1 INTRODUCTION

1.1 ENDOTOXIN

Endotoxin is a lipopolysaccharide (LPS) and its molecular weight is >100,000 Daltons [1]. LPS is composed of two major parts, the hydrophobic lipid A portion and the hydrophilic polysaccharide portion (commonly called the "O" region) [2]. The structure of the lipid A portion is fairly well conserved, but the nature (length and chemical composition) of the polysaccharide side chain varies between genera, species, and even strains of Gram-negative bacteria. The lipid A portion of the molecule has been shown to be responsible for numerous in vivo and in vitro effects of endotoxin [3].

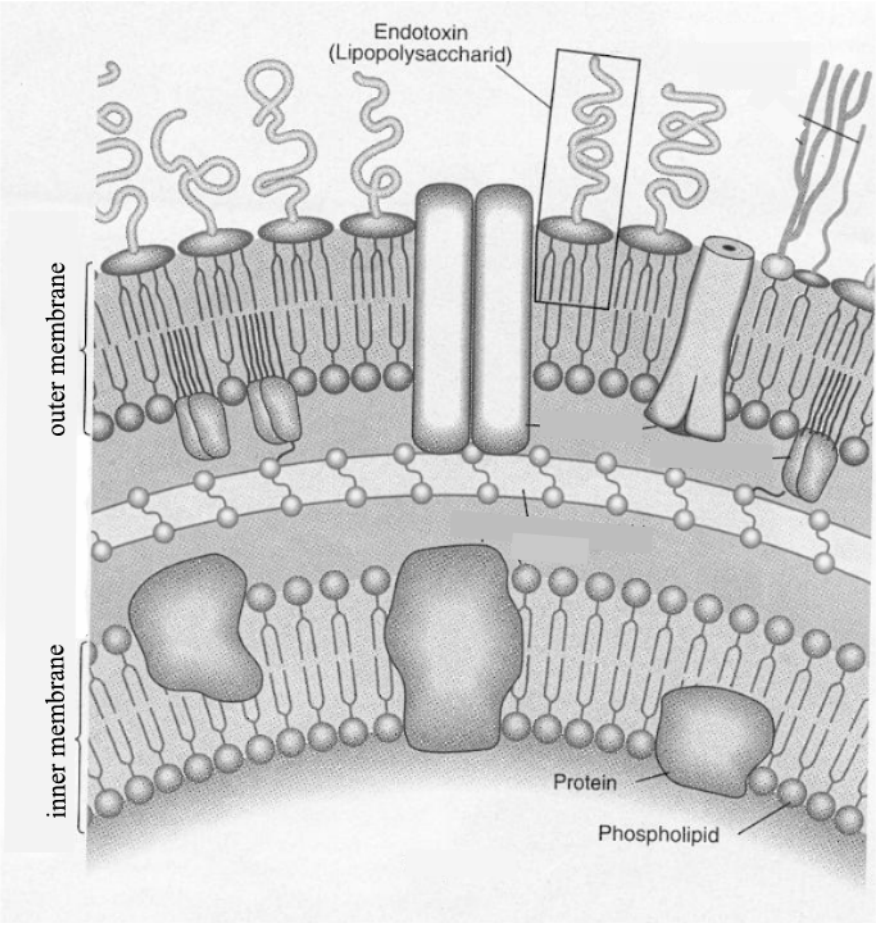

FIGURE 1-1 LIPOPOLYSACCHARIDE IN CELL WALL MODIFIED FROM RIETSCHEL [4] ET AL.

The lipopolysaccharide is part of the outer wall of Gram-negative bacteria (Figure 1-1) and is released when the bacterias die or grow [3-5].

1.1.1 ENDOTOXIN IN CLINICAL MEDICINE

Endotoxin is playing a very important role in developing sepsis. When it enters the bloodstream it will be phagocytosed by macrophages. The macrophages will produce mediators like tumor necrosis factor, interleukins 1 + 6 + 8, platelet activating factor, thromboxane A2 and reactive oxygen species [3-8]. These mediators induce the septic syndrome with fever, hypothermia, tachycardia, tachypnoea, oliguria, acidosis and hypotension. This severe illness could lead to septic shock, disseminated intravascular coagulation (DIC) and multiple organ failure (MOF) [4, 9].

The Source of endotoxinaemia can be endogenous from the bacterial flora of the gut [5]. Every time when the bacteria die fragments of the cells penetrates the wall of the bowel and enter the bloodstream. The liver will filter the endotoxin off the blood [10]. Low activities of endotoxin stimulate the immune response [11, 12], higher activities can lead to septic shock [4, 13]. Other sources may be exogenous like catheter or surgery that can transport endotoxin or gram negative bacteria into the body. The lung [13] is an other way to convey endotoxin into the organism.

