitate detoxification. Although it is well established that whole blood and serum can inhibit the detection of endotoxin, the specific participants as well as the mechanism of this inhibition is controversial. This study used a chromogenic Limulus amebocyte assay to demonstrate the ability of triglyceride-rich lipoproteins to inhibit the detection of bacterial endotoxin *in vitro*. Human chylomicrons and very low density lipoproteins were isolated via sequential ultracentrifugation using sterile technique and apyrogenic glassware. Soyacal, a commercially available triglyceride-rich synthetic lipid emulsion, was also obtained. These triglyceride-rich particles were mixed with increasing concentrations of *E. coli* (055:B5) endotoxin, 10% lipoprotein-deficient plasma, and phosphate buffered saline, placed in a 37°C shaking water bath and incubated for four hours. The Limulus assay was then employed to detect the presence of endotoxin in the test mixtures. The results revealed that all three triglyceride-rich particles (chylomicrons 1.0 mg triglyceride; very low density lipoproteins 1.0 mg triglyceride; and Soyacal 2.5 mg triglyceride) significantly increased (10-100x) the ability of 10% lipoprotein-deficient plasma to inhibit the detection of bacterial endotoxin. These data demonstrate the isolated ability of triglyceride-rich lipoproteins, and the synthetic lipid emulsion, to inhibit the detection of bacterial endotoxin *in vitro*.

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Preface

The following work resulted in two publications --- in press *Journal of Laboratory and Clinical Medicine,* and in press *Journal of Surgical Research.* The thesis presented is a compilation of these data.

Acknowledgements

I would like to thank Judy Tweedie and Roland Tung for their ever present technical advice and company. To Hobart W. Harris, M.D., and Joseph Rapp, M.D., whom I cannot thank enough for their constant assistance and support beginning the day I entered the laboratory.

Introduction

Bacterial endotoxin, or Lipopolysaccharide (LPS), is an essential component of the cell wall of gram-negative bacteria. Lipopolysaccharides of all gram negative bacteria conform to a common structural principal: they consist of a hydrophillic heteropolysaccharide component covalently linked to a hydrophobic lipid portion, termed Lipid A (1). The polysaccharide portion is separated into two subcomponents: the O-specific chain, which carries the main serologic specificity, linked to the core polysaccharide, which is common to groups of bacteria (1, 2, 3). It is widely accepted that the Lipid A region contains the endotoxic properties of Lipopolysaccharides (1-2, 4). Endotoxemia causes the variety of pathophysiologic responses including fever (5), leukocytosis (5), hypotension (6), left ventricular dysfunction (7), induction of acute phase reactants (4), and disturbances in lipid metabolism (8, 9, 10). The earliest and most dramatic of these disturbances in lipid metabolism is an increase in circulating plasma triglyceride, mainly in the form of very low density lipoproteins (VLDL) (11, 12). This increase in circulating VLDL has generally been thought to be the mobilization of fat stores to fuel the body's response to the infectious challenge. However, an alternative hypothesis is that this increase not only involves fat mobilization, but may serves as a defensive mechanism against endotoxemia (13, 14).

Presently, the most commonly employed assay used for detecting the presence of bacterial endotoxin in biological fluids and preparations for parenteral administration is the Limulus amebocyte gelation assay. In the past, the methods used for detection of the presence of bacterial endotoxin has ranged from rabbit pyrogenicity (15), to mice lethality (16), to the Limulus

amebocyte gelation assay (17-18). In general, rabbit pyrogenicity and mice lethality have primarily been used to test for gross contamination with bacterial LPS. However, these methods are not only poor indicators of the quantity of LPS present, but they are also inconvenient. The discovery that the blood of *Limulus polyphemus*, the horshoe crab, gels in the presence of bacterial endotoxin allowed for the use of a relatively sensitive and very convenient assay (19-20). The blood of the Lumulus horshoe crab is easily attainable and has only one type of circulating cell, the amebocyte. Several studies have demonstrated that lysis of the amebocyte is the required for gelation to occur (18-20). It is the biologically active Lipid A region which reacts with this amebocyte lysate in two stages: a proenzyme present in the amebocyte is activated by Lipid A and this activated proenzyme then cleaves peptide bonds in a coagulant protein causing visible gelation (20). This temperature (37°C) and pH (7.5) dependent process is very similar to human blood platelet coagulation (19). Even though the Limulus amebocyte gelation assay affords quantification of LPS in the nanogram range, recent investigators have demonstrated that an increase in assay sensitivity to the picogram range can be achieved by using a chromogenic substrate to bypass the gelation reaction and thus measure the activated proenzyme directly (21).

The ability of whole blood and serum/plasma to inactivate bacterial lipopolysaccharide has been well established (22). Suggested constituents of serum that inactivate LPS include an alpha-globulin protein with (23) and without esterase activity, complement C4, C5, C6, (24) as well as high density lipoproteins (HDL) (24). Consequently, these serum proteins and lipid particles have the ability to inhibit the *in vitro* detection of bacterial endotoxin by the Limulus gelation assay. Ulevitch and his laboratory have extensively studied this interaction between bacterial LPS and cholesterol

ester-rich plasma lipoproteins (HDL). Their studies have demonstrated that the addition of bacterial LPS to serum/plasma, whether *in vitro* or *in vivo,* not only results in a marked reduction of the hydrated buoyant density of the parent LPS molecule (from $d = 1.44\ g/cm^3$ to $d < 1.2\ g/cm^3$), but also results in a close association between the LPS and plasma HDL (23-26). This reduction in hydrated buoyant density of LPS, and its interaction with plasma HDL occurs only in the presence of both lipid deficient plasma and the cholesterol ester-rich lipoproteins (25). Once the LPS-HDL complex is formed, several modifications of the parent lipopolysaccharide's biologic properties occur. These include a markedly decreased ability to activate complement (by 100 fold), a markedly reduced ability to produce pyrogenicity in rabbits; and to a lesser degree, a decrease in mitogenic stimulation in murine B cells, macrophage activation, and ability to produce hypotension, disseminated intravascular coagulation and death in experimental animals (13). This process of inactivation is termed *detoxification*.

Although most studies have centered on the cholesterol ester-rich HDL, the interaction between bacterial endotoxin and the other lipoproteins has yet to be fully examined. As previously stated, one of the earliest and most dramatic disturbances in lipid metabolism, secondary to endotoxemia, is a rise in circulating triglyceride-rich VLDL. Intuitively, it would seem plausible to assume that one of the explanations leading to this hypertriglyceridemia is the body's attempt to thwart the lipopolysaccharides's toxic sequelae (14). Perhaps the triglyceride-rich lipoproteins are released into the vascular space in an effort to "bind" the LPS and *detoxify* it. It is the purpose of this study to examine the interaction of triglyceride-rich lipid particles (i.e., chylomicrons, VLDL, and the commercial lipid emulsion Soyacal), and bacterial lipoplysaccharide *in vitro* -- can triglyceride-rich lipid

particles inhibit the detection of bacterial endotoxin *in vitro*, using a chromogenic modification of the Limulus assay?

