Endogenous Endotoxemia in Patients with Liver Cirrhosis

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INTRODUCTION

Since the limulus test, a method for the evaluation of endotoxin levels using extract from horsehoe crabs (limulus, spp), was developed in the year 1969 by Levin and Bang, there have been reports on endotoxin from portal blood without negative gram bacterial infection,[1,2] a condition known as endogenous endotoxemia.[3]

Up to now, endogenous endotoxemia in patients with liver cirrhosis (LC) is still controversial. This can be seen from the great variation in the prevalence of endotoxemia in peripheral vein in patients with liver cirrhosis, ranging from 0 to 80%.[4] The main cause for this difference is the methodological factor, reflecting a difference in examination technique. Most studies conducted without a specific method demonstrate endotoxemia in patients with liver cirrhosis.[4,5,6,7,8,9,10,11] While studies using a sensitive method of evaluation for endotoxin found the opposite, detecting no increase in endotoxin in patients with liver cirrhosis.[12,13]

ENDOTOXIN

Endotoxin is the outer most layer of gram negative microorganism, also known as Lipopolysaccharide (LPS).

Endotoxin is made up of the following 3 structures:

1. Polysaccharide consisting of specific O chains
2. A middle polysaccharide layer divided into inner and outer layers
3. Lipid A layer

All negative gram microorganisms have a similar middle structure and Lipid A layer, but differ in the specific O polysaccharide chains. Lipid A layer is an important layer in endo-toxicity and is responsible for multiple organ failure due to various pathophysiologic responses induced during negative-gram sepsis. Lipid A molecules with the strongest toxicity are those with 6 fatty acid chains, while those having only 1 or 2 fatty acids have weak toxicity.[14,15]

The lipid A portion of endotoxin is implanted within the cell membrane. In order to be effective, endotoxins must be released from the bacterial surface. Endotoxins are released from the cell wall during bacterial multiplication as well as when bacteria is destroyed by antibiotics or complement. Released endotoxins do not directly destroy host cells or cause an immediate response, but instead do so indirectly through mediators released by the host cell itself.[14,16,17]

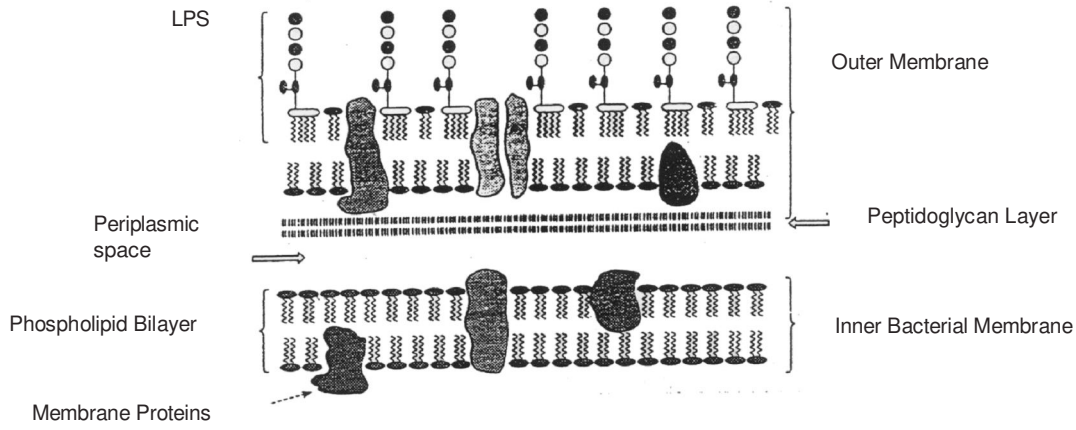

Figure 1. Structure of negative gram bacteria.[15]

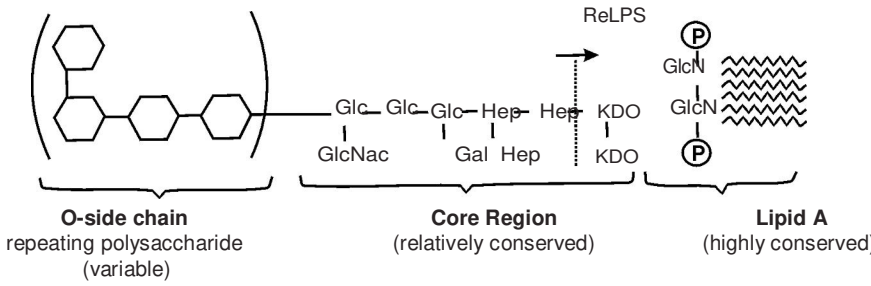

Figure 2. Lipopolysaccharide structure[15]