1.1.2 ENDOTOXIN IN THE ENVIRONMENT

When endotoxin is inhaled into the lung it is phagocyted by macrophages, resulting in the release of tumor necrosis factor, interleukins, platelet activating factor, thromboxane A2 and reactive oxygen species [13-15]. These mediators are source of fluid flooding into the intraalveolar space and inflammation of the epithelium. This may cause airway obstruction [9, 13, 15] and asthma [16-19]. Sources for environmental endotoxin are dust [13, 15-17, 19-45], humidifier systems [9, 13, 15, 18, 22, 46-51], machining fluids [9] and water pools [13] because of the ubiquitous nature of gram-negative bacteria and their fragments.

1.1.3 ENDOTOXIN AND SICK BUILDING SYNDROME

The symptoms of endotoxin inhalation are the same like described of the World Health Organization (WHO) for the Sick Building Syndrome (SBS) in accordance to the symptoms of the lung [52, 53], namely headache, exhaustion and inflammation of the mucous membrane. These manifestations are found in so-called "sick buildings" and are probably related to heating, ventilation and air-conditioning systems (HVAC) [54]. Investigations of Teeuw [55] in 19 Dutch office buildings with heating, ventilation and air-conditioning systems showed a very high correlation of endotoxin and SBS. He found a more than 7 fold higher activity of endotoxin in buildings where the workers are suffering under these problems (see discussion).

1.2 MEASUREMENT OF ENDOTOXIN

Endotoxin is suspected in some environmental investigations as a possible cause for the sick building syndrome. The hypothesis is that gram negative bacteria were sucked onto the filters of HVAC systems. When they die fragments of their cell walls (LPS) are released and contaminate the indoor air. For indicating the time for changing the filter a method is needed to determine the endotoxin activity on used filters. Therefore the three most common filter types of HVAC systems in Germany were investigated. Because endotoxin seems to attend to the filter material [21] the recovery rate when extracting the filter is unclear. A reliable and precise method is needed to introduce endotoxin activity limits.
There are several methods to detect endotoxin and different extraction protocols and investigators are using different methods (Table 4-2).

1.2.1 RABBIT TEST

A former method to detect endotoxin is by injecting the agent into a rabbit's blood vessel and measure it's temperature increase [56-58].

1.2.2 GCMS

Gas chromatography-mass spectrometry is used to detect fatty acids (FA) from the LPS. One mole of LPS contains 4 moles of 3-OH-FAs and the average molecular weight of LPS is 8,000. It is possible to calculate the total activity of LPS (no matter if active or inactive) out of these variables. [26]

1.2.3 THE LIMULUS AMEBOCYTE LYSATE TEST

Novel methods are based on the Limulus Amebocyte Lysate test. The different assays are listed below (Table 1-1).

TABLE 1-1 DIFFERENT ASSAYS WITH ITS DISTINCTION

	Rabbit Test	Clotting Test	Chromogenic Endpoint Test	Chromogenic Kinetic Test
Qualitative	yes	yes	yes	yes
Quantitative	no	semi quantitative	yes	yes
Range	no	no	0.15 to 1.2 EU/ml or 0.015 to 0.12 EU/ml	0.005 to 50 EU/ml
Time*	Days	1 hour	15 minutes	1 ½ hours

*) Without the time for preparing the samples

1.2.3.1 GEL-CLOT METHOD

FIGURE 1-2 *LIMULUS POLYPHEMUS*

This method developed by Levin & Bang [59-61] in 1964 is based on the clotting properties of the horseshoe crabs (*Limulus polyphemus* Figure 1-2) blood upon getting into contact with endotoxin.