Methods

Sample Collection. Six healthy, normolipidemic young men contributed four fasting and two postprandial venous blood samples. These samples were drawn from vigorously cleansed antecubital fossae into sterile plastic syringes containing endotoxin-free heparin (Sigma Chemical Co., St. Louis, MO.), producing a final concentration of 10 IU/ml, and immediately placed on ice. Blood was placed in depyrogenated glass tubes and the plasma separated by centrifugation at 2000 *g* for 15 minutes. Plasma was stored at $4^{o}C$ until analyzed.

Lipoproteins. Rigorous precautions were taken to avoid exogenous contamination of the lipoproteins with endotoxin. All glassware and materials used in the processing and assay of the lipoproteins were rendered sterile and endotoxin-free by steam autoclaving followed by dry heating at $180^{o}C$ for a minimum of 4 hours (22, 27). The isolation of endotoxin-free lipoproteins included the use of depyrogenated stainless steel ultracentrifuge tubes (Beckman Instrument Co., Palo Alto, CA) with custom-crafted silicone O-rings. To remove any adherent endotoxin, the dialysis tubing (Spectrapor 3, Spectrum Medical Industries, Inc., Los Angeles, CA) was autoclaved in 3% H_2O_2, rinsed, and then stored in apyrogenic saline at $4^{o}C$ (28). For all

experiments described the lipoprotein fractions were used within 7 days of their isolation.

Lipoproteins were isolated from normal human plasma by ultracentrifugation (29). Briefly, very low density lipoproteins (VLDL) were isolated from fasting, normolipidemic plasma by ultracentrifugation at plasma density (d = 1.006 g/cm^3) for 18 hours, 12°C, at 36,000 rpm in a Beckman L5-50 ultracentrifuge (Beckman Instruments, Palo Alto, CA) using a Beckman 40.3 or a Sorval 50.38 Ti rotor (DuPont Co., Wilmington, DE). Chylomicrons were initially isolated from postprandial plasma by ultracentrifugation at plasma density for 30 minutes, 12°C, at 20,000 rpm using a 40.3 rotor and then washed by flotation through 1-2 volumes of endotoxin-free 50 mM phosphate buffered saline (PBS, pH = 7.4). Lipoprotein-deficient plasma was isolated from a d = 1.21 g/cm3 separation of the volunteers' pooled plasma, reconstituted with PBS to its original volume, and then stored in 3 ml aliquots at -70°C until its use. Lipoprotein-deficient plasma was exhaustively dialyzed against sterile, apyrogenic 0.9% NaCl. The triglyceride and cholesterol content of lipoproteins was determined using standard enzymatic assays (Sigma Chemical Co., St. Louis, MO and Wako Chemicals USA, Inc., Dallas, TX).

Endotoxin. *Escherichia coli*, (055:B5) endotoxin purchased from Difco Laboratories, Detroit, MI, was reconstituted with sterile, apyrogenic water to the concentration of 1 mg/ml, and stored in 1 ml aliquots at -70°C until its use. This preparation of endotoxin had a specific activity of approximately 15 endotoxin units/ng (USP reference endotoxin).

Chromogenic Limulus Assay. To assess the ability of lipoproteins to inhibit endotoxin activity in vitro, a chromogenic modification of the Limulus assay was used (21). Individual lipoprotein classes suspended in lipoprotein-deficient plasma (LPDP) were incubated at 37°C for 4 hours with increasing concentrations of endotoxin and then assayed for detectable endotoxin activity. As a control, the inhibition of endotoxin activity by lipoproteins plus plasma was reversed in a parallel series of samples by a standard dilution and heating technique (21). Specifically, following the four hour incubation at 37°C, all lipoprotein-endotoxin-LPDP mixtures were then diluted 1:9 (vol:vol) with endotoxin-free PBS, and one-half of the samples were heated at 75°C for 5 minutes. These heated samples served as controls, whereas the experimental samples were only diluted and not heated so as to retain their inhibitory property. The diluted test samples (0.1 ml) were added to 0.1 ml Limulus lysate (Sigma), reconstituted to twenty fold less than the manufacturer's recommended concentration (secondary to very high costs) with endotoxin-free PBS and incubated at 37°C for 60 min. Thereafter 0.4 ml of 0.5 mM chromogenic substrate S-2222 (Helena Laboratories, Beaumont, TX), reconstituted using endotoxin-free H_2O, was added, and the mixture incubated at 37°C for an additional 30 minutes before the reaction was quenched by the addition of 0.4 ml 60% acetic acid. Absorption of the final mixture was measured at 405 nm using a Cary Model 219 spectrophotometer and distilled H_2O as a zero. Lipoproteins and lipid emulsions have an intrinsically higher optical density than saline controls. Hence, to record the optical density of endotoxin alone and enable comparisons between saline samples versus lipoprotein/lipid emulsion samples at equal endotoxin concentrations, the following maneuver was performed. The mean optical density of the saline sample with 0 pg/ml of endotoxin was subtracted from

the mean optical density of each respective lipoprotein/lipid emulsion sample at 0 pg/ml of endotoxin. This difference was then subtracted from the optical densities throughout the range of endotoxin concentrations tested for in each respective lipoprotein/lipid emulsion. VLDL was assayed at a single concentration due to the difficulty of obtaining samples minimally contaminated with endotoxin. Despite stringent apyrogenic technique, ultracentrifugally-isolated human VLDL is frequently contaminated with endotoxin, presumably of an endogenous origin (14). Conversely, chylomicrons were more readily isolated free of detectable endotoxin, thereby allowing for testing at various concentrations.

To quantitate the percent recovery of endotoxin after the standard dilution and heating technique, we performed a series of experiments with radioiodonated endotoxin prepared by the method of Ulevitch (30). Briefly, the endotoxin was first derivatized by reacting *p*-OH methylbenzimidate at alkaline pH, and then radiolabeled with $Na^{125}I$. Various concentrations of 125I-labeled endotoxin (100-10,000 pg/ml, specific activity = 2.65 µCi/µmg) were incubated with endotoxin-free VLDL (approximately 1.0 mg TG/ml) and 50% lipoprotein-deficient plasma at 37°C for 3 hours in a shaking water bath. To determine the percent recovery of endotoxin we compared the radioactivity of the incubated mixtures by gamma scintillation spectrometry before and after the mixtures were diluted and heated.