CELL RESPONSE TO ENDOTOXIN

The body response towards endotoxin is very complex. It involves interaction between endotoxin, serum components that might strengthen or inhibit endotoxin action, and receptors on the surface of specific cells.[15]

Free endotoxins within the blood circulation would quickly bind to form complexes with various proteins and lipids, including with its specific carrier, the LPS binding protein (LBP). LBP concentration increases within the body's circulation as a response towards inflammatory stimuli. These LPS-LBP complexes have strong affinity towards CD14 receptors, especially those on the surface of monocytes and macrophages, including Kupffer cells. CD14 is a protein that binds with 55-kDa glycophosphatidil (GPI).[15,18]

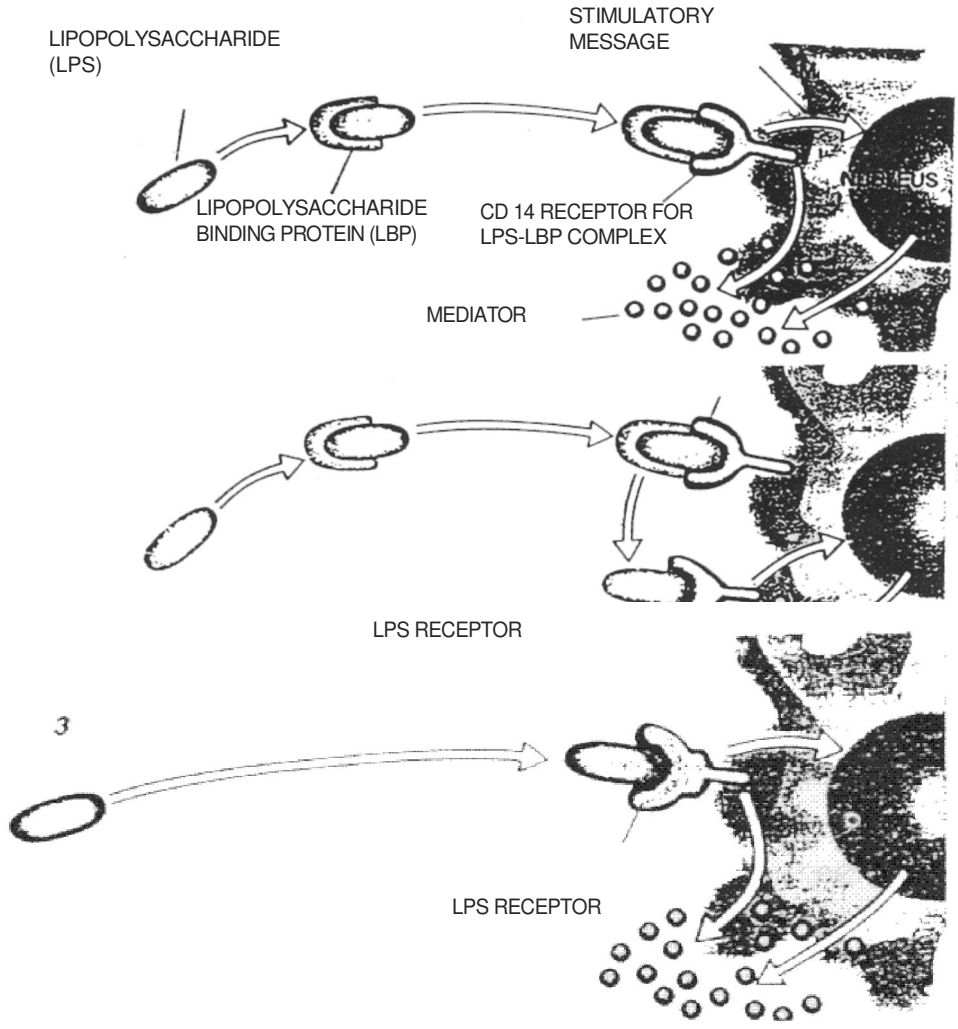

Figure 3. Macrophage activation by LPS mediated by CD14.[14]

There are three pathways to activate macrophages and monocytes to release mediators due to stimuli from endotoxin. The first pathway is where endotoxins bind with LBP in the body's circulation. These complexes then activate CD14 receptors, which instruct macrophages to release mediators. In the second pathway, LPS-LBP-CD14 complexes are the only factors that allow endotoxins to interact with other receptors, which stimulate macrophages separately or together. The third pathway is where endotoxins directly activate macrophages without assistance from LBP or CD14.[14]

