Detection of Microbial Translocation in HIV and SIV Infection Using the Limulus Amebocyte Lysate Assay is Masked by Serum and Plasma

The Harvard community has made this article openly available. Please share how this access benefits you. Your story matters

Citation

Published Version
doi:10.1371/journal.pone.0041258

Citable link
http://nrs.harvard.edu/urn-3:HUL.InstRepos:10576040

Terms of Use
This article was downloaded from Harvard University’s DASH repository, and is made available under the terms and conditions applicable to Other Posted Material, as set forth at http://nrs.harvard.edu/urn-3:HUL.InstRepos:dash.current.terms-of-use#LAA
Detection of Microbial Translocation in HIV and SIV Infection Using the *Limulus* Amebocyte Lysate Assay is Masked by Serum and Plasma

Ashwin Balagopal¹*, Lucio Gama², Veronica Franco¹, Julia N. Russell², Jeffrey Quinn¹, Yvonne Higgins¹, Laura M. Smeaton³, Janice E. Clements², David L. Thomas¹, Amita Gupta¹, for the NWCS 319 and ACTG 5175 study team

¹ Department of Medicine, Johns Hopkins University, Baltimore, Maryland, United States of America, ² Department of Molecular and Comparative Pathobiology, Johns Hopkins University, Baltimore, Maryland, United States of America, ³ Center for Biostatistics in AIDS Research, Harvard School of Public Health, Boston, Massachusetts, United States of America

Abstract

**Objective:** Microbial translocation (MT) is thought to be a major contributor to the pathogenesis of HIV-related immune activation, and circulating lipopolysaccharide (LPS) from Gram-negative bacteria is the principle measurement of this process. However, related research has been impeded by inconsistent LPS test results.

**Methods:** Specimens were obtained from HIV-infected adults enrolled in the PEARLS study (ACTG A5175) and HIV-HCV co-infected participants enrolled in a study of liver disease staging using MRI elastography. Pig-tailed macaque specimens were obtained from SIV-infected and −uninfected animals. Samples were tested for LPS using the LAL assay with diazo-coupling modifications to improve sensitive detection.

**Results:** When exogenous LPS was added to macaque plasma, >25% inhibition of LPS detection was found in 10/10 (100%) samples at 20% plasma concentration compared to control; in contrast 5/10 (50%) samples at 2% plasma concentration (p = 0.07) and 0/10 (0%) at 0.1% plasma concentration (p = 0.004) showed >25% inhibition of LPS detection. Similarly, when LPS was added to human serum, >25% inhibition of LPS detection was found in 5/12 (42%) of samples at 2% serum concentration compared to control, while 0/12 (0%) of samples in 0.1% serum showed >25% inhibition of LPS detection (p = 0.07). Likewise, LPS detection in human sera without exogenous LPS was improved by dilution: LPS was detected in 2/12 (17%) human samples in 2% serum, ranging from 3,436–4,736 pg/mL, compared to 9/12 (75%) samples in 0.1% serum, ranging from 123 pg/mL –60,131 pg/mL (p = 0.016). In a separate validation cohort of HIV-HCV co-infected participants sampled at two different times on the same day, LPS measured in 0.2% plasma and with diazo-coupling was closely correlated between the first and second samples (R = 0.66, p<0.05).

**Conclusions:** Undiluted serum and plasma mask LPS detection. The extent of MT may be substantially underestimated.


Editor: Johan K. Sandberg, Karolinska Institutet, Sweden

Received June 29, 2011; Accepted June 25, 2012; Published August 1, 2012

Copyright: © 2012 Balagopal et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The project described was supported by Grant Numbers K08 AI081544 (A.B.) from National Institute of Allergy and Infectious Diseases (NIAID), P01 MH070306 (J.C.) from National Institute of Mental Health (NIMH), U01 AI068634, U01 AI068636 (AIDS Clinical Trials Group), R01 DA016078 (D.T.), and R01 AI080417 (A.G.) from National Institute of Allergy and Infectious Diseases (NIAID). The contents herein are solely the responsibility of the authors and do not necessarily represent the official views of the National Institutes of Health (NIH). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: abalago1@jhmi.edu

