CONCISE COMMUNICATION

Antibody and B cell responses may control circulating lipopolysaccharide in patients with HIV infection

A. Lim\textsuperscript{a}, A. Amini\textsuperscript{a}, L. D’Orsogna\textsuperscript{b}, R. Rajasuriar\textsuperscript{c,d}, M. Kramski\textsuperscript{e}, SR. Lewin\textsuperscript{c,f,g}, D. Purcell\textsuperscript{e}, P. Price\textsuperscript{a,b} and MA. French\textsuperscript{a,b}

Objectives: To examine the relationship between plasma markers of microbial translocation and antibodies to lipopolysaccharide (LPS) and circulating memory B cells in patients with HIV infection.

Design: Cross-sectional study in antiretroviral therapy (ART)-naïve (n = 23) and ART-treated (n = 27) HIV patients.

Methods: Antibodies to LPS and immunoglobulins, assayed in stored serum, and matched memory B cell counts were correlated with levels of LPS and bacterial 16S ribosome DNA (16S rDNA), assayed in stored plasma.

Results: In ART-naïve patients, plasma LPS levels correlated inversely with serum levels of IgG and IgA antibodies to LPS (\(P = 0.03\) and 0.006, respectively), serum levels of IgA anti-LPS correlated with total IgA (\(P < 0.0001\)) and levels of IgG anti-LPS correlated with IgM\textsuperscript{+} memory B cell counts (\(P = 0.025\)). In ART-treated patients, plasma LPS levels were not related to levels of LPS antibodies but were related to CD4\textsuperscript{+} T cell and switched memory B cell counts. There were no correlations with plasma levels of 16S rDNA.

Conclusions: Plasma LPS levels were associated with antibody and possibly B cell responses to LPS in ART-naïve HIV patients whereas they were associated with the degree of immune reconstitution in ART-treated patients.

© 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins

Objectives: To examine the relationship between plasma markers of microbial translocation and antibodies to lipopolysaccharide (LPS) and circulating memory B cells in patients with HIV infection.

Design: Cross-sectional study in antiretroviral therapy (ART)-naïve (n = 23) and ART-treated (n = 27) HIV patients.

Methods: Antibodies to LPS and immunoglobulins, assayed in stored serum, and matched memory B cell counts were correlated with levels of LPS and bacterial 16S ribosome DNA (16S rDNA), assayed in stored plasma.

Results: In ART-naïve patients, plasma LPS levels correlated inversely with serum levels of IgG and IgA antibodies to LPS (\(P = 0.03\) and 0.006, respectively), serum levels of IgA anti-LPS correlated with total IgA (\(P < 0.0001\)) and levels of IgG anti-LPS correlated with IgM\textsuperscript{+} memory B cell counts (\(P = 0.025\)). In ART-treated patients, plasma LPS levels were not related to levels of LPS antibodies but were related to CD4\textsuperscript{+} T cell and switched memory B cell counts. There were no correlations with plasma levels of 16S rDNA.

Conclusions: Plasma LPS levels were associated with antibody and possibly B cell responses to LPS in ART-naïve HIV patients whereas they were associated with the degree of immune reconstitution in ART-treated patients.

© 2011 Wolters Kluwer Health | Lippincott Williams & Wilkins

Keywords: HIV, IgA, lipopolysaccharide antibodies, memory B cells, microbial translocation

Introduction

Immune hyperactivation is central to the pathogenesis of immune dysfunction in patients with chronic HIV infection. Possible causes include translocation of microbial products, such as lipopolysaccharide (LPS) and bacterial 16S ribosome DNA (16S rDNA), from the intestine into the circulation following depletion of memory CD4\textsuperscript{+} T cells from the intestinal mucosa [1–6]. However, the microbial content of the gut and amount of
microbial products in the circulation are also affected by B cell and antibody responses. Thus, IgA antibodies are important in the intestine [7] and circulating marginal zone-like B cells respond to LPS and transport antigens to follicular regions of lymphoid tissue [8,9]. B cells with a marginal zone phenotype (CD27+, IgM+, IgD−) comprise the largest proportion of circulating non-switched IgM+ memory B cells [10]. We have shown that IgM+ memory B cells are more numerous in antiretroviral therapy (ART)-naive HIV patients with high CD4+ T cell counts than in patients with low CD4+ T cell counts [11], and suggested that IgM+ memory B cells may have a beneficial effect in HIV infection. Here, we describe the relationship of plasma levels of LPS and 16S rDNA with serum levels of antibodies to LPS and immunoglobulins and with nonswitched (CD27+, IgM+) and switched (CD27+, IgM+) memory B cell counts in ART-treated and -naive patients.