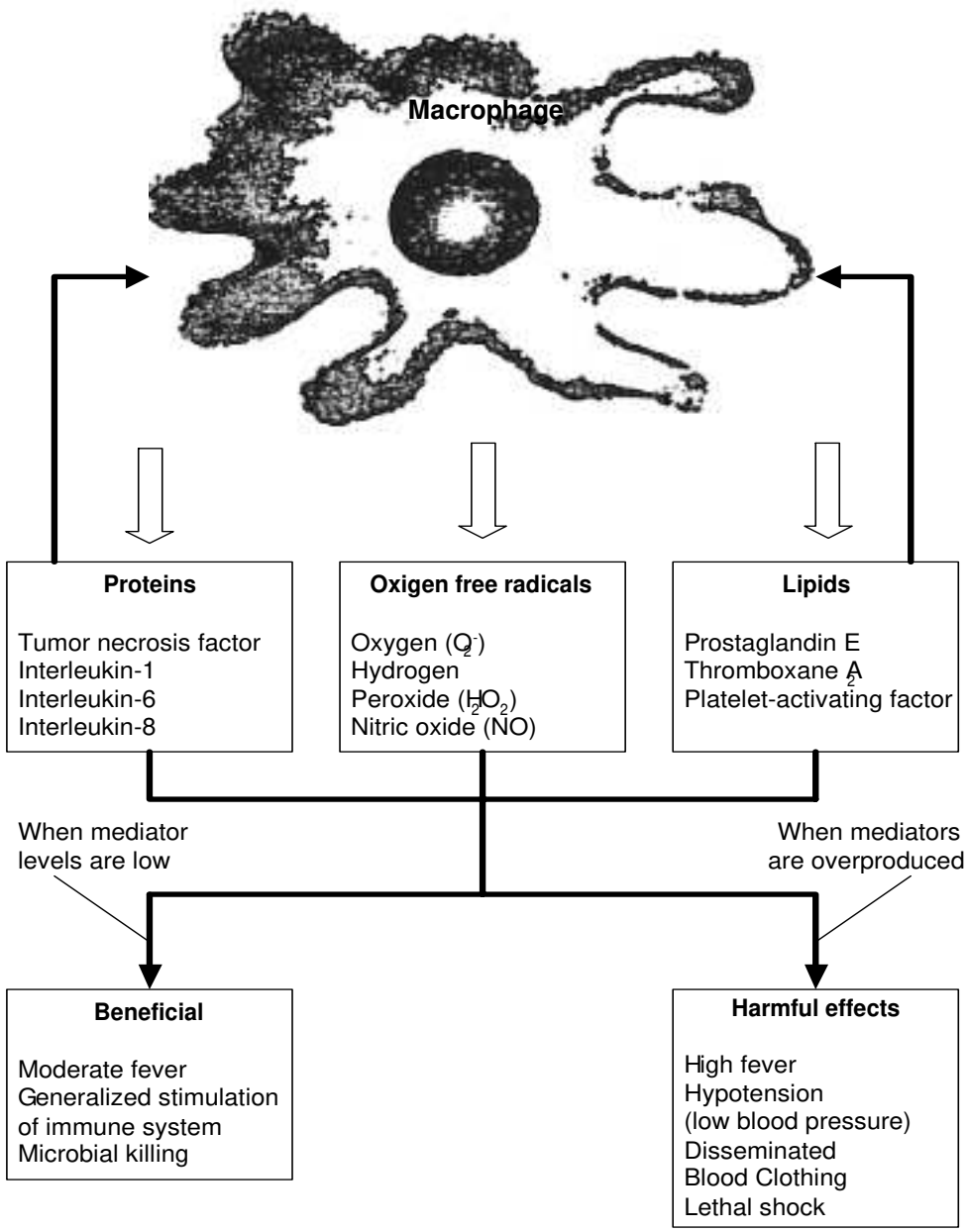

Figure 4. LPS effect on monocytes/macrophages.[14]

