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Endotoxin and Other Microbial Translocation Markers in the Blood: A Clue to Understand Leaky Gut Syndrome

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Abstract

Gut affects various systems in the human body. The leaky gut hypothesis tells us that gut microbial products cause systemic low-grade inflammation, which enhances the progression of various human diseases. Microbial translocation attributable to intestinal barrier dysfunction and hyperpermeability have been proven in major human diseases. Among these microbial products, endotoxin/lipopolysaccharide is most extensively studied in clinical situations. However, its detection in the blood and its impact in the clinical course still arouse much discussion. Overviewing the long-standing controversies in the assay system and the main results in various clinical situations, this review regards plasma endotoxin level as a feasible microbial translocation marker. Although the detection of bacterial DNA in the blood has been gradually accepted among other microbial products, uniformity of analytical methods and usefulness in the clinical site should be established. The results on peptidoglycan, flagellin, lipoteichoic acid, and (1→ 3)-β-D-glucan in the blood are still scarce. The analysis of Toll-like receptors has suggested that several microbial products act concomitantly as pathogen-associated molecular patterns (PAMPs) in the progression of various diseases. Collective efforts to read the whole story on leaky gut and its sequences from the side of circulating microbial products may lead future progress both in patient's care and preventive medicine.

Keywords: Endotoxin; Bacterial DNA; Peptidoglycan; Flagellin; Lipoteichoic acid; (1→3)-β-D-glucan; Microbial translocation; Leaky gut; Toll-like receptors

Introduction

Pathogenic roles of gut and gut dysbiosis have been proposed in various human diseases. The passage of viable bacteria from the intestinal lumen through the mesenteric lymph nodes and other sites are defined as bacterial translocation (BT). The concept of BT was later broadened to microbial products or their fragments, such as endotoxin, peptidoglycan, lipopeptides, and bacterial DNA [1]. On the other side, intestinal epithelial barrier dysfunction and increased permeability have been described in various human diseases, such as inflammatory bowel disease (IBD) [2-7], irritable bowel syndrome [8,9] alcoholic liver disease [10-13] nonalcoholic steatohepatitis (NASH) [14,15] liver cirrhosis [16-19], primary biliary cholangitis (PBC) [20], acute pancreatitis [21-23], type 1 and type 2 diabetes [24-27], chronic kidney disease [28-30], chronic heart failure (CHF) [31,32] and depression [33,34]. Detrix et al. [35] has focused on the delicate role of gut mucosal interface in patients with various surgical or traumatic insults. In general, the leaky gut hypothesis explains that the intestinal barrier dysfunction provokes the chronic low-grade inflammation and functional disturbances in various target organs in virtue of microbial products, which play major roles in the development and progression of these diseases [36]. Several useful markers of gut wall integrity have been proposed. Intestinal fatty acid binding protein (I-FABP), primarily limited to mature enterocytes of the small and large intestine, is a plasma/urinary marker for early enterocyte cell death [35]. It rises rapidly after episodes of acute intestinal ischemia and inflammation [35]. Measurement of tight junction (TJ) proteins such as claudin-3 in the urine and zonulin levels in the blood reflect the dysfunction of TJ causing leaky gut [35-37]. Increased level of D-lactate, a product of bacterial fermentation, is correlated with bacterial overgrowth, short bowel syndrome, and mesenteric ischaemia [35]. Plasma D-lactate was high in patients with end-stage renal disease and positive blood bacterial DNA [38].

The microbial products are translocated from the intestinal lumen to the circulation through disrupted TJs. They react to toll-like receptors (TLRs) in the macrophages, lymphocytes and variable cells and induce inflammation in various tissues. Each TLR detects a specific pathogen-associated molecular pattern (PAMP), including endotoxin/lipopolysaccharide (detected by TLR4), bacterial lipoprotein and peptidoglycan (detected by TLR2), flagellin (detected by TLR5), unmethylated CpG DNA (detected by TLR9), double-stranded RNA (detected by TLR3), and single-stranded RNA (detected by TLR7 and 8). TLR1 and TLR6 recognize tri-acyl lipopeptides and di-acyl lipopeptides, respectively, in cooperation with TLR2 [39,40]. Endotoxin binds to TLR4 with co-receptor CD14 and MD-2. TLR2

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heterodimerizes with TLR1 or TLR6 to recognize lipoprotein and peptidoglycan derived from Gram-positive bacteria [41]. Intracellular TLR3 and TLR9 are activated by microbe-derived nucleic acids including double stranded RNA and CpG motif containing unmethylated DNA, respectively [41]. Taken together, TLRs 1, 2, 4, 5 and 6 seem to specialize in recognizing mainly bacterial products, whereas TLRs 3, 7 and 8 seem to specialize in viral detection [40]. TLR 9 are related both of them.