Establishing Parameters for the Chromogenic Modification of the Limulus Assay for Lipoprotein Containing Solutions. Past studies have demonstrated that using the chromogenic modified Limulus assay over the standard Limulus gelation assay can increase sensitivity in detection of endotoxin from the nanogram to picogram range. Even though this

detection of picogram levels of endotoxin is possible, to actually attain consistent results in lipoprotein containing solutions is unpredictable and very difficult. Therefore it was essential to establish parameters for using the chromogenic modified Limulus assay in these samples. Multiple manipulations of the sample constituents (endotoxin, lipoproteins, lipoprotein-deficient plasma, and phosphate-buffered saline), the Limulus reagents/hardware (lysate, chromogen, glassware, steel ultracentrifuge tubes), as well as other variables (incubation time of the samples) were performed.

Initially, samples of increasing concentrations of LPS suspended in saline were tested by the manufacture's suggested concentrations of Limulus lysate and chromogen. This highly concentrated lysate solution was very sensitive to very low levels of LPS, but if used at this concentration the cost of adequate experimentation would have been extraordinarily high. Therefore, sequential dilutions of the lysate revealed that equally sensitive and reproducible results were obtainable with 20 fold less than the manufacture's recommended concentration.

Following trials at various volumes, a total sample volume of 1.0 ml was set. This final 1.0 ml sample consisted of 0.1 ml of endotoxin-lipoprotein-LPDP-PBS mixture, 0.1 ml of Limulus lysate, 0.4 ml of chromogen, and 0.4 ml of acetic acid.

Once lipoprotein and Soyacal triglyceride contents were measured, the concentration of lipoprotein/Soyacal in the 0.10 ml portion was sequentially diluted down to the physiologic concentrations. This dilution was first performed with endotoxin-free saline, but subsequently changed to endotoxin-free phosphate-buffered saline in order to optimize the sensitivity of the Limulus assay. Increasing concentrations of endotoxin were then added to the above samples and assayed for activity. Ten percent lipoprotein-

deficient plasma was added to the lipoprotein-endotoxin-PBS mixtures to facilitate lipoprotein-endotoxin interaction. This final combination achieved consistent and reproducible results, as long as strict sterile/apyrogenic techniques were maintained.

Reagents and Solutions. HPLC grade methanol and chloroform; reagent grade o-H_3PO_4, KBr, glacial acetic acid (Fisher Chemical Co., Fair Lawn, NJ) and NaOH (J.T. Baker Chemical Co., Phillipsburg, NJ); apyrogenic, preservative-free 0.9% NaCl (Kendall McGraw Labs, Inc., Irvine, CA) and H_20 (Elkins-Sinn, Inc., Cherry Hill, NJ); SOYACAL 20% intravenous fat emulsion, containing 20% soybean oil (triglycerides), 1.2% egg yolk phospholipid, and 2.21% glycerin, (Alpha Therapeutic Corp., Los Angeles, CA); were used as indicated in Methods.

Statistical Analysis. Each test sample was assayed in quintiplicate. Comparisons were assessed by paired t-test. Statistical significance was assigned at a $p \leq 0.05$.

Results

The mean recovery of radioloabeled endotoxin added to the incubation mixtures following the standard dilution and heating technique used in these experiments was 100 ± 8 % (± S.E.M.). The chromogenic Limulus assay was

linear over a range of 5-100 pg endotoxin/ml saline producing a maximal optical density of approximately 2.0 (Figure 1).

Initially it was very difficult to perform these experiments as several variables contributed to inconsistencies in the chromogenic Limulus assay. These variables included: the concentration of triglyceride-rich particles, the concentration of the Limulus reagents (i.e., lysate and chromogen), the concentration of the lipoprotein-deficient plasma, the amount of dilutional volume required in each sample, as well as the optimal incubation period. For all these reasons, the special parameters (described in methods) were set. For example, the dilutions were performed with endotoxin-free PBS versus saline so as to increase the pH of saline to approximately 7.4. The chromogenic Limulus assay has been shown to be both temperature and pH dependent (20). Also, the Limulus lysate was diluted to 20 fold less than the manufacture's recommendation as this gave equally sensitive as well as reproducible results at a fraction of the cost.

It was of extreme importance to strictly abide by sterile/apyrogenic technique in performing the assays. Bacterial endotoxin is ubiquitous and contamination of the sample, and subsequent false positive assay results, could occur at any step in the procedure--from the initial blood sample collection, to the handling of the centrifuged plasma, to the use of all glassware and reagents. Only when these techniques and steps had been mastered, could true testing proceed.

Varying incubations times were tested to examine the shortest time required for optimal inhibition to occur. Sequential decreases in incubation times demonstrated that four hours was the shortest period that allowed for maximal inhibiton.

To study the capacity of lipoproteins to inhibit endotoxin activity *in vitro* I initially examined VLDL, chylomicrons, and the synthetic lipid emulsion (Soyacal) alone (suspended in endotoxin-free PBS without any lipoprotein-free plasma present). VLDL, chylomicrons, and the lipid emulsion demonstrated no capacity to inhibit endotoxin activity when incubated with increasing concentrations of endotoxin in saline (data not shown). Over the range of endotoxin concentrations tested, approximately equivalent quantities of endotoxin activity were detected in samples containing PBS plus lipoproteins or the synthetic lipid emulsion as compared to those containing PBS alone.

Since lipoproteins or Soyacal alone in PBS demonstrated no significant inhibition of endotoxin activity, we then added lipoprotein-deficient plasma to the test mixtures. We were interested in examining the capacity of just lipid particles to inhibit endotoxin activity, but our results stated in the previous paragraph plus past studies (13, 24-26) revealed that lipoprotein-deficient plasma is required for lipoproteins to interact with endotoxin. In fact, lipoproteins are never isolated *in vivo*. However, lipoprotein-free plasma can effectively inhibit the *in vitro* detection of endotoxin by the Limulus assay. After examining increasing concentrations of lipoprotein-deficient plasma, ten percent was determined to be the lowest concentration plasma that facilitated the interaction between endotoxin and lipoproteins yet readily revealed nanogram quantities of endotoxin by *in vitro* assay (Figure 2). This semi-log figure demonstrates that detection of increasing log concentrations of endotoxin is increasingly less sensitive in 20 and 50% lipoprotein-deficient plasma. In the presence of 50% lipoprotein-free plasma the Limulus test failed to detect up to 100 ng (or 10,000 pg) of added endotoxin/ml. Only in the case of 10% lipoprotein-deficient plasma was the

inhibitory capacity of plasma overcome, allowing for the detection of nanograms of added endotoxin. Therefore, to distinguish the capacity of lipoproteins to inhibit endotoxin activity from that of lipoprotein-deficient plasma, we kept the concentration of plasma in our control and experimental mixtures constant and at a minimum (10%).