Macrophages/monocytes including Kupffer cells and endothelial cells activated by endotoxins release alpha tumor necrosis factors (alpha-TNF), interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8) and platelet activating factor (PAF). After these mediators are released, arachidonic acid is metabolized into leukotrienes, thromboxane A2 and prostaglandins (especially prostaglandin E 2 – PGE2) and prostaglandin I 2 (PGI-2). IL-1and IL-6 activate T cells into producing gamma interferon, interleukin 2 (IL-2), interleukin 4 (IL-4), and granulocyte-monocyte colony-stimulating factor (GM-CSF). All these substances have direct effect on vascular endothel.[19]

Alpha TNF plays a role in various aspects of the inflammatory response, including induction of inflammatory cytokines (IL-8). Alpha TNF also induces changes in the vascular wall, activates monocytes and neutrophilles, and destroys infected cells. Alpha TNF is also the chief mediator in septic shock. This cytokine also influences lipid metabolism and storage, causing cachexia.[20]

These days, IL-1 is known as an endogenous pyrogen. If IL-1 is administered to humans, hypothalamic effects in the form of rigor, chills, fever, and malaise ensue. Administration of high doses of IL-1induces hypotension. Aside from stimulating acute phase reactions, IL-1 could also cause leukocytosis and changes in the plasma protein.[20]

IL-6 is a hepatocyte-stimulating factor. IL-6 enhances the secretion of immunoglobulines, increasing the levels of liver acute phase proteins up to 100 times, but reducing albumin synthesis up to 50%.[20]

Tilg H**,** et al in their study demonstrated increased levels of proinflammatory cytokines (alpha TNF, IL-1 beta, IL-6), as well as significant increases in gamma-IFN, in patients with chronic liver disease, changes of which do not depend on the primary liver disease.[21]

Kakumu, et al also demonstrated increased productions of IL-6 in primary billiary liver cirrhosis compared to control subjects. However, the increase was not statistically significant.[22] In the meantime, Deviere, et al also found increased alpha TNF and IL-6 levels in patients with cirrhosis due to alcohol, who were in stable condition.[7]

Le Moine, et al, compared alpha TNF levels in normal control subjects to patients with liver cirrhosis Child A, B, or C. There was a significant increase in the alpha TNF levels in patients with liver cirrhosis compared to normal control subjects, but no significant difference in alpha-TNF levels among Child A, B, and C patients was identified.[23]

The coagulation cascade and complement system are also activated, even though it is still unclear whether it is caused directly by endotoxin, stimulated by alpha-TNF, or other mediators or a combination of either. The complement system is activated through alternative pathways. Under normal conditions, complement components are inactive. During complement reaction, each component is activated serially, creating a cascade of reactions. Complement activation could induce cell destruction, inflammation and immunological effects.[20]

Complement influences chemotaxis, anaphylaxis, vasodilatation, and cytokines. C3a and C5a are anaphylatoxins that cause mast cell degranulation. Mast cell degranulation causes the release of histamine and other mediators that in turn cause vasodilatation. C5a induces TNF and IL-1 release increases inflammatory response. Most complements are synthesized by hepatocytes, although mononuclear cells, endothel, fibroblasts, and epithelial cells of the gastrointestinal tract and urinary tract could also synthesize complement. Liver cirrhosis and other chronic liver diseases could cause abnormalities such as decreased complement synthesis.[20]

Activation of XII factors (Hageman factor) and production of tissue factors due to stimulation of macrophage and endothelial cells by endotoxin could induce disseminated intravascular coagulation (DIC). In addition, alpha-TNF is suspected to be able to stimulate the development of DIC.[24]

Increased nitric oxide (NO) levels are also found in endotoxemia. This is becaue the production of inducible NO-synthase (iNOS) from macrophages, neutrophils, alpha-TNF, and IL-1 is also increased. Increased NO levels create a hyperdynamic circulation and peripheral vasodilatation.[25,26]

If the above condition and endothelial destruction continues, organ failure. occurs Endothelial destruction continues and finally causes hypotension (septic shock). Aside from the process mentioned above, hypotension is thought to occur due to direct effect of endotoxins, cytokines such as alpha-TNF, IL-1, and myocardial depressant substances, on myocardiac muscles, causing depression of muscle cells.[27]

Counter-regulatory mechanisms to limit endotoxin activity

LPS forms a complex with serum lipoproteins, including low density lipoproteins (LDLs), high density lipoproteins (HDLs), and apolipoprotein A, which reduces toxicity. The LPS complex would then be eliminated from the body circulation. LBP binds free endotoxin or re-

leases endotoxins from lypopolisaccharide complexes to be brought to sCD14 or mCD14. Within serum, we also find anti-LPS antibodies that act as buffers towards biological effects of endotoxin. Neutrophil also has specific anti-LPS activity. Proteins that neutralize LPS, such as bactericidal/permeability-increasing protein (BPI) and neutrophil enzymes such as acyloxyacylhydrolase, are released during phagocytosis. A balance between these contradictory factors determine the development of sepsis and shock.[15]

It turns out that endotoxin also stimulates the production of the anti-inflammatory cytokines IL-10 and IL-4, which increases significantly up to 28 times and 3 times normal levels.[18] IL-10 acts to protect the liver from destruction by alpha-TNF, as well as inhibit the synthesis of alpha TNF and IL-1-beta.