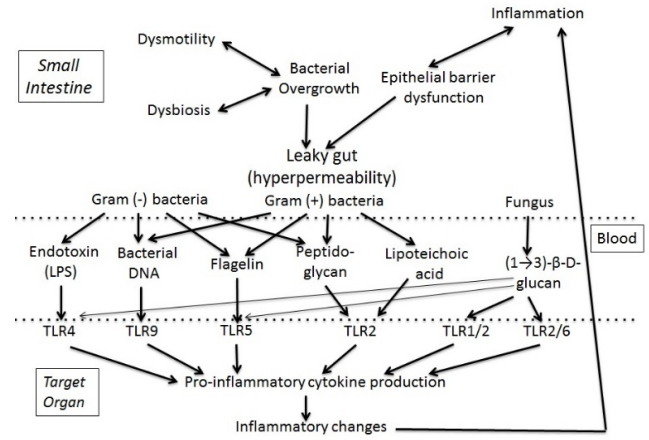

Figure 1 Intestinal dysfunction, main microbial translocation markers in the blood and subsequent inflammatory changes in the target organs. In the small intestine, dysmotility, bacterial overgrowth, dysbiosis and epithelial barrier dysfunction are closely connected to each other and causally linked to the pathogenesis of leaky gut, which subsequently leads to a variety of microbial translocation. Translocated microbial products of Gram negative and Gram positive bacteria and fungus in the blood concomitantly act on Toll-like receptors of various cells, which provokes innate immune responses and promotes proinflammatory signaling inducing inflammatory changes in the target organs. LPS: lipopolysaccharide, TLR: Toll-like receptor.

The levels of these PAMPs in the blood are increased in various clinical situations. Although endotoxin is the most popular circulating marker reflecting gut microbial translocation, the detection of circulating endotoxin is still not complete with several methodological difficulties [1]. Other microbial products have not yet been established as universal inflammatory mediators in the blood. We should reconsider the assay method of these substances and evaluate their relationships to intestinal permeability, leaky gut markers, and pro-inflammatory cytokine and chemokines before we admit them as gut-derived inflammatory mediators with pathogenic relevance in the leaky gut syndrome.

This review will focus on these microbial products as translocation markers and inflammatory moderators in relation to gut leakiness and various inflammatory changes to understand the concept of leaky gut syndrome and to find out whether they are useful circulating markers reflecting gut-derived inflammatory changes. **Figure 1** summarized the intestinal dysfunction, main microbial translocation markers in the blood and subsequent inflammatory changes in the target organs.

Endotoxin and Related Markers

History of blood endotoxin assay and their problems

Lipopolysaccharide (LPS) known as endotoxin is a major component of the Gram-negative bacterial wall. It can cross the deranged paracellular TJ or can be taken up by the enterocytes coupled with damaging lipoproteins, because it has a strong affinity for chylomicrons [42]. Its detection in the blood has a long and evolving history but is still not complete with several methodological difficulties [1]. Endotoxemia was first demonstrated by the Limulus amoebocyte lysate (LAL) test and later by several quantitative assays, such as the chromogenic Limulus assay and the turbidometric endotoxin assay. Although these assays have been shown to give reliable results for minute amount of endotoxin in water, measurements of endotoxin in blood present some difficulties. Major problems include the way of preparing standard curves [43-45], the optimal method for eliminating plasma inhibitors of endotoxin [45-47], and specificity of LAL for endotoxin [48,49]. We have insisted that a standard curve should be prepared for each individual plasma sample in the endotoxin determination, because ideal 100% recovery of endotoxin is not validated in any trial of plasma pretreatment [45]. The internal standard is especially necessary in the acid pretreatment, where strict adjustment of pH (i.e. neutralization) for the chromogenic assay is sometimes difficult [50,51]. However, the influence of slight pH shift can be overcome by the existence of internal standard. We discovered hidden extra portion of endotoxin in plasma of patients with chronic liver diseases, using new way of plasma pretreatment either by Tween 80 in the dilution and heating method or by triethylamine in the perchloric acid method [50]. Finally, the LAL reagents widely used in the world react not only endotoxin but also (1,3)-β-D-glucan, component of cell wall of fungus [48], because they contain factor G reacting (1,3)-β-D-glucan in addition to factor C reacting endotoxin. Although endotoxin-specific reagents were produced early in Japan by removing G-factor from the lysate [48] proposed methods of sample pretreatment thereafter [52-54] were not adequate for the detection of minute amount of endotoxin in the blood of patients without bacteremia. Although some Japanese investigators have doubted the existence of portal endotoxemia, spillover endotoxemia or metabolic endotoxemia because of the negative results in their studies using the endotoxin-specific LAL tests, I disagree with them because we did detect endotoxemia in patients with chronic liver diseases by our improved chromogenic assay using endotoxin-specific substrate Endospecy (Seikagaku Kogyo Co., Tokyo, Japan) after the pretreatment of sample by perchloric acid and triethylamine [49,51]. A final pH of the sample was always adjusted to 7 by careful titration of triethylamine and the reactivity of each sample containing internal standard was confirmed by kinetic analysis [51]. The results of our