Introduction

Human immunodeficiency virus (HIV) progression to AIDS is associated with immune activation. [1] One proposed pathway for immune activation in HIV/AIDS is HIV-induced intestinal CD4+ T-cell depletion that alters intestinal immunity and enhances microbial translocation (MT) of commensal bacterial products (i.e., the “leaky gut” hypothesis). The cardinal expression of MT is blood lipopolysaccharide (LPS), the cell wall component of Gram-negative enteric organisms. Brenchley et al. found that persons with HIV infection and CD4+ T-lymphocyte depletion had higher levels of plasma LPS than controls using the *Limulus* Amebocyte Lysate (LAL) assay. [2] This finding was confirmed by some, but not all, studies of HIV and simian immunodeficiency virus (SIV) infection.[2–8] Inconsistencies in this literature do not appear to be explained by differences in the research subjects or study design, raising the hypothesis that limitations in the LPS assay itself might contribute.

Conventional LPS detection exploits an enzymatic clotting reaction in the horseshoe crab, *Limulus polyphemus*. The LAL assay couples the reaction with catalysis of a colorimetric substrate. The LAL assay is sensitive at detecting 1–10 pg/mL LPS, although this
sensitivity is compromised by mammalian serum. [9,10] In this study, we demonstrate LPS masking by serum and plasma from HIV- and SIV-infected hosts and uninfected controls using the LAL assay. We identified that LPS detection is only interpretable at concentrations far below the range typically used, a finding that has important implications for all LPS-related research including studies of the pathogenesis of HIV and SIV infection.

Methods

Plasma and serum were derived from three sources. The first source was plasma from 10 pigtailed macaques that were collected pre- and post-SIV infection (42 days post-infection) as part of ongoing studies of SIV progression and the use of antiretroviral therapy (ART). The second source was human serum obtained from the ACTG study A5175, a completed HIV treatment trial assessing responses to ART in diverse international settings. To validate that LPS masking is a generalized phenomenon in HIV infection, a third source of plasma was obtained from HIV-Hepatitis C virus (HIV-HCV) co-infected subjects who were recruited for a study of magnetic resonance elastography to determine liver disease stage. In this study plasma was obtained and tested for LPS at two different times during the same day in 10 subjects, before and after magnetic resonance elastography. All macaque and human specimens were stored at $-80\degree\text{C}$ on-site or in the testing laboratory. Aliquots were thawed on the day of testing and tested in batch.

LPS was measured using the LAL assay (LONZA, Walkersville, MD) as previously published but with the following modifications, unless otherwise stated. [3] A) Samples were diluted in LPS-free 10 mM MgCl$_2$ (LONZA, Walkersville, MD) in series from 1:5 (20% concentration) to 1:1000 (0.1% concentration) and divided into two aliquots; a known amount of LPS (25 or 50 pg/mL) was added to the first tube (referred to as “exogenous LPS”), whereas none was added to the second (referred to as “naïve”). B) Samples were heated to 80\degree\text{C} for 12–15 minutes, transferred to LPS-free flat-bottomed 96-well plate (LONZA, Walkersville, MD) and kept at 37\degree\text{C} for testing. Background values were obtained at 405 and 540 nm. C) The assay was performed according to the manufacturer’s protocol but stopped by the addition of diazo-
coupling reagents that derivatize p-nitroaniline, inducing a colorimetric change that is best detected at 540 nm (OD 540). [11] Samples were then measured at 540 nm and LPS quantities were derived from a standard curve of known LPS concentrations. Spearman estimation was used to correlate the background and corrected final absorbance. The proportion of inhibited samples at different concentrations was compared using McNemar’s exact test for non-parametric data, and paired t-tests were used to compare mean inhibition in the same samples at different concentrations.

### Ethics Statement

Study approval was obtained from the Institutional Review Boards (IRB) at sites of sample collection and testing (Table S1). Informed consent was obtained for the collection and storage of serum and plasma specimens from ACTG study A5175 participants and from magnetic resonance elastography study participants. In ACTG A5175, the Johns Hopkins University Institutional Review Board allowed a waiver for additional purposes. In ACTG A5175, the Johns Hopkins University Institutional Review Board allowed a waiver for additional purposes. In the parent study, informed consent was obtained for the collection and storage of serum and plasma specimens from ACTG study A5175 participants and from magnetic resonance elastography study participants. In ACTG A5175, the Johns Hopkins University Institutional Review Board allowed a waiver for additional purposes. In the parent study, informed consent was obtained for the collection and storage of serum and plasma specimens from ACTG study A5175 participants and from magnetic resonance elastography study participants. In ACTG A5175, the Johns Hopkins University Institutional Review Board allowed a waiver for additional purposes. In the parent study, informed consent was obtained for the collection and storage of serum and plasma specimens from ACTG study A5175 participants and from magnetic resonance elastography study participants. In ACTG A5175, the Johns Hopkins University Institutional Review Board allowed a waiver for additional purposes.