Endotoxin cleansing mechanism

Mimura, et al studied biliary excretion of endotoxin using endotoxin labeled with flourescin isothiocyanate (FITC), demonstrating that after intravenous injection, FITC-LPS was detected in macrophages in the spleen, pulmonary dust cells, and on Kupffer cells. Hepatocytes were also proven to play the same role as Kupffer cells in processing endotoxins from circulation, and then secreting it into the bile. The remaining endotoxin would then be eliminated by phagocytic cells such as granulocytes, monocytes, and macrophages.[28]

Tolerance mechanism towards endotoxin

Jarvelainen et al studied the effect of chronic administration of endotoxin and ethanol on liver pathology and cytokine of rats, demonstrating that chronic administration of endotoxin did not result in steatosis, focal inflammation or necrosis of liver cells, even though there were high levels of endotoxin within the bloodstream. This demonstrates the development of a condition of tolerance towards high levels of endotoxin circulating for long periods of time. The mechanism for the development of such tolerance is still unclear. The absence of liver destruction may be due to the presence of anti-inflammatory cytokines IL-10 and IL-4.[18]

METHOD FOR ENDOTOXIN EVALUATION

Studies to measure plasma endotoxin levels that have been conducted up to date use two methods of the limulus test. The first method to be used is the kinetic turbidimetry method, which evaluates plasma gel-clot formation after interaction with amebocyte lyzate. Evaluation is determined based on visual observation alone, and is considered positive if a persistent solid sediment is found when the tube is placed horizontally.[29,30,31] Evaluation using this method produces inconsistent results, due to a variation of the preparation and sensitivity of the lyzate used, the method used to prepare plasma samples, patient population, and statistical analysis.[32,33]

In the year 1978, Iwanaga et al developed a quantitative method for the evaluation of endotoxin, using synthetic chromogenic peptides as substrates for endotoxin-sensitive limulus enzymes. This evaluation provides quantitative evaluation with a higher specificity and sensitivity compared to the previous gelation evaluation method.[6]

Evaluation using the kinetic turbidimetry method that had become unpopular along with the development of the chromogenic method, regained popularity in 1985, when Oishi, et al invented the toxinometer, which computerized turbidity level reading, thus creating a more standardized and sensitive method.[34]

Factors within the plasma that influence the limulus test, including inhibitors and non-specific amydase, must be eliminated prior to testing. For this cause, several methods can be used, such as dilution and heating, as used in the kinetic turbidimetry method. With the chromogenic method, the perchloric acid (PCA) or new-PCA is used.[35]

In the meantime, it becomes clearer that the specificity of limulus examination for the detection of endotoxin is inadequate.[32,33,36] It has been proven that extracts coming from the blood of limulus polyphemus (horshoe crabs) used in the conventional limulus test as well as the synthetic chromogenic test, also produces a positive result through another pathway, activated by 1-3-beta-d-glucan (beta-glucan), a component of fungal cell walls. Thus, other than the known B and C factors activated by endotoxin, there seems to be another factor, the G factor, activated by beta-glucan and beta-glucan-like activity (BGLA).[13,36,37]

Lately, many methods have been found to obtain a lyzate specific only to endotoxin, by eliminating the G factor from the lyzate. Specificity towards the endotoxin is acquired by adding excessive amounts of glucan into the amebocyte lyzate. Such addition would prevent activation of the G factor, making the test more specific for endotoxins.[37]

Up to now, there are two specific methods for the evaluation of endotoxin. The first method is the calorimetric limulus test (endospecy) method. In this test, a synthetic substance is added as a clotting enzyme. Paranitroalanine (pNA) is released from the enzyme substrate, changing the pNA into red azodyes by means of diazocoupling. The content of the red azodye is then

measured using photometry at 550 nm. The endotoxin is then determined using a standard curve.[37]