Once these baseline variables for the incubation of lipoproteins with endotoxin were established, a first set of experiments examined the ability of each lipoprotein class and the synthetic lipid emulsion together with 10% lipoprotein-deficient plasma to inhibit the detection of endotoxin (Figure 3). The inhibitory capacity of 10% lipoprotein-deficient plasma was significantly increased by the addition of VLDL, chylomicrons, and the synthetic lipid emulsion. When triglyceride-rich lipoproteins, or the synthetic lipid emulsion were suspended in 10% lipoprotein-deficient plasma, the curve representing detectable endotoxin activity in 10% lipoprotein-deficient plasma was shifted to the right. Quantitatively, the addition of triglyceride-rich lipid particles increased the inhibitory capacity of 10% lipoprotein-deficient plasma at least 10 to 100-fold. The inhibitory capacity of samples containing VLDL, chylomicrons, and the synthetic lipid emulsion was exceeded at an endotoxin concentration between 1,000 and 10,000 pg/ml (chylomicrons 1.0 mg triglyceride, $p < 0.0001$; VLDL 1.0 mg triglyceride, $p < 0.0001$; Soyacal 2.5 mg triglyceride, $p < 0.0001$).

In a second set of experiments, we examined the ability of increasing concentrations of chylomicrons plus 10% lipoprotein-deficient plasma to inhibit endotoxin activity (Figure 4). As the concentration of these large, triglyceride-rich lipid particles increased, endotoxin activity was inhibited in a dose-dependent fashion.

Discussion

The interaction between bacterial endotoxin and human blood (plasma/serum) is a complex interaction yet to be fully understood. The results presented demonstrate that triglyceride-rich lipid particles can inhibit the *in vitro* detection of bacterial endotoxin by the modified chromogenic Limulus assay. Both triglyceride-rich lipoproteins, chylomicrons (1 mg triglyceride) and very low density lipoproteins (1 mg triglyceride), in the presence of ten percent lipoprotein-deficient plasma were able to inhibit the detection of up to 10,000 pg endotoxin/ml. The same was true for the synthetic lipid emulsion (Soyacal, 2.5 mg triglyceride), a commercial lipid emulsion used for parenteral fat feeding. This is a significant amount as the chromogenic Limulus assay has been shown to be able to detect between 1 pg endotoxin/ml (31) and 10 pg endotoxin/ml (20). Four ng endotoxin/kg has been shown to elicit fever, leukocytosis, tachycardia, elevated cardiac output, and elevated levels of catecholamines and tumor necrosis factor in the plasma of healthy volunteer (7, 32). Normal fasting plasma triglyceride content ranges between 0.40 mg TG/ml to 1.0 mg TG/ml, and in some cases up to 2.0 mg TG/ml. Therefore, each mililiter of plasma could theoretically conceal 10 ng of endotoxin by triglyceride content alone. These data reveal that the triglyceride-rich lipoproteins, as well as the synthetic lipid emulsion, can decrease the detection of endotoxin by at least 10-100 fold. Although other published reports have demonstrated that the cholesterol ester-rich lipoproteins, high density lipoproteins (HDL) and low density lipoproteins

(LDL), significantly interact with bacterial endotoxin, they have been unable to demonstrate the same effect (if any) with the triglyceride-rich lipoproteins (13, 24-26, 33). They have postulated that the ability of lipoproteins to interact with endotoxin is directly proportional its cholesterol content. Since triglyceride-rich VLDL and chylomicrons have a low concentration of cholesterol, they concluded that VLDL and chylomicrons could not interact with endotoxin. Contrary to the above theory, not only can VLDL and chylomicrons, but the synthetic lipid emulsion, composed of only triglycerides and phospholipids (no cholesterol), can also interact with endotoxin. This study is one of the first to demonstrate this significant interaction between triglyceride-rich lipoproteins and/or the synthetic lipid emulsion with bacterial endotoxin *in vitro*. In fact, chylomicrons were able to interact with endotoxin and inhibit its detection in a dose dependent manner. Perhaps an explanation to the discrepancy lies in the methods used by the other studies to isolate their lipoproteins. As previously stated, bacterial endotoxin is ubiquitous in the environment. Hence, the isolation of endotoxin-free lipoproteins requires strict adherence to apyrogenic techniques. These techniques include use of depyrogenated glassware, KBr, and centrifuge tubes during the sequential isolation of the different subfractions of plasma. Even minor breaks in technique can result in contamination of the lipoprotein preparation. Theoretically, as the level of lipoprotein contaminated with endotoxin increases, the lipoprotein capacity to *detoxify* additional endotoxin could be reduced. Perhaps in the previous studies mentioned, the VLDL they tested (for the ability to interact with endotoxin) was contaminated prior to experimentation, and thereby relatively incapable of detoxifying much additional endotoxin. In this study, both isolated triglyceride-rich lipoproteins and the synthetic lipid emulsion

samples were tested for contamination with endotoxin using the heating-dilution technique describe under methods (control samples). We found that occasional VLDL were contaminated even after strict sterile/apyrogenic technique in both isolation and testing, suggesting endogenous contamination. This would support the theory that VLDL binds endotoxin *in vivo* (14). If any of these lipoprotein or lipid emulsion samples were contaminated with endotoxin, they were excluded from experimentation.

Insight into the exact mechanism of this interaction may stem from the fact that bacterial endotoxin, or lipopolysaccharide, is an amphipathic molecule. This is defined as a molecule which is comprised of both hydrophobic and hydrophilic regions with a low solubility in water. The Lipid A portion of bacterial lipopolysaccaride is the hydrophobic region, whereas the long polysaccharide chain is the hydrophilic region. Subsequently, in water-based solutions (i.e., plasma) the hydophobic Lipid A portion orients itself into the oil phase of an oil-water interface, while the hydrophilic polysaccharide projects into the water phase. Both cholesterol ester-rich and triglyceride-rich plasma lipoproteins have similar structures such that they are transported in plasma as sphere shaped microemulsions, having a hydrophobic cholesterol ester- or triglyceride-rich core surrounded by hydrophilic phospholipids and free cholesterol arranged around the core in a unilamellar membrane (34). It is this structural similarity between bacterial endotoxin and plasma lipoproteins which may be the major factor allowing these macromolecules to interact and "bind". As it is the Lipid A moiety that initiates the cascade of Limulus amebocyte enzymes that terminate in formation of a gel (or yellow color in the modified chromogenic assay), it follows that the triglyceride-rich lipid particles "hide" the Lipid A from the Limulus proenzyme. Most likely it is a lipid-lipid (lipid particle-

lipopolysaccharide) interaction occurring by one of the following mechanisms: 1) the glycolipid endotoxin molecule adjusts its conformation so that the Lipid A moiety associates itself with the phospholipids in the lipoproteins' outer membrane; 2) the Lipid A becomes incorporated into the lipid-rich core; or 3) a combination of 1 & 2.