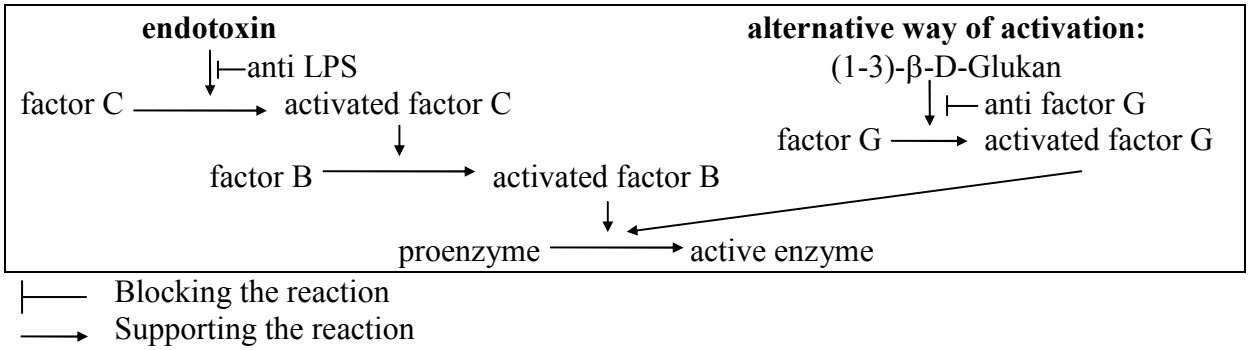

FIGURE 1-3 THE LIMULUS AMEBOCYTE LYSATE ENZYME CASCADE

The clotting cascade of *Limulus Polyphemus* (Figure 1-3) is comparable to the blood clotting cascade in human beings [61]. Factor C is activated in presence of endotoxin. This can be prevented by anti LPS, which has to be removed by the manufacturer when creating the assay. The activated factor C in turn activates factor B. This form of factor B will make an active enzyme out of a proenzyme. This cascade can also be activated by an alternative way. (1-3)-β-D-Glukan a part of fungi is able to activate factor G that can activate the proenzyme to the active enzyme [62, 63]. Because of this possibility to activate the assay the manufacturers have to avoid eliminating the anti factor G that blocks the activation of factor G, however the Associates of Cape Cod do not block this way of activation [64].

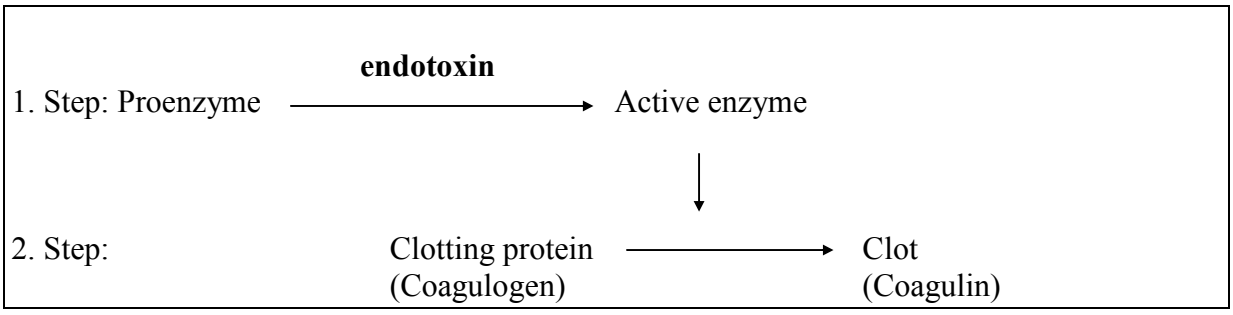

FIGURE 1-4 REACTION SEQUENCE OF THE GEL-CLOT METHOD

In the gel-clotting test the proenzyme is activated by endotoxin like described above (Figure 1-4). The active enzyme hydrolyzes specific bonds in the protein coagulogen. In this reaction it will develop coagulant that is a gelation [3, 65].

1.2.3.2 CHROMOGENIC TEST

Principle of the chromogenic test:
Endotoxin turns a proenzyme into an active enzyme. The active enzyme releases coloring para-Nitroanillin (pNA) out of a colorless substrate.

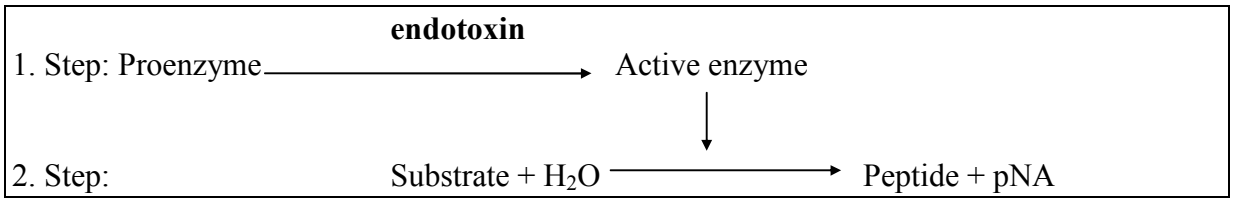

FIGURE 1-5 REACTION SEQUENCE OF THE CHROMOGENIC METHOD

The pNA release produces a yellow color, which is measured photometrically at 405 nm. The intensity of the color corresponds to the activity of endotoxin present in the system (Figure 1-5).