Methods

Study participants
Serological investigations were undertaken on cryopreserved serum and plasma from 23 ART-naive and 27 ART-treated HIV patients enrolled into a study of memory B cells [11]. Plasma from 12 non-HIV subjects was also assayed for LPS. The project was approved by the Ethics Committee of Royal Perth Hospital (RPH) and all patients provided informed consent.

Measurement of serum levels of LPS-specific antibodies and plasma levels of LPS
IgA, IgG and IgM LPS-specific antibodies were detected by an in-house enzyme-linked immunosorbent assay (ELISA) based on the method of Péterfi and Kocsis [12]. Polystyrene Costar microplates (Corning Inc., Corning, NY) were precoated with 10 µg/mL poly-L-lysine (Sigma-Aldrich, Sydney, Australia) diluted in 0.01 M phosphate buffered saline (PBS) pH 7.5. Plates were washed once with PBS and coated with LPS from the rough mutant Shigella sonnei Re4350 (1 µg/mL in PBS) for 1 hr at 37°C. Following four washes with PBS containing 0.05% Tween (PBS-T) they were blocked with normal goat serum (Sigma-Aldrich) diluted 1/20 with PBS (30 min, 37°C), followed by 4 washes with PBS-T. Serial dilutions of patient serum were incubated for 1 hr at 37°C followed by 4 washes with PBS-T. Peroxidase-conjugated antihuman IgA, IgG or IgM (Sigma-Aldrich) diluted 1/1000 were added (1 hr, 37°C), followed by 4 washes with PBS-T. TMB substrate (Sigma-Aldrich) was added for 10 min (room temperature in the dark). Reactions were stopped with 1 M sulfuric acid and optical densities measured at 450 nm. Standard curves were generated by serially diluting a serum pool with high levels of LPS-specific IgA, IgG or IgM. The top OD was assigned an arbitrary value of 100 units. Patient sera generating OD readings above the highest standard were diluted until at least 2 wells yielded OD values in the linear part of the curve.

LPS levels were assayed in plasma collected within 4 weeks of the CD4+ T cell count using a commercial chromogenic limulus amebocyte lysate assay (Lonza, Walkersville, MD) as described elsewhere [13]. All samples were analysed in duplicate.

Real-time quantitative PCR assay of 16S rDNA
Total DNA was extracted from 200 µL EDTA plasma (DNeasy Kit Qiagen, Valencia, CA), eluted in 50 µL AE buffer and stored at -80°C until analysed for 16S rDNA by a novel and sensitive real-time qPCR assay (Kramski M et al., submitted). Each sample was assayed in triplicate. The assay had a detection range of 10–10⁶ copies per 5 µL plasmid 16S rDNA and samples lacking added plasma template showed no amplification at Ct = 40.

Enumeration of memory B cells and assay of serum immunoglobulins
Switched (CD27+ IgM+) and IgM+ (CD27+, IgM+) memory B cells were enumerated as described elsewhere [11]. Serum immunoglobulins were assayed by immunoturbidimetry in the Core Service laboratory of RPH.

Statistical analyses
The Kruskal-Wallis test with Dunnett’s T3 posttest was used to compare groups of individuals. Spearman’s test was used to assess nonparametric correlation coefficients. For all analyses, a P value of <0.05 was considered to be significant.