Ulevitch and his co-investigators have suggested a two step method for the detoxification of endotoxin in plasma (13, 24-26). The first step involves plasma factors interacting with the native macromolecular bacterial lipopolysaccharide and producing modified LPS monomers. These modified LPS monomers are then able to freely partition into lipid environments, such as in the cholesterol ester, triglyceride-rich lipoproteins, and synthetic lipid emulsion. Other investigators have looked at interactions only between LPS and membranes (35-38). One if these studies have shown that particles of endotoxin associate with membranes by edge attachment suggesting that this process probably allows the lipids of LPS to interact with membrane receptors or lipids (38). Another study demonstrated that the LPS molecule associates itself with phospholipid monolayers and bilayers by a combination of penetration and adsorption to the surface (35); while others have suggested that lipopolsaccharide and phosphatidylethanolamine form a complex in which each LPS molecule is surrounded by several phophatidylethanolamine molecules (36). An alternative method allowing for lipoprotein-lipopolysaccharide interaction may involve the lipid transfer protein found in plasma. It is well documented that this lipid transfer protein facilitates exchange of cholesterol esters, triglycerides, and phospholipids between plasma lipoproteins (39), such as VLDL, chylomicrons, and HDL. Perhaps this lipid transfer protein facilitates this interaction between lipopolysaccharide and the plasma lipoproteins. For example, the Lipid A moiety may be

transferred into the lipid-rich core of lipoproteins, thereby hiding the moiety from the Limulus proenzyme and subsequently inhibiting its detection. This may explain the requirement of plasma both in the samples of this study, as well as others (13, 24-26).

As mentioned previously, bacterial endotoxn can cause several pathophysiological effects culminating in death if not treated sufficiently. Just as the Lipid A moiety of endotoxin initiates the Limulus assay cascade, it also initiates the body's physiologic response to endotoxin. Studies of radioactive labeled LPS reveal that the liver serves as the major organ of endotoxin clearance, with the Kupffer cell (the liver's macrophage) almost exclusively clearing this macromolecule (40). The majority of endotoxin's biological effects are mediated through the cytokine tumor necrosis factor (TNF) released by endotoxin stimulated macrophages (7, 32). Once released, TNF causes several pathophysiologic effects including a disturbance in lipid metabolism. The earliest and most striking disturbance in lipid metabolism is hypertriglyceridemia, mainly in the form of VLDL. Whether the hypertriglyceridemia is secondary to increased production, decreased break down, or increased peripheral mobilization is controversial, and most likely a combination of the three. Some investigators have demonstrated an increase in hepatic lipid synthesis secondary to TNF (41) and other cytokines (42), while other investigators have found that endotoxemia (11) and infection (15) cause a decrease in breakdown (i.e., marked decrease in lipoprotein-lipase activity). It has been generally thought that this marked increase in circulating triglyceride was the mobilization of fat energy stores to fuel the body's response to the infectious challenge. Although this explanation is plausible, there is an alternative theory. Perhaps not only is this a mobilization of body energy stores to aid in combating the invader, but also a

defensive mechanism in itself against endotoxemia. The ability of triglyceride-rich lipid particles to bind and detoxify significant amounts of bacterial endotoxin *in vitro* has been demonstrated in this present study whereas *in vivo* detoxification and decreases in endotoxin's toxicity and biologic activities by plasma HDL has been demonstrated elsewhere (13, 24-26). Furthermore, recent investigations have revealed that *in vitro* incubation of plasma VLDL, chylomicrons, or the synthetic lipid emulsion with a lethal dose of endotoxin can significantly reduce endotoxin lethality in mice (14). The exact mechanism is not understood, but a possible a explanation could be that the circulating triglyceride-rich lipoproteins (VLDL and chylomicrons) bind the bacterial lipopolysaccharide, specifically at the Lipid A moiety, and travel to the liver in a lipoprotein-LPS complex to be cleared. But instead of binding to and activating the liver Kupffer cells, the lipoprotein-LPS complex is cleared via the lipoprotein clearance mechanism--by the hepatocyte. This complex in taken up by hepatocytes, perhaps by receptor-mediated endocytosis (43), then enters the degradative pathway (44) and is degraded. The shunting of the LPS to the hepatocyte rather than the Kupffer cell inhibits the release of TNF and all of its sequellae. This theory could also account for the absence of signs or symptoms of endotoxemia (i.e., fevers, chills, rigors, etc.) arising from daily activities such as tooth brushing and bowel movements (45), or minor procedures such as tooth extraction or colonoscopy (46), which have been shown to cause transient bacteremias.

Numerous investigators have studied the clearance of labelled triglyceride-rich particles in healthy human volunteers (47-50). Published reports invariably pay careful attention to the biochemical characteristics of the particles infused, but frequently provide cursory information as to what precautions were taken to avoid infusing contaminating bacterial endotoxins.

Although the infusion of a small quantity of endotoxin-contaminated lipoproteins may cause no overt symptoms, the volumes of some bolus infusions have exceeded 600 ml, delivered within a few minutes (48). These data presented in this study further highlight the the potential for significant quantities of endotoxin to be undetected in lipoprotein-containing solutions when assayed by the standard Limulus assay. The data suggest that the assay of postprandial plasma preparations by the standard Limulus assay can fail to detect the presence of 1,000-10,000 pg of endotoxin for each mg chylomicron of VLDL triglyceride in the solution. Therefore, an infusion of postprandial plasma with triglyceride content of 300 mg/dl could conceal up to 30 ng of endotoxin per milliliter of infusate. This level of contamination is significant, since 4 ng of endotoxin/kg body weight has been shown to elicit fever, tachycardia, leukocytosis, elevated cardiac output, and elevated concentrations of catecholamines and tumor necrosis factor in the plasma of healthy volunteers (7, 32). It is uncertain if this amount could be toxic, but we first came upon the notion that endotoxin could bind to lipoproteins when a volunteer in our laboratory experienced an episode of fever, chills and rigors following an autologous plasma enriched chylomicron infusion of approximately 100 ml. Although the working hypothesis is that triglyceride-rich lipoproteins are protective against endotoxemia, the former event demonstrated an endotoxic reaction even though the endotoxin was bound to chylomicrons. One explanation for this endotoxic event could be that the binding capacity of the chylomicrons had been overwhelmed and hence their protective effect exceeded. Alternatively, the chylomicron-endotoxin molecule may have not been toxic, but following triglyceride breakdown by the normal catabolism pathway of chylomicrons with peripheral tissue lipoprotein lipase, the bound endotoxin was released into the circulation.