The second method is the kinetic turbidimetry method. This method requires the use of computers, but the procedure is fairly simple. The principle of the instrument is as follows: if an endotoxin-containing sample is mixed with limulus reagents, a gellatinous sediment is formed. The passage of light would then be reduced. A reduction up to the pre-programmed cut-off value is then calculated using the instrument. The percent of light transmittance to reach the cut-off value is called the gel sedimentation time or gelation time. Endotoxin could then be quantitatively determined as log [log(Tg)]. If Tg of the sample to be measured has been determined, the level of endotoxin could be calculated using the standard curve.[38]

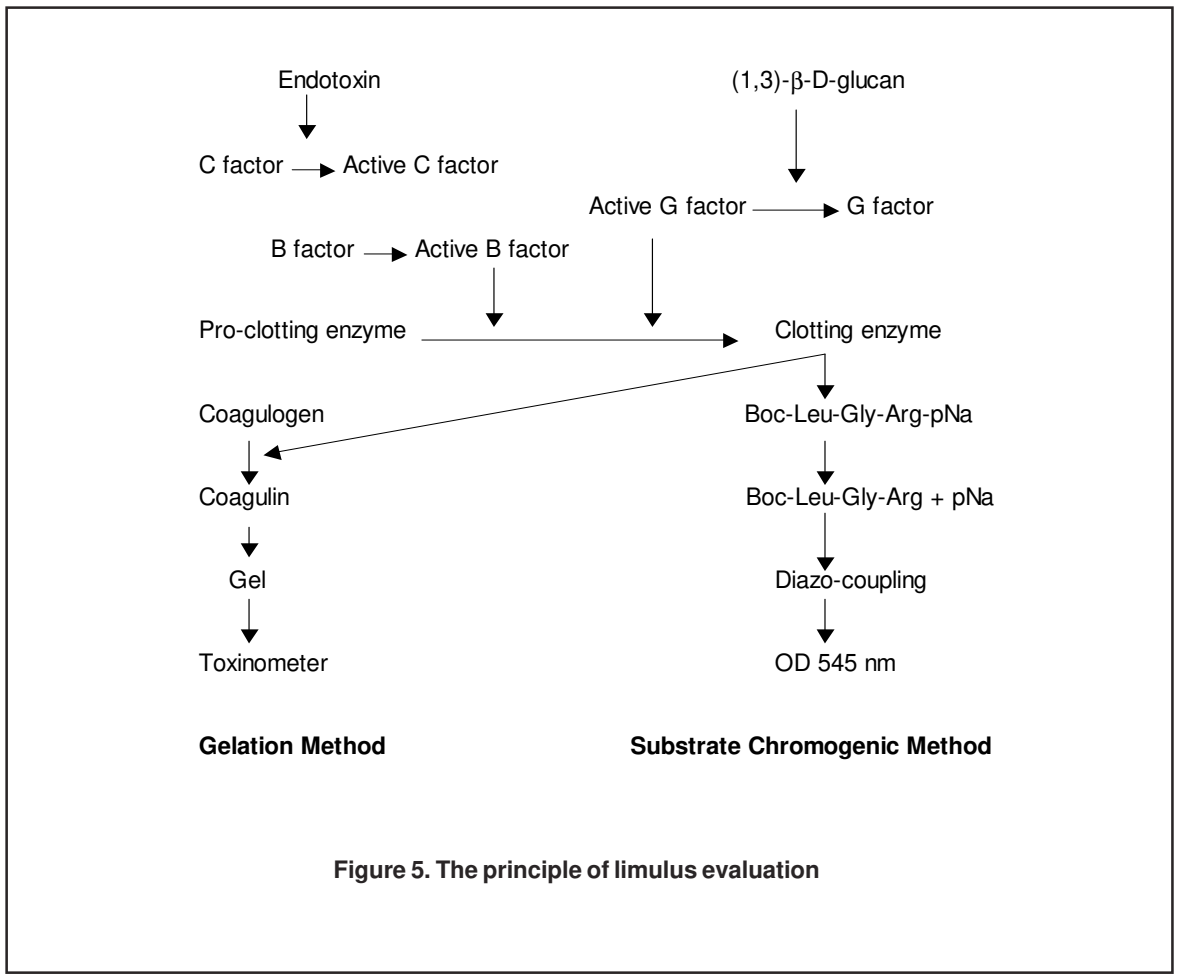

Figure 5. The principle of limulus evaluation

THE PATHOPHYSIOLOGY OF ENDOGENOUS ENDOTOXEMIA

Endogenous endotoxemia is a condition where endotoxins are found within the circulation unaccompanied by negative gram bacteremia.[39] The intestinal mucosal barrier in normal mammals allow the possibility of a small number of macromolecules to pass through the mucosa of the small intestines. This has been demonstrated in laboratory animals, where a small number of endotoxin from the intestinal mucosa normally passes through the normal mucosa into the portal blood vein.[39,40] In conditions of bowel inflammation, alcoholism, hypoperfusion, or anoxia, liver cirrhosis and extrahepatic obstruction, there is a significant increase of endotoxin absorption through the intestinal wall.[3,41]