measurement of plasma endotoxin in 90 patients with liver cirrhosis and 11 patients with chronic hepatitis with this method were summarized as follows: 1) There was an increase of plasma endotoxin with the progression of chronic liver disease. 2) In patients with bleeding from esophageal varices, plasma endotoxin increased for 3 days after the bleeding and thereafter decreased. 3) Endotoxin level increased as the progression of Child-Pugh grades and was negatively correlated to prothrombin time [1,49]. However, this improved method has not been generally accepted because of its methodological complexity despite its precise setting and reliability for plasma endotoxin assay. The prevailing endotoxin-specific turbidometric assay also has a drawback of poor sensitivity for the clinical use. Obata et al. [55] developed a new detection method using laser scattering photometry to analyze the formation of small particles of clotted enzyme in the process of LAL gelation. This endotoxin scattering photometry (ESP) has been proved to be more sensitive than the conventional turbidimetric LAL assay and proposed to be useful in discriminating between sepsis and septic shock in patients undergoing gastrointestinal emergency surgery [56].

Significance of endotoxemia in various diseases

Most studies on endotoxin measurement in the world have used non-specific LAL reagents after the dilution and heating of samples, ignoring the above problems. There still remains various problems on the overall assay validation as described in a recent extensive review [57]. However, accumulating results on clinical endotoxemia tested by this simple modification of LAL test are generally acceptable for clinicians, because they reflected clinical findings well and were closely associated with inflammatory reactions in various diseases. I would like to list up positive results in relation to leaky gut hypothesis in various diseases.

Endotoxemia in liver diseases: Among all liver diseases, alcoholic liver injury has been admitted as the typical entity in close association with leaky gut and endotoxemia. There is strong evidence to support the concept that gut-derived endotoxin plays a central role in the initiation and progression of alcohol-induced liver injury [58]. Once endotoxin reaches various organs, it powerfully stimulates TLR4 both in hepatic macrophages (Kupffer cells) and extrahepatic macrophages, which activate downstream signalling pathways responsible for overproduction of pro-inflammatory cytokines such as TNF-α, IL-6, and IL-8. Plasma endotoxin levels were increased with the progression of alcoholic liver injury and reached the maximal level in patients with alcoholic cirrhosis and severe alcoholic hepatitis who showed marked hypercytokinemia [59]. Plasma endotoxin level was reported to be positively correlated to TNF-α [60] and IL-8 levels [59]. Parlesak et al. [12] further found significant correlation between the plasma endotoxin levels and the intestinal permeability measured by the PEG Mr 4000 method.

In patients with cirrhosis, the plasma endotoxin levels progressively increased in relation to the severity of liver dysfunction [49,61]. Lumsden et al. [62] and Tachiyama et al. [63] further found significant increase of endotoxin levels in the portal blood from cirrhotic patients. This suggested an increased intestinal production and/or absorption of endotoxin in cirrhotics, which later opened the discussion on bacterial overgrowth and leaky gut in liver cirrhosis. Major complications of liver cirrhosis such as renal disturbance [64,65] gastrointestinal bleeding [51,65,66] and infection are reported to be related to endotoxemia. Recently TJ proteins occluding and claudin-1 expression were reported to be inversely correlated to endotoxin levels, which suggests that altered intestinal TJ expression leads to intestinal hyperpermeability and endotoxemia in liver cirrhosis [67].

Significant endotoxemia was also detected in patients with early stage of primary biliary cholangitis (PBC), and increased immunohistochemical expression of TLR4 and CD14 was found in the liver of PBC patients [68].

Recent clinical studies have brought the attention of clinicians to the role of endotoxemia in patients with nonalcoholic fatty liver disease (NAFLD), the hepatic manifestation of the metabolic syndrome. NAFLD includes a spectrum of pathological changes ranging from the simple fatty liver (NAFL), nonalcoholic steatohepatitis (NASH) to fibrosis and cirrhosis [69]. NAFLD patients reveal gut hyperpermeability characterized by disruption of the intercellular TJs with decreased ZO-1 expression, which may enhance translocations of bacteria and their products [14]. Several groups reported elevated serum endotoxin in NAFLD and NASH patients [70-72]. NASH patients showed endotoxemia and overexpression of TLR4 in the liver [73,74] associated with pro-inflammatory cytokine production and inflammation. Elevated endotoxin level was further correlated with insulin resistance and several inflammatory cytokines in NAFLD patients [75,76]. NAFLD patients with small intestinal bacterial overgrowth (SIBO) showed significantly higher endotoxin levels and TLR4 expression in the liver [74].

Serum lipopolysaccharide-binding protein (LBP), which has longer half-life than endotoxin, is evaluated as another useful surrogate marker of BT and endotoxemia [76]. LBP levels were associated with pro-inflammatory state and hemodynamic derangement in liver cirrhosis, which were proved to be mitigated by selective intestinal decontamination with norfloxacin [77]. In non-infected cirrhotics with ascites, increased serum LBP was selected as the only factor independently associated with first severe bacterial infection in a multivariate analysis [78].