The infusion of endotoxins could also interfere with the interpretation of a lipoprotein kinetic study. Through the induction of elevated cytokine and hormone levels, endotoxemia can cause alterations in lipid metabolism (8, 50) and in hepatic blood flow and ultrastructure (9-11, 15, 41-42, 52-53). Thus experimental protocols that involve the infusion of humans with postprandial plasma and/ or lipoproteins should include detailed testing for the presence of endotoxin.

Even though the chromogenic modification of the Limulus gelation assay improves its convenience as well as its sensitivity to detect endotoxin (up to 1000 fold), it still has the problem of inconsistency. In this study, only after performing multiple manipulations of each variable in experimentation were the results consistent as well as reproducible. This included varying the reagent and sample concentrations, modifying reagents and samples, varying incubation periods, and adhering to strict sterile/apyrogenic technique throughout experimentation. In view of these results, the chromogenic Limulus assay can be a reliable assay in the detection of endotoxin in plasma-lipoprotein containing solutions, but only if experimental parameters are carefully set and strict adherence to sterile/apyrogenic technique is maintained.

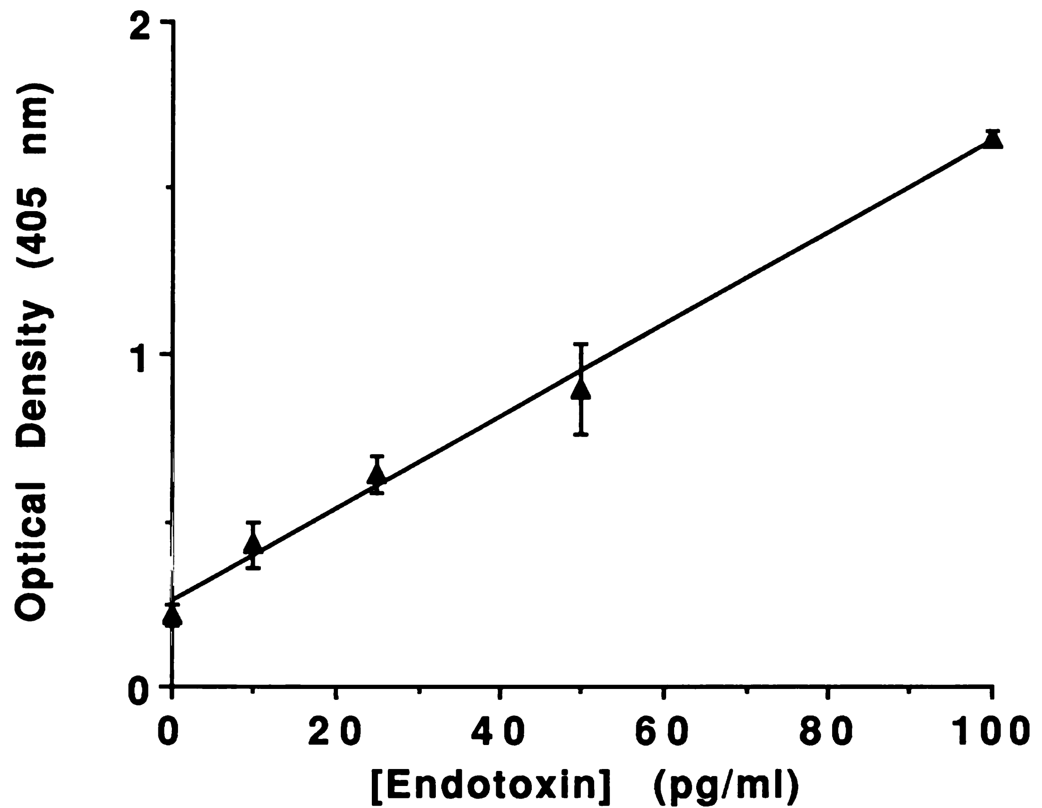

Figure 1. The detection of increasing concentrations of *E. coli* (055:B5) endotoxin activity in apyrogenic saline by a chromogenic modification of the standard Limulus assay. Optical density of the test mixture was read at 405 nm with a maximum of 2.0. The data are the mean ± S.D. of at least five determinations. A linear equation is generated which best describes the data points. $Y = 0.25 + 0.01X$, $r = 0.99$.