Endogenous endotoxemia should be differentiated from bacterial translocation. Bacterial translocation is a condition where live bacteria from the gastrointestinal tract passes through the epithelial mucosa to infect mesenteric lymph nodes (MLNs) and systemic organs.[42]

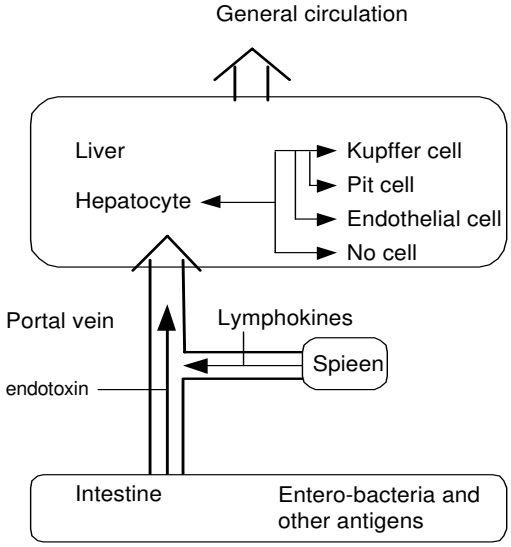

Figure 6. The schematic relationship between liver, spleen, and bowel

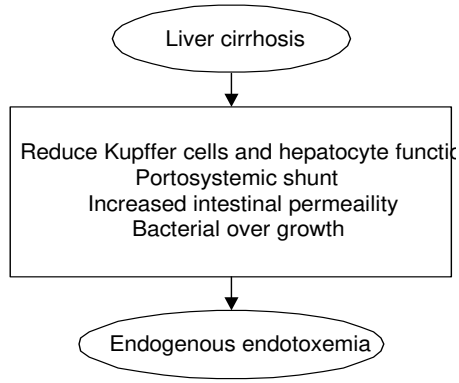

Figure 7. Factors that play a role in the development of endogenous endotoxemia

Several reports state that endogenous endotoxemia is closely related with disorders that reduce liver function,[2] and intestinal wall,[13] trauma,[42] and reduced immune system.[13] The concept of endogenous endotoxemia has become widely accepted.

The presence of a portosystemic shunt and reduced phagocytic function of the rethiculoendothelial system are important factors for the development of endotoxemia in patients chronic liver disease.[4] Liver is the main plase for the cleansing of endotoxin performed by Kupffer cells. In liver cirrhosis, there is reduced Kupffer cell function,r esulting in inadequate detoxification of endotoxins. Portosystemic shunting also results in the passage of endotoxin directly into the systemic circulation. Other factors that contribute to the high level of endotoxin in alcoholic liver disease is increased permeability of the intestinal mucosa towards macromolecules. Such increase in permeability facilitates the passage of endotoxin from the gastrointestinal tract.[4] In addition to the points mentioned, there is also bacterial overgrowth in the small intestines of patients with liver cirrhosis, particularly due to alcoholism. Such bacterial overgrowth increases endotoxin production in the gastrointestinal tract.

Takinawa et al demonstrated a significant increase of endotoxin absorption in laboratory rats injected with lypopolisaccharide (LPS) in the colon. This is found in rats with liver cirrhosis, alcoholism, and extra-hepatic obstruction. Through the electron microscope, endotoxin particles were observed to pass through the colon epithelial cell.