### Results

#### LPS Detection in Macaque Plasma Samples

All macaques were male with median age (IQR) of 4 (3-4.5) years. The median (IQR) CD4+ T-lymphocyte counts at the time of testing were 1394 (1086-1584) cells/μL for uninfected macaques and 373 (343–496) cells/μL after SIV infection. Median (IQR) SIV RNA for infected macaques at time of testing was 6.7 (3.8–7.3) log10 copies/ml. Initial LPS testing of SIV-uninfected and -infected plasma was performed in 20% plasma, as was described previously. [2] Visual inspection of the samples at this concentration revealed heightened turbidity. In the absence of diazo-coupling, the median background values were closely correlated to the absorbance values at the completion of the assay (r = 0.97, p<0.0001, Fig. 1A), and appeared to explain much of the corrected final absorbance values (r = 0.74, p<0.0001, Fig. 1B). Serially diluting each sample diminished some but not all background readings. After performing the diazo-coupling modification, the dependence of final on background absorbance values reduced in all plasma concentrations except the highest (20%), where it persisted (r = 0.87; p<0.0001). The signal-to-noise ratio at this concentration mask detection of LPS in SIV-infected and -uninfected samples in spite of the diazo-coupling modification, prompting further study.

Exogenous LPS (50 pg/mL) was added to different concentrations of plasma (0.1% to 20%) from uninfected macaques and SIV-infected macaques, and compared to a control sample with the same amount of LPS added to diluent alone. Serially, 10/10 (100%) samples in 20% plasma had >25% lower OD 540 values compared to control (Fig. 2A). In contrast, 5/10 (50%) of samples in 10% plasma had >25% inhibition (p = 0.07), and 0/10 samples in 0.2% plasma showed >25% inhibition (p = 0.004). Importantly, relative differences in inhibition that were found between samples in 20% plasma were not preserved in 0.2% plasma. To validate that LPS masking in SIV infection can be generalized, plasma samples from 10 SIV-infected macaques were tested in a separate laboratory and with a second operator. Compared to a control sample with a known amount of LPS, 9 of 10 samples in 20% plasma had >25% inhibition; in contrast, 0 of 10 samples in 0.2% plasma had >25% inhibition (p = 0.008; Fig. 2B). Naive samples (without exogenous LPS) were tested in triplicate for LPS and results were found to be consistently more sensitive at paradoxically lower concentrations: samples tested in 0.2% plasma had mean (SD) LPS concentrations of 633 (118) pg/mL, compared to the same samples tested in 20% plasma that had mean (SD) LPS concentrations of 18 (15) pg/mL (p<0.001, data not shown).

---

**Table 1.** Characteristics of twelve randomly selected HIV-infected adult participants from the ACTG A5175 cohort and ten HIV-HCV co-infected adult participants from the Magnetic Resonance Elastography (MRE) study.

<table>
<thead>
<tr>
<th></th>
<th>ACTG 5175</th>
<th>MRE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Median Age, years (IQR)</td>
<td>31.5 (27.5–35.3)</td>
<td>47.5 (44.5–52.3)</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>4 (33.3)</td>
<td>5 (50)</td>
</tr>
<tr>
<td>Black, n (%)</td>
<td>8 (66.7)</td>
<td>9 (90)</td>
</tr>
<tr>
<td>On Antiretrovirals, n (%)</td>
<td>7 (58.3)</td>
<td>10 (100)</td>
</tr>
<tr>
<td>Median CD4+ T lymphocytes/mm³ (IQR)</td>
<td>244 (212–384)</td>
<td>269 (220–334)</td>
</tr>
<tr>
<td>Median HIV-1 RNA log10 copies/mL (IQR)</td>
<td>2.6 (2.3–4.0)</td>
<td>Median &lt;50</td>
</tr>
<tr>
<td>Median Baseline ALT (U/L) (IQR)</td>
<td>25 (20–30)</td>
<td>42 (36–69)</td>
</tr>
<tr>
<td>HCV RNA log10 IU/mL</td>
<td>NA</td>
<td>6.58 (5.84–6.76)</td>
</tr>
</tbody>
</table>

All MRE patients were HCV genotype 1; 2 (20%) had biopsy-proven cirrhosis. doi:10.1371/journal.pone.0041258.t001
Conclusion

In this study we demonstrate that serum and plasma in HIV and SIV infection mask the detection of LPS using the LAL assay. Additionally, we report a method that enhances LPS detection. These results may have significant implications for studies of the pathogenesis of HIV infection and others for which LPS quantification is essential.