Metabolic endotoxemia: In type 2 diabetes together with metabolic syndrome, the gut barrier dysfunction is again considered to induce BT and so-called metabolic endotoxemia, which subsequently predispose to systemic low-grade inflammation and insulin resistance [79]. Increased intestinal permeability and its relation to systemic inflammatory markers were reported in these patients [27]. Although baseline endotoxin level was already high in patients with type 2 diabetes [80,81], high-fat meal further augmented this endotoxemia [81]. Hawkesworth et al. [82] reported that endotoxin levels were highest in the obese-diabetic women compared with the obese non-diabetic and lean women. On the contrary, anti-core endotoxin IgM antibody (EndoCab IgM)

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levels were markedly lower in the obese and obese diabetic women compared with their lean counterparts. This could be explained by degradation of IgM-LPS complex that constantly neutralizes a persistent endotoxin leakage from the gut [82]. In type 2 diabetes, endotoxin activity was further positively correlated with the serum levels of TJ protein ZO-1 and pro-inflammatory TNF-α and IL-6 [83]. In the feces of type 2 diabetics, relatively higher abundance in endotoxin producing Gram-negative bacteria and lower abundance in butyrate-producing bacteria were reported [84].

Endotoxemia in inflammatory bowel diseases: The relation of endotoxemia to active Crohn's disease (CD) was noted early in the period of LAL test [85,86]. Gardiner et al. [87] found endotoxemia in 88% patients with ulcerative colitis (UC) and 94% patients with CD even during clinical relapse and that endotoxemia was correlated with anatomic extent and clinical activity of UC. They further showed that plasma IgG endotoxin core antibody concentrations were significantly high in patients with CD, highest in patients with small intestinal CD, and correlated with endotoxemia and the soluble TNF receptor p55 [87]. They concluded that endotoxemia and its correlation with disease activity, disease extent, and circulating TNF supported a pathogenic role of endotoxin in IBD [87]. Pastor Rojo et al. [88] later found the elevation of plasma endotoxin levels in 48% patients with CD and in 28% patients with UC, where endotoxin, LBP and sCD14 levels were correlated with disease activity and normalized after treatment. Lakatos et al. [89] further reported that LBP and sCD14 levels were significantly correlated to high-sensitivity C-reactive protein (hs-CRP) and admitted that they were useful markers of active disease like hs-CRP.

Endotoxemia in pancreaticobiliary diseases: Endotoxemia was found in 51%~85% patients with severe acute pancreatitis [90,91] and was more common in non-survivors compared with survivors [90]. Necrotizing pancreatitis is accompanied by persistent marked endotoxemia [92]. The presence of endotoxin in blood and peritoneal fluid was associated with the disease severity, systemic complications, and mortality rates of acute pancreatitis [93]. EndoCab IgM levels conversely decreased early in patients with severe acute pancreatitis, although it remained relatively unchanged in those with mild pancreatitis [91,94,95]. In patients with acute pancreatitis, intestinal permeability was proved to be increased [21-23] which was correlated with plasma endotoxin [21-23,96] serum TNF-α [21,23] IL-6, and CRP levels and with the severity index estimated by computed tomography [23]. Mucosal function was injured early in those with organ dysfunction [21]. The expression of TJPs (occludin and ZO-1) in the colonic mucosal tissue was decreased in patients with severe acute pancreatitis, 62% of which showed BT (positive bacterial DNA in the peripheral blood) [97]. It is thus plausible that the failure of intestinal barrier predisposes to translocation of bacteria and inflammatory products through the intestinal wall, which can induce the infection of necrotic pancreas and systemic inflammatory response [98]. Plasma endotoxin was positively correlated with the abundance of Enterococcus, whereas serum IL-6 levels were positively correlated with the abundance of *Enterobacteriaceae* and *Enterococcus* and negatively correlated with that of *Bifidobacterium* in the feces, which suggested that the intestinal *dysbiosis* may be involved in endotoxemia and inflammation of acute pancreatitis [99].

The relation between obstructive jaundice and endotoxin was detected early in 1976, when Bailey [100] detected endotoxemia in 67% of portal blood and 54% of peripheral venous blood from patients with obstructive jaundice and thought that this endotoxemia was mostly gut-origin. He additionally noted that patients with endotoxemia showed evident decrease in endogenous creatinine clearance postoperatively, while those without endotoxemia did not [100]. The following studies further confirmed that malignant obstructive jaundice caused endotoxemia and inflammatory cytokinemia [101,102]. Several authors [101,103,104] reported increased intestinal permeability, which was also associated with BT [105]. Altered TJP, decreased expression of occluding, claudin-1 and -7, was further found in the duodenal epithelium of patients with obstructive jaundice [106]. The significance of endotoxemia as a key mediator of obstructive jaundice was thus established by the LAL test and its modification. Nevertheless, Kimmings et al. [101] reported that baseline plasma endotoxin levels in patients with obstructive jaundice were unexpectedly low and were not affected by the biliary drainage, while increased endotoxin binding proteins (LBP, sCD14 and BPI) were reduced after the drainage. As they stated, the endotoxin assay is potentially influenced by the yellow color of jaundiced plasma [101]. Other methods (i.e. turbidimetric assay or endotoxin acitivity assay) may solve this problem in jaundiced patients.