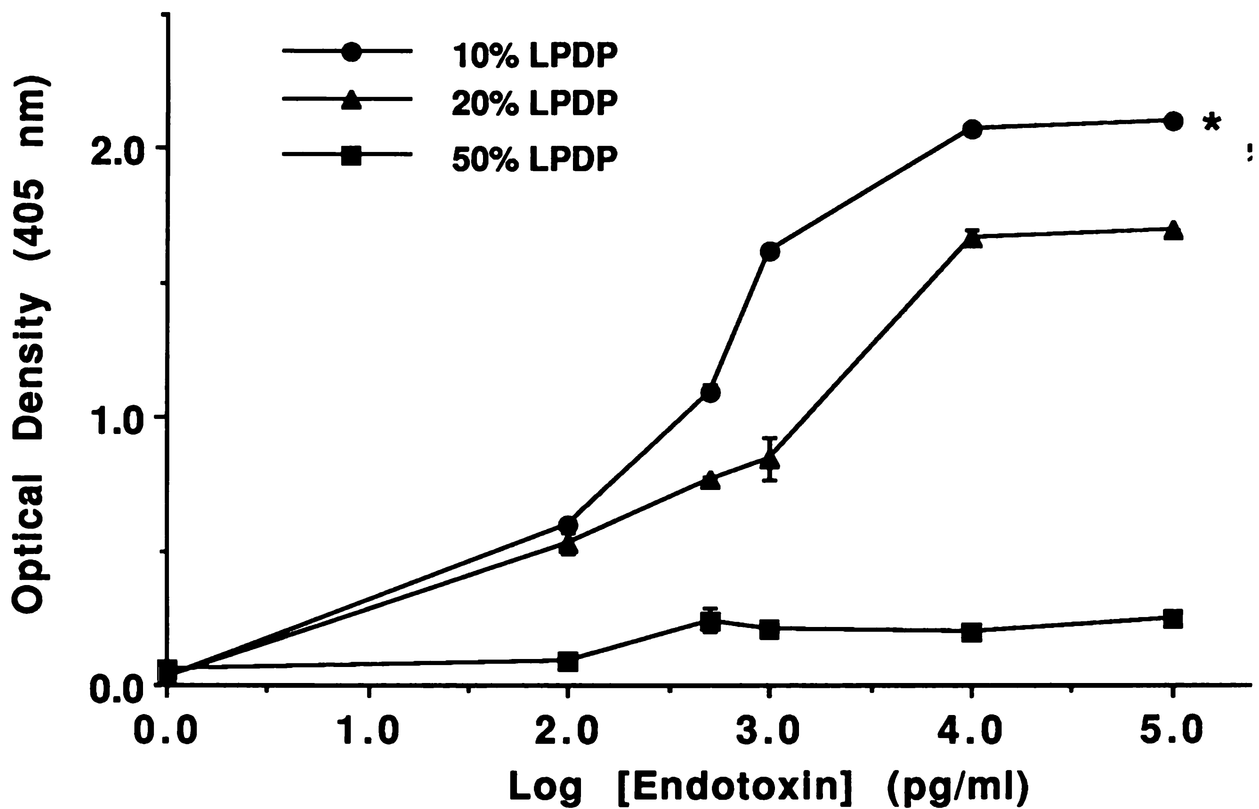

Figure 2. Detection of increasing concentrations of *E. coli* (055:B5) endotoxin activity as measured simultaneously by the chromogenic Limulus assay in test mixtures containing 10%, 20%, and 50% lipoprotein-deficient plasma. Optical density was measured at 405 nm. The data are the mean ± S.E.M. of at least five determinations, presented as a semi-log plot. The S.E.M. is omitted when it is smaller than the data point. LPDP, lipoprotein-deficient plasma. * 10% LPDP curve versus 20% LPDP curve, $p = 0.030$, ** 10% LPDP curve versus 50% LPDP curve, $p < 0.02$.

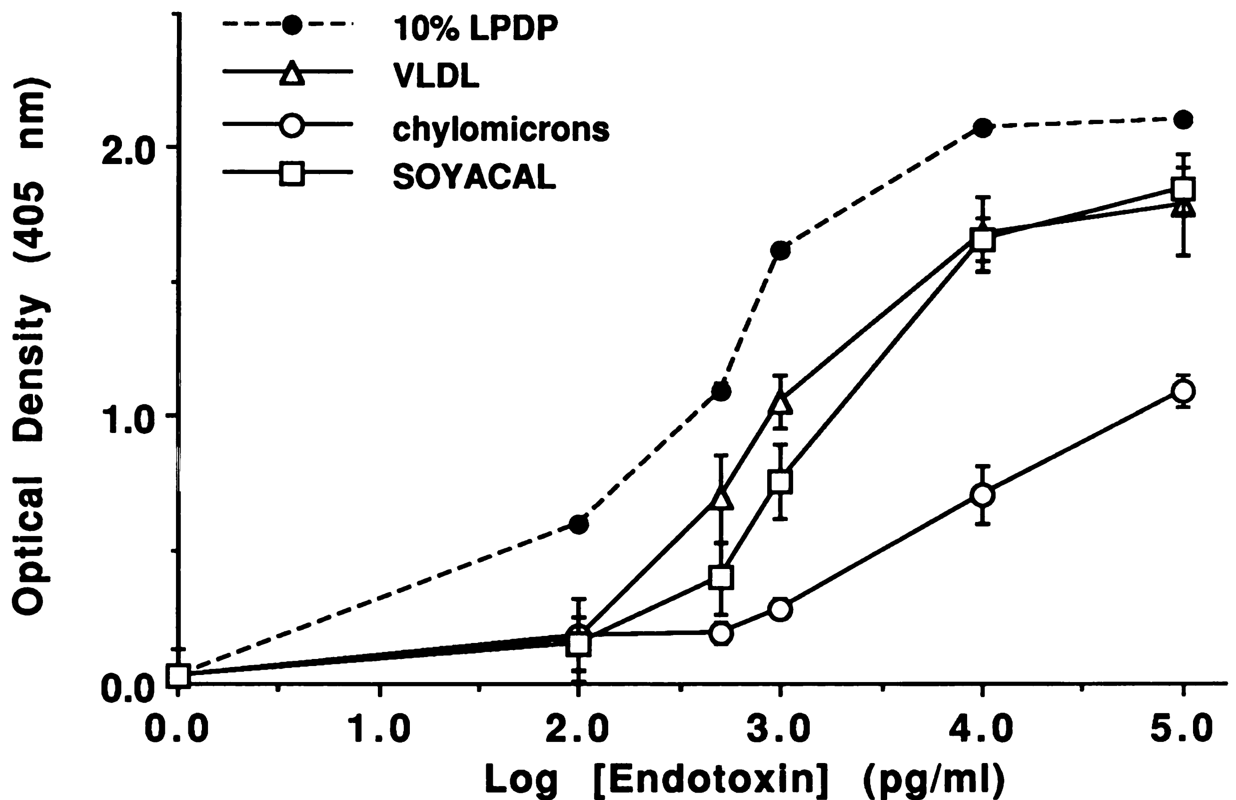

Figure 3. Detection of increasing concentrations of *E. coli* (055:B5) endotoxin in test mixtures containing 10% lipoprotein-deficient plasma alone or with a specific triglyceride-rich particles. Chylomicrons were assayed at a concentration of 1.0 mg triglyceride, VLDL at 1.0 mg triglyceride, and SOYACAL at 2.5 mg triglyceride. Optical density was measured at 405 nm. The data are the mean ± S.E.M. of at least five determinations, presented as a semi-log plot. The S.E.M. is omitted when it is smaller than the data point. $p < 0.005$ for the 10% LPDP curve versus VLDL, chylomicrons or SOYACAL curves. VLDL, very low density lipoproteins, LPDP, lipoprotein-deficient plasma.