LPS is a heterogeneous collection of molecules that is found in the cell wall of most Gram-negative bacteria. [12] The fundamental structure of LPS, however, is conserved; it is composed of an inflammatory Lipid A domain, a core oligosaccharide, and a polysaccharide O-antigen. [13] Circulating LPS has been found in hosts with Gram-negative bacterial infections, and can act as a pathogen-associated molecular pattern that stimulates an innate immune response by binding to the Toll-like receptor 4 (TLR4) complex. [13] TLR4 binding triggers a downstream cascade that results in the up-regulation of pro-inflammatory cytokines such as tumor necrosis factor-α, and can be critical for priming an adaptive immune response. [12]

In HIV-infected patients there is mounting evidence that continued disease progression despite ART is due to systemic immune activation, a state wherein levels of inflammatory cytokines and markers of cellular activation are elevated. [1,2,14] Brenchley et al. linked intestinal CD4+ T-lymphocyte depletion in HIV infection to immune activation by demonstrating increased amounts of LPS circulating in individuals with chronic HIV infection and AIDS compared to uninfected persons and persons with early HIV-1 infection; the authors found similar evidence of MT in SIV-infected Rhesus macaques. [2] In contrast, Redd et al. did not find any association of LPS levels with AIDS progression in a longitudinal African cohort of HIV-infected persons, despite the presence in that cohort of persons with rapid disease progression. [4] Understanding these discordant results will require further testing, but already concerns have been raised that the conventional detection of LPS may lead to inconsistent results. [6] Our findings illustrate the difficulty in measuring LPS in HIV and SIV infection unless LPS masking is considered.

Inhibition of LPS detection has been described previously. [9,10] While attempts have been made to identify the assay-inhibiting agent, none have been revealing. It is clear, however, that LPS recovery from serum and plasma can be improved at paradoxically low sample concentrations. Past reports have demonstrated that 1–10% serum concentrations have been sufficient to mitigate LAL inhibition, but those studies were not performed in HIV or SIV-infected samples. [9,10] In the present study inhibition was diversely found in macaques and in humans, and concentrations as low as 0.2% were required for reliable quantification of LPS in serum and plasma. In contrast, recent studies of LPS detection in HIV or SIV-infected samples have used serum and plasma concentrations of up to 20% (1:5 dilution) and did not assess for inhibition.[2–4] Although dilution of samples to 0.1% concentration reduces the lower limit of detection, this is balanced by the increased sensitivity of detection. In this study, serum and plasma dilution improved both the number of samples with detectable LPS and the total amount of LPS found in each sample.

Relative differences between LPS measurements might still be valid if inhibition occurred to the same degree between samples at a dilution, but are less interpretable if the amount of inhibition was specimen-dependent. In this study we found that the range of LPS inhibition in human serum varied from ~4% to 98% at higher concentrations, but decreased to ~11% to 13% at concentrations <0.5%, and similar results were found in plasma (Fig. 3). These findings suggest that relative differences in LPS detection at higher serum and plasma concentrations cannot be used as surrogates for LPS amounts observed at lower concentrations.
There are a variety of methods used to minimize LAL inhibition, including heating samples before assay performance as was done in our study. In addition, chloroform-based serum extraction can reduce the amount of inhibition if sufficient starting material is available. Diazo-coupling, specimen heating, and sample dilution attenuated LPS masking inhibition in this study.

In this report we illustrate that serum and plasma from hosts with HIV or SIV infection mask the detection of LPS more than has been previously described in uninfected persons. Therefore it is likely that earlier studies may have underestimated microbial translocation. We recommend that future studies of microbial translocation in HIV and SIV infection use a combination of techniques to minimize LPS masking, including tested diluted samples and employing diazo-coupling modifications.

Supporting Information

Table S1  International Testing Sites for ACTG study A5175. Shown is the full list of each testing site and its associated IRB that was responsible for approving the use of participants’ samples for study.

References