Endotoxemia in cardiorenal diseases: Plasma endotoxin and inflammatory cytokine levels were elevated in patients with CHF [107,108] and in adult patients with congenital heart disease [109], which were related to cardiac functional status. Increased intestinal permeability was again reported in patients with CHF [31]. These results suggest that disturbed intestinal microcirculation may induce gut barrier dysfunction and translocations of bacteria and their products, which may predispose to cytokine generation, thereby contributing to further impairment of cardiac function [110].

In the early 1980s [111,112] intestinal mucosal changes such as shortening of the villi, elongation of the crypts, and infiltration of lamina propria was reported in patients with chronic kidney disease (CKD). Intestinal hyperpermeability was reported later in these patients [28]. Recent quantitative endotoxin assay further revealed that circulating endotoxin levels were elevated along with the progression of CKD stages and serum CRP levels [113-115]. Vaziri et al. found marked disintegration of the colonic epithelial barrier structure and significant colonic dysbiosis in patients with advanced CKD [116] and concluded that this disruption of the intestinal epithelial barrier can induce systemic inflammation by enabling influx of endotoxin and other noxious luminal contents [117].

Other Microbial Translocation Markers

Peptidoglycan and flagellin

Peptidoglycan and flagellin, which bind to TLR2 and TLR5, respectively, have been implicated in the pathogenesis of inflammation of the liver and intestine [118]. Peptidoglycan is a common component of both Gram-positive and Gram-negative bacteria, accounting for about 70% and 20% of bacterial cell walls, respectively [119]. The silkworm larvae plasma (SLP) test was developed as a quantitative assay for peptidoglycan and β-glucan in plasma and dialysate [119,120]. Elevated SLP activity, which was noted in experimental hemorrhagic shock and alcohol intoxication [121,122], was reported also in infected patients during the postoperative period of gastrointestinal surgery [123]. It was also elevated in 83.6% patients with severe bacterial infection [119]. Transient rise of SLP activity noted in patients at the termination of cardiopulmonary bypass is considered attributable to hypoperfusion of small intestinal mucosa and resultant BT [124]. Kim et al. later developed a new tool to detect circulating peptidoglycan-like structure using a NOD2-transfected cell line and reported elevated levels of circulating NOD2 agonist in 90.5% patients with abdominal aortic surgery [125]. This circulating NOD2 agonist levels were correlated to IL-10 and cortisol levels and were more sensitive as a marker of BT than plasma endotoxin levels [125]. However, the measurement of peptidoglycan has been adopted by restricted surgical institutions, which makes its evaluation as a general BT marker difficult.

Flagellin is a monomeric subunit of flagella found on motile bacteria [126]. It is detected by an ELISA that recognizes a broad array of Gram-negative flagellin [127]. Flagellin interacts with basolateral TLR5 on gut epithelial cells leading to the secretion of inflammatory cytokines and chemokines [128]. Serum flagellin was detected either with or without endotoxemia in adult patients with short bowel syndrome (SBS) [127]. Flagellin-specific serum IgM, IgA, and IgG levels were also markedly increased in these SBS patients [127]. Elevated serum IgA and IgG titers against flagellin derived from *Escherichia coli* and other commensal bacteria were found in some patients with CD as well [129-131], which was associated with an increased adaptive immune response to flagellin [129-131], immune dysregulation and gut barrier dysfunction [127]. In addition, the level of antibodies to flagellin in the blood was higher in patients with diarrhea-predominant irritable bowel syndrome [132]. Human immunodeficiency virus (HIV) infection induces depletion of mucosal $CD4^+$ lymphocytes, which is linked to disruption of gut epithelial integrity and increased mucosal translocation of bacteria and their products from the intestinal lumen to the systemic circulation [133,134]. The levels of anti-flagellin antibodies were also used as a microbial translocation markers after the treatment in patients with HIV-infection [135]. Munukka et al. [136] found that the expression of bacterial flagellin-recognizing TLR5 in the adipose tissue was associated with liver fat content and insulin sensitivity in healthy women, while no such associations were found for LPS-recognizing TLR4 expression. Together with their experimental results, they thought that the adipose tissue TLR5 expression may promote liver fat content in humans [136]. The evaluations of flagellin and its antibodies as serum BT markers have been thus very limited until now. Further studies in different clinical situations are needed.