1.2.3.2.1 CHROMOGENIC ENDPOINT TEST

In end-point measurements, the Limulus Amebocyte Lysate reagent and the endotoxin containing sample are mixed and incubated a predetermined period of time at 37°C. The chromogenic substrate S-2423 is then added and free pNA is produced during a second incubation period. After stopping the reaction with acetic acid the yellow color (absorbance) is measured photometrically at 405 nm. Linear standard curves, showing the relation between the absorbencies and the endotoxin activity of unknown samples are determined from the standard curve [66].

1.2.3.2.2 KINETIC CHROMOGENIC TEST

Kinetic measurements are carried out by means of a microplate reader equipped with adapted software. Here, the Limulus Amebocyte Lysate reagent and the endotoxin containing sample are mixed and incubated at 37°C in a microplate reader. After continuously measuring the increasing absorbance during the reaction, the time (onset time) required to increase the optical density (OD) to 0.200 absorbance units (onset OD) is registered. Alternatively the rate (OD_{max}/min.), the maximum velocity of the reaction, can be registered instead of the onset time. A standard curve, showing the linear correlation between the log onset time and log activity of the standard endotoxin, may be constructed in the range 0.005 EU/ml to 50 EU/ml and used for the measurement of the endotoxin activity in unknown samples. [66]

1.2.3.3 ENHANCEMENT AND INHIBITION OF THE TEST

The *Limulus* Amebocyte Lysate reagent reaction is influenced by substances such as sodium-, calcium-, magnesium ions [67, 68], and circumstances as suboptimal pH conditions, aggregation [69] or adsorption of control endotoxin spikes, unsuitable cation concentrations, enzyme or protein modification and non-specific *Limulus* Amebocyte Lysate activation [1, 70]. They can enhance and / or inhibit the test. Probably interference is not only due to presence of interfering ions alone but is also a consequence of the chemical structure of LPS. LPS form micelles with hydrophilic polysaccharides facing the water. The lipid portion of LPS is responsible for the activation of the *Limulus* lysate the reaction will be further inhibited [68].

1.2.3.3.1 DETERMINATION OF INFLUENCING FACTORS

To detect any factors that might influence the test other than endotoxin there are two methods:
1. Spiking
2. Serial dilution

1.2.3.3.1.1 SPIKING

Given a sample A which is measured in duplicate there were two more samples A added to the microplate. The additional samples were mixed with a certain activity of standard endotoxin (positive product control (PPC) see 2.1.1.2.2) and its recovery was determined. In case of inhibition a lower added activity would be found. If enhancement were present the regaining activity would be higher than the certain activity.
The recovery of the spike should equal the known activity of the spike ± 50% to be considered to neither enhance nor inhibit the assay [71]. This large range is required because the activity of every sample and every spike is allowed to vary 25% from one measurement to another. Thus we have twice 25%, resulting in a total variation of the spike recovery of ± 50% [71].

1.2.3.3.1.2 SERIAL DILUTION METHOD

Serial dilutions of standard endotoxin and test samples were performed and measured with the LAL test. The analysis of covariance (ANCOVA) of these dilution sequences was computed. It shows correlation at least for part of the lines if there were no or dilution dependent influencing factors. For this method special software is needed. [70, 72, 73] With kinetic Limulus assay with resistant-parallel-line estimation (KLARE) it is possible to calculate a coefficient of variation (CV) for every single probe [74].

1.2.3.3.2 OVERCOMING THE DILUTION DEPENDENT INHIBITION OR ENHANCEMENT

The most common way to overcome the influence is to dilute the sample 1:10 and measure it again [75]. Alternative ways are heating the sample [76], pH adjustment [77], perchloric acid, chloroform extraction [78, 79], divalent cations, gel filtration [80] and ultrafiltration [65].

1.2.3.3.3 OVERCOME DILUTION INDEPENDENT INHIBITION OR ENHANCEMENT

Dilution independent inhibition could be related to assay factors because it does not occur repeatedly in samples. Other sources could be lot of glassware, microplates or pipette-tips. It was shown by Milton et al. 1992 that TAP buffer (Triethylamin Phosphate) helps to protect against dilution independent inhibition [74, 81]. Milton et al. 1997 also found that a higher frequency of dilution independent inhibition is associated with the use of onset time. The way to overcome the influence is to measure the sample once again [28].

1.3 STUDY AIMS

❶ To investigate the most reliable and effective method to retrieve endotoxin on filters of HVAC systems.
❷ To show differences between the most common filter types in retrieving endotoxin.
❸ To show differences of endotoxin retrieval between various types of endotoxin.