Endogenous endotoxemia in liver cirrhosis

In the year 1972, Caridis et al reported two cases of liver cirrhosis accompanied by increased levels of endotoxin, detected using the limulus test, without bacteremia.[43] This was the first report on endogenous endotoxemia. In the year 1976, Wilkinson et al reported 45 cases of cirrhosis where endotoxemia was detected in 84% (21/25) patients accompanied by renal failure, and in only 10% (2/20) of endotoxemia in patients without renal failure.[30] Clemente et al also reported 43 cases of liver cirrhosis with ascites where endotoxemia was detected in 41% (8/22) of patients with renal failure, while endotoxemia was not detected patients without renal failure (0/21).[31] On another occasion Tarao et al reported that out of 29 patients with liver cirrhosis and ascites, 23 (80%) were found with endotoxin in their ascites fluid, while 22 (76%) was positive for endotoxin within the plasma.[29] Only 23% (4/17) were positive among those without ascites.[29] Among the many studies that found endotoxemia in patients with liver cirrhosis, Fulenwider et al found the opposite, and reported no endotoxin in the peripheral vein, portal vein, nor the ascites fluid of 38 study subjects with liver cirrhosis.[44] All of the studies mentioned utilized the conventional limulus amebocyte lysate (LAL) test which is considered positive if gel formation is visually observed.

Lumsden et al used the non-specific chromogenic method to study the incidence of endotoxemia in the portal vein, hepatic vein, and peripheral vein in patients with liver cirrhosis. They found the highest levels of endotoxin in the portal vein, hepatic vein, and peripheral vein, respectively.[6]

Lin et al, using the same method as Lumsden, et al, demonstrated that the level of plasma endotoxin in-

creased progressively according to the severity of liver dysfunction.[10] Fukui et al (1991), in comparing the endotoxin levels of patients with liver cirrhosis due to alcoholism and non-alcoholic liver cirrhosis patients, found a higher level of endotoxin in patients with liver cirrhosis due to alcoholism.[4] Even though there have been many data that demonstrated increased endotoxin levels in liver cirrhosis using the synthetic chromogenic method, Le Moine et al did not identify such increase in patients with liver cirrhosis classified as Child-Pugh A, B, and C in the 49 study subjects.[23]

In two other studies using the endospecy instrument (an endotoxin-sensitive chromogenic method), Fukui et al conducted measurement of endotoxin levels in patients with chronic hepatitis and liver cirrhosis in patients with liver cirrhosis and bleeding from esophageal varices. Increased endotoxin levels were found in accordance with the progressiveness of liver disease and there is an increase in patients with liver cirrhosis and variceal bleeding compared to those without.[45,46] Results of the study by Fukui is not supported by other studies using the same method. In the study, Fukui modified the standard curve calculation to produce a positive result.

Yajima et al in a study on 8 patients with liver cirrhosis complicated by disseminated intravascular coagulation (DIC) conducted endotoxin evaluation using the convensional method and the endospecy method, demonstrating positive results by conventional evaluation while no increased levels of endotoxin was not detected using the endotoxin-sensitive endospecy. This finding was supported by Kikuchi et al, that studied 9 patients with liver cirrhosis and esophageal varices undergoing the esophageal operation transaction operation and splenectomy, finding no increase in endotoxin levels either in the portal vein or the peripheral vein using the endospecy method.

There is still the possibility that the endotoxin was not detected using a specific method because the endotoxin binds with various proteins and undergo changes within the body, even though this hypothesis has not been proven and may not be correct, recalling the difference between the specific and non-specific method lies only on the G factor, while the other principles of evaluation is the same.

Why endotoxemia does not occur in liver cirrhosis

The presence of endogenous endotoxemia in liver cirrhosis theoretically poses a serious condition, since the endotoxin would activate macrophages/monocytes to release mediators that would then stimulate the inflammatory cascade, resulting in sepsis and septi shock. This is rarely found, except in patients with negativegram infection.

Gabe et al demonstrated that patients with end-stage chronic liver disease (liver cirrhosis with portal hypertension) has a normal intestinal permeability, preventing excessive passage of endotoxin through the intestinal mucosal barrier.[47] However, there has been many studies that demonstrated otherwise, identifying increased intestinal permeability. Studies on Kupffer cell function in patients with liver cirrhosis also found contradictory results, some stating reduced Kupffer cell function and some stating that Kupffer cell function was still adequate despite liver cirrhosis.

CONCLUSION

Evaluations of endotoxin levels that have been conducted up to now have produced controversial results, with studies using the conventional method mostly producing positive results. Further evaluation using the specific method did not detect increased endotoxin levels in stable liver cirrhosis patients. Such finding produces the believe that there is another substance that produces a positive result in the conventional limulus test.

These new findings cast a doubt on the current assumption of the concept of endogenous endotoxemia in patients with liver cirrhosis. There needs to be a reevaluation to explain a more precise concept of endotoxemia in patients with liver cirrhosis.

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