Lipoteichoic acid

Lipoteichoic acid (LTA) is a cell wall component exclusive to Gram-positive bacteria and is shed during bacterial replication and after antibiotic administration [137]. Although LTA is the functional equivalent to endotoxin from Gram-negative bacteria, they induced differential cytokine/chemokine release via effects distal to activation of NF-κB/AP-1 [137] via TLR2 cluster [138]. LTA, as well as peptidoglycan, is released spontaneously into the culture medium during growth of Gram-positive bacteria, which was enhanced by incubation with antibiotics [139]. Like endotoxin, HDL has the highest binding capacity for LTA in the blood [140]. A continuous redistribution of LTA primarily from HDL to LDL occurs under simulated physiological conditions, which suggests that lipid transport proteins recognize and process endotoxin and LTA in a similar fashion [140]. However, unlike endotoxin, circulating level of LTA has not been reported in relation to clinical inflammation. Histologically, LTA-containing mononuclear cells were frequently detected in the portal tracts, particularly around the bile ducts and in hepatic sinusoids in patients with primary biliary cholangitis (PBC) [141]. LTA was detected at the sites of inflammation around damaged bile ducts in the livers of PBC, and sera from PBC patients showed higher levels of anti-LTA titers compared with those from chronic hepatitis C patients or from healthy subjects [142].

(1→3)-β -D-glucan

(1→3)-β-D-glucan (BG) is a polysaccharide found in the cell wall of fungal organisms such as *Aspergillus, Candida*, and *Pneumocystis* [143]. BG is highly immunogenic. It activates macrophages, neutrophils, and T-cells and stimulates release of pro-inflammatory cytokines [143]. (1-3)-β-D-glucan powerfully co-stimulate cytokine production (IL-6/IL-8) induced by ligands for TLR1/2, TLR2/6, TLR4, and TLR5 [144]. Circulating BG is measured by the modification of quantitative LAL test: removal of bacterial endotoxin-sensitive factor C from the LAL reagent. So far, several specific tests for BG detection have been developed, namely Fungitell kit, FungitecG test, Wako BG assay and GKT-25M set. BG might be a useful indicator of gut mucosal barrier impairment [145]. Serum BG levels are increased in patients with active CD and positively correlated with the severity of the disease [146]. Higher blood BG levels were significantly related to higher global deficit scores in HIV-infected adults, reflecting worse neurocognitive performance among patients with suppressed viral loads [145]. Translocation of gut microbial products into the systemic circulation is likely an important driver of immune

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dysfunction and persistent inflammation in the pathogenesis of neurocognitive dysfunction during HIV-infection [145].

Bacterial DNA

The detection of bacterial DNA in the blood by polymerase chain reaction (PCR) is considered as a surrogate marker for the diagnosis of BT showing higher sensitivity than blood cultures and longer half-life than endotoxin [76]. DNA is extracted from blood and PCR techniques used to amplify genes from *E. coli, Bacteroides fragilis* and 16SrRNA found in many Gram-positive and Gram-negative bacteria [147,148].

TLR9 is activated by bacterial DNA containing unmethylated CpG-dinucleotides in a particular base context called CpG-motif [149]. Bacterial DNA is present in 39% of the portal venous blood sample and in 43% of hepatic venous blood sample, suggesting that no major hepatic elimination of bacterial DNA occurs in cirrhosis [150]. Bacterial-DNA induces a marked immune reaction in vivo in patients with advanced cirrhosis and ascites [151]. Presence of bacterial DNA is also associated with aggravation of peripheral vasodilation and with worsening of intrahepatic endothelial dysfunction [152]. Cirrhotic patients with translocation of bacterial DNA from Gram-positive microorganisms showed increased pro-inflammatory cytokine levels unrelated to endotoxin [153]. In this meaning, bacterial DNA seems to be superior to evaluate BT as a whole compared with endotoxin.

Ortiz et al. [154] demonstrated that the translocation of bacterial products into the blood of morbidly obese patients characterizes a subgroup of patients who are not able to reduce their systemic inflammatory cytokine levels after following a massive weight reduction protocol consisting of a fasting period followed by bariatric surgery. Their multivariate analyses revealed bacterial DNA as an independent significant factor, explaining the systemic cytokine response and the insulin resistance levels [154].

Bacterial DNA was also found in 44% of patients with active CD versus 23% of patients with remitting disease [155]. Bacterial DNA in the blood was the only independent factor associated with relapse at 6 months by the multivariate analysis [155]. Preoperative bacterial DNA translocation into the blood increased the incidence of postoperative adverse outcomes in patients with CD who underwent abdominal surgery [156].

Gut bacterial DNA fragments are detectable in the bloodstream from both CKD and dialysis patients, and correlate with severity of systemic inflammation [30]. Circulating bacterial DNA fragment level is a strong predictor of cardiovascular event, need of hospitalization, as well as the progressive change in arterial stiffness in new peritoneal dialysis patients [157]. The plasma bacterial DNA level was also correlated with serum CRP and endotoxin levels in peritoneal dialysis patients [158]. In 20.7% of hemodialysis patients, bacterial DNA fragments were present in the whole blood. Serum CRP and IL-6 levels were significantly higher in chronic hemodialysis patients with bacterial DNA fragments than in those without [159].