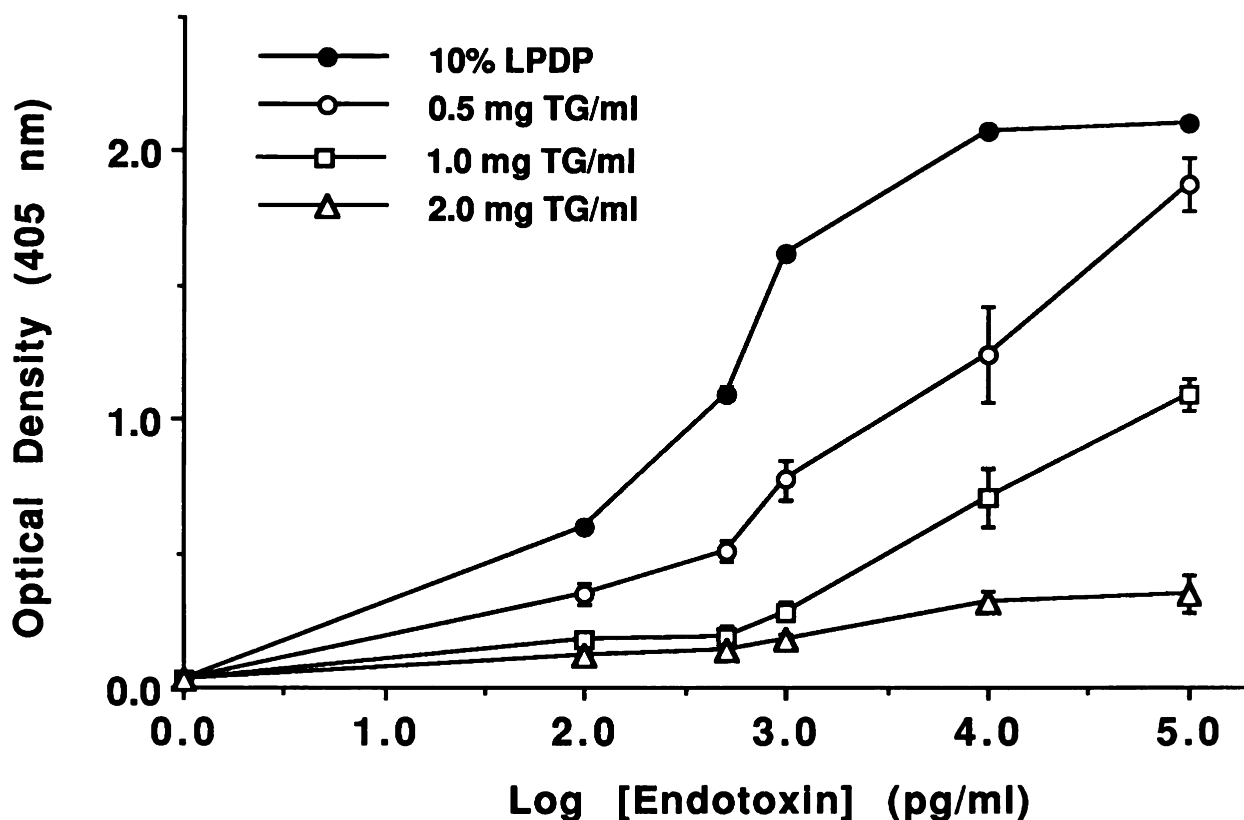

Figure 4. Detection of increasing concentrations of *E. coli* (055:B5) endotoxin in test mixtures containing 10% lipoprotein-deficient plasma, and increasing concentrations of chylomicrons (0.5 mg, 1.0 mg, and 2.0 mg triglyceride). Optical density was measured at 405 nm. The data the mean ± S.E.M. of at least five determinations, presented as a semi-plot. The S.E.M. is omitted when it is smaller than the data point. $p < 0.005$ for 10% LPDP curve versus 0.5, 1.0 or 2.0 mg chylomicron triglyceride curves. TG, triglyceride, LPDP, lipoprotein-deficient plasma.

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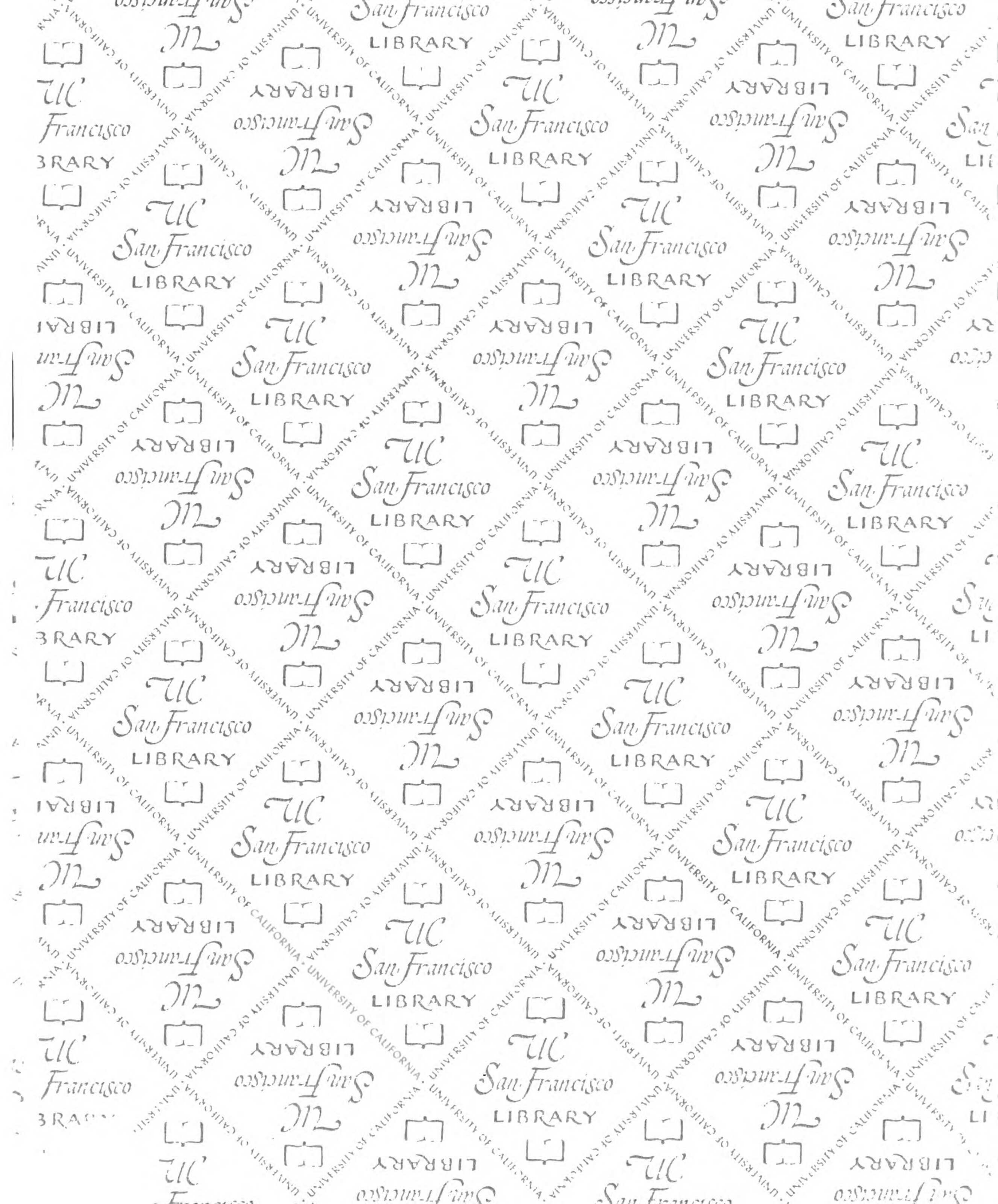
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