The prevalence of positive bacterial DNA was significantly higher in HIV–infected patients with chronic hepatitis C than in healthy blood donors [160]. Patients with markers of advanced liver disease (F3/F4, and A2/A3) and high fibrosis progression rate (FPR >0.15) had higher plasma values of bacterial DNA than did patients without these markers of liver disease [160]. Surprisingly, Bala et al. [161] reported that a single alcohol binge (2 ml vodka 40% v/v ethanol/kg body weight in a total volume of 300 ml orange/strawberry juice) resulted in increased serum endotoxin and 16S bacterial rDNA levels in healthy individuals. This interesting observation further supports the usefulness of bacterial DNA as a marker of leaky gut.

In spite of these accumulating promising results, uniformity of analytical methods of bacterial DNA is still needed to ascertain its real value in routine clinical setting [76].

The Expression of TLRs in Various Diseases

Table 1 summarized the changes in circulating microbial translocation markers in relation to expression of TLRs in various clinical situations. Several TLRs are sometimes concomitantly affected in aforementioned diseases. For example, hepatic TLR 1-5 mRNAs expression was upregulated in NAFLD patients [162]. Although TLR4 plays a cardinal role in the pathogenesis of alcoholic liver disease [163], upregulation of TLR3 and TLR7 in the liver tissue is associated with end-stage alcoholic liver disease [164]. In alcohol-fed animals, most of hepatic TLRs expression (TLR1, TLR2, TLR4, TLR6, TLR7, TLR8 and TLR9) was further upregulated [165].

TLRs expression in patients with liver cirrhosis are conflicting as summarized by Yang et al [166]. Although increased TLR4 expression in the peripheral blood mononuclear cells (PBMCs) was reported by one group [167], other investigators reported that TLR4 expression was decreased and TLR2 expression was increased in cirrhotic PBMC [168-170]. Testro et al. [171] noted that decreased TLR4 expression and hyporesponsiveness to LPS in PBMCs were recovered after antibiotics treatment targeting enteric Gram-negative bacteria in patients with advanced Child C alcoholic cirrhosis. In contrast, hepatic expression of TLR2 and TLR4 was increased or unchanged in patients with cirrhosis [166,168,172]. Soares et al. [172] thought that upregulation of TLR2, TLR4 and their pro-inflammatory mediators were associated with virus-induced hepatic inflammation-fibrosis-carcinoma sequence.

In PBC patients, TLR3 is highly expressed on macrophages surrounding portal tract and hepatocytes [173], while TLR4 expression is significantly elevated in biliary epithelial cells, periportal hepatocytes [68], and PBMCs [174].

Table 1 Changes in circulating microbial translocation markers in relation to expression of Toll-like receptors in various clinical situations.

	LPS	Peptidoglyan	Flagellin	LTA	BG	Bact-DNA	TLRs
Alcoholic liverdisease	↑					↑	Liver TLR3,4,7↑
Liver cirrhosis	↑					↑	PBMC TLR2,4↑ TLR4↓ recovered by antibiotics liver TLR2,4→↑
PBC	↑			anti-LTA↑			Liver TLR3,4↑
NAFLD	↑						Liver TLR1-5↑
Severe obesity	↑					↑	Adipose tissue TLR2,3,4,5↑ Wounds TLR1,2,4,6↑
Type 2 diabetes	↑						PBMC TLR2,4↑ adipose tissue TLR2,4↑ wounds TLR1,2,4,6↑
Active Crohn's disease	↑		anti-F (CBir-1)↑		↑	↑	Colon epithelium TLR2,4↑ ileal epithelium TLR4↑
Ulcerative colitis	↑		anti-F (CBir-1)↑				Colon epithelium TLR2,4↑
severe acute pancreatitis	↑						
obstructive jaundice	↑						
chronic heart failure	↑						Myocardium TLR4↑
chronic kidney disease	↑					↑	PBMC TLR2,4↑ neutrophil TLR4↑ diabetic nephlopathy glomerular endothelium TLR2↑ tubular epithelium TLR4↑
Short bowel syndrome	↑		↑ anti-F↑				
HIV-infection	↑		anti-F↑		↑	↑	PBMC TLR6,7,8↑ advanced infection PBMC TLR2,3,4↑
depression	anti-LPS↑					↑	Brain TLR3,4↑
Cardiopulmonary bypass	↑	↑					

LPS: lipopolysaccharide (endotoxin), LTA: lipoteichoic acid, BG : (1→3)-β-D-glucan, anti-F: anti-flagellin ↑: increased, ↓: decreased

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Colonic crypt epithelial cells isolated from active CD and ulcerative colitis revealed significantly higher expression of TLR2 and TLR4 compared with control cells [175]. Enhanced expression of TLR4 was also noted in crypt cells from ileal CD [175].

Type 2 diabetic subjects showed significantly increased TLR2, TLR4 mRNA and proteins in PBMCs [176]. As for diabetic complications, TLR1, 2, 4, and 6 mRNA expressions were increased significantly in wounds of diabetic patients compared with non-diabetic wounds [177]. Recent studies have further highlighted the critical role of TLRs, mainly TLR2 and TLR4, in the pathogenesis of diabetic nephropathy [178]. TLR2 and TLR4 are upregulated in monocytes, and TLR4 is upregulated in neutrophils of end-stage renal disease patients [179].

Chronic, untreated HIV-1 infection was significantly associated with increased mRNA expression of TLR6, TLR7, and TLR8 in PBMC and when analysis was limited to those with advanced disease (CD4 cell count <200 cells/ml) TLR2, TLR3, and TLR4 were additionally elevated [180]. In the postmortem brain of depressed suicide victims, TLR3 and TLR4 expressions were increased in the dorsolateral prefrontal cortex [181]. In case of cardiopulmonary bypass surgery, a 29% reduction in monocyte TLR4 expression was noted at the end of operation and more than 120% increase in monocyte TLR2 and TLR4 expression was noted in the following day [182].

Taken together, these results suggest that several microbial products might be concomitantly increased as pathogen-associated molecular patterns (PAMPs) and reacting to separate TLRs, which may work together and strongly promote the progression of major diseases. Although the additional effects of endogenous damage/danger-associated molecular patterns (DAMPs) unrelated to microbiome cannot be neglected especially in metabolic disorders such as NASH or type 2 diabetes, the whole figures of circulating PAMPs in clinical situations cannot be depicted at present.

In line with the leaky gut hypothesis, outlines of gut leakiness, endotoxin, TLRs and inflammatory mediators in various clinical situations were summarized also in my previous review [36].

Revisit to the Endotoxin Assay

The main issue of this article was to understand the concept of leaky gut syndrome from clinical microbial translocation markers. The detection of endotoxin in the blood is regarded as the most reliable tool for this purpose, although there still remain several problems in the methodology and the interpretation of results. We have previously compared endotoxin-specific chromogenic substrate with the conventional chromogenic substrate and could not find out any superiority of the endotoxin-specific test for evaluation of BT. Both tests were well correlated to clinical course and considered to be useful markers in patients with chronic liver diseases. It should be noted that (1-3)-β-D glucan powerfully co-stimulate cytokine production (IL-6/IL-8) induced by ligands for TLR1/2, TLR2/6, TLR4, and TLR5 [144]. Elevated plasma glucan levels in patients with bacterial infections and low plasma glucan levels in normal individuals may be attributable to the movement of glucan from the gut into the blood and not necessarily to the presence of pathogenic fungus [1,183]. Although most prevalent quantitative LAL test reacts (1-3)-β-D-glucan and is not endotoxin-specific, both endotoxin from Gram-negative bacteria and (1-3)-β-D-glucan from fungus are microbial translocation products [1] and strongly co-stimulate innate immune system and induce the production of inflammatory mediators. While the additive influence of (1-3)-β-D-glucan on the measured endotoxin level cannot be neglected, its close correlations to systemic inflammatory markers and clinical findings seems to validate the usefulness of the assay as a general marker of microbial translocation.

To confirm the usefulness of these assays, we can further try other new endotoxin assays and compare the results. Endotoxin activity assay [184] is an indirect method for evaluation of endotoxemia measuring the chemiluminescence of superoxide produced by neutrophils that incorporate endotoxin combined with anti-lipid A antibody and complements. This assay has been proved to be useful for assessing disease severity and predicting outcome in critically ill patients [185-187], liver transplant recipients [188] and patients with biliary tract infection [31]. We ourselves have noted its clinical usefulness for various hepatobiliary diseases (unpublished data). Its simplicity and rapidity may further broaden the potential clinical applications.

Two breakthroughs in the endotoxin assay, the factor C system [189] and the cell-based biosensor system [190,191], have been recently introduced, although they have not been intended to detect endotoxin in the blood. Both of them have a merit to protect horseshoe crab resources. If hTLR4A-MD2-CD14 cells reported by Jiang et al. [191] can be reconstituted for the detection of endotoxemia removing the interferences with responses by cytokines, this may become a promising alternative to endotoxin assay in the blood.

Conclusion

This review concludes that plasma endotoxin determination is considered as a feasible microbial translocation marker in spite of methodological controversies. Although the detection of bacterial DNA in the blood may be useful and promising, the detection method should be more simple and unified for routine clinical use. In order to determine an ideal setting of BT markers, we should continue further comprehensive studies on these and other microbial markers and their relations to host reactions. Our goal is to read the whole story on the leaky gut and its consequences in various clinical scene. Intestinal bacterial overgrowth and dysbiosis may additionally predispose to gut leakiness and BT. A rational attempt to modulate intestinal microbiome and permeability is a cornerstone for the prevention of pathological BT. The clinical approaches to the gut disturbances along with the leaky gut hypothesis appears to be a promising and innovative tool in controlling various diseases in the future.

Conflict of Interest

The author declares that there is no conflict of interest regarding the publication of this paper.

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