Results

Study participants
ART-naive and -treated patients were matched for sex and age. Plasma LPS levels were unexpectedly higher in ART-treated than ART-naive patients (P = 0.08), possibly reflecting higher rates of male-to-male HIV transmission in ART-treated (70%) than ART-naive (26%) patients consequent upon the changing demographics of patients referred to our clinic. Plasma HIV RNA was <50 copies/mL in none of the ART-naive patients and 96% of ART-treated patients, who had received ART for at least 6 months. Median CD4+ T cell counts were 325/µL (range 60–425) in ART-naive patients and 540/µL (342–748) in treated patients.

LPS and LPS-specific antibody levels were inversely correlated in ART-naive HIV patients
In ART-naive patients, plasma LPS levels were higher than in non-HIV controls (median [IQR] = 44.6 [39.2–64.2] vs 37.1 [25.1–44.3] pg/ml, P = 0.006), and correlated inversely with serum levels of IgG and IgA
antibodies to LPS (Fig. 1A, B). There was also a striking positive correlation between serum levels of IgA anti-LPS and IgA (Fig. 1C). Consequently, plasma LPS levels correlated negatively with serum levels of IgA (Fig. 1D). No correlations were found between plasma LPS levels and serum levels of IgM anti-LPS or IgG or IgM (P > 0.05 for all).

Circulating memory B cell counts did not correlate with plasma levels of LPS. However, IgM⁺ memory B cell counts correlated positively with serum levels of IgG anti-LPS (r = 0.5, P = 0.025, data not shown) but not with levels of IgA anti-LPS (r = 0.18, P = 0.4) or IgM anti-LPS (r = -0.07, P = 0.7). Switched memory B cell counts did not correlate with levels of LPS-specific antibodies of any isotype (P > 0.5 in all cases).

Plasma 16S rDNA levels did not correlate with levels of LPS (r ≤ -0.27, P ≥ 0.24) nor with antibodies to LPS, CD4⁺ T cell counts or memory B cell counts in ART-naïve or -treated patients (data not shown).

Levels of LPS and LPS-specific antibodies in patients on ART were related to the degree of immune reconstitution

In ART-treated patients, plasma LPS levels were also higher than non-HIV controls (median [IQR] = 66.7 [49.4-72.0] vs 37.1 [25.1–44.3] pg/ml, P < 0.001). In contrast to ART-naïve patients, levels of LPS did not correlate with LPS-specific antibodies or serum immunoglobulin levels (P > 0.2 for all). However, there was a negative correlation between LPS levels and CD4⁺ T cell counts (Fig. 2A). Serum levels of IgG anti-LPS correlated negatively with current CD4⁺ T cell counts, nadir CD4⁺ T cell counts and switched memory B cell counts (2B-D, respectively). In addition, serum levels of IgA anti-LPS correlated negatively with current CD4⁺ T cell count (Fig. 2E).

Discussion

Increased plasma LPS levels LPS in HIV patients have been related to increased gut permeability resulting from depletion of mucosal CD4⁺ T cells [2–6,14]. A weak negative correlation has been demonstrated between plasma LPS levels and serum levels of IgM endotoxin core antibody in ART-naïve HIV patients [2,4], suggesting that antibodies to LPS might decrease plasma LPS levels. Here, we show that plasma LPS levels correlated negatively with serum levels of both IgG and IgA antibodies to LPS in ART-naïve HIV patients (Fig. 1A, B), providing further evidence that antibodies might decrease LPS levels.

Serum levels of IgG anti-LPS also correlated with IgM⁺ memory B cell counts, possibly reflecting a role for marginal zone-like B cells in the induction of antibody responses against LPS [9,10]. We also demonstrated a striking positive correlation between serum levels of IgA anti-LPS and IgA in ART-naïve HIV patients (Fig. 1C). Increased serum IgA levels have been recognized as a manifestation of HIV infection for over 20 years [15], and more recently have been proposed as an indicator of persistent immune dysfunction and/or immune activation in patients receiving long-term ART [16,17]. Increased serum IgA levels might result from an IgA antibody response to LPS but could also reflect LPS

![Fig. 1. Correlations between levels of plasma LPS, serum antibodies to LPS and serum IgA in ART-naïve HIV patients.](image-url)
driven polyclonal B cell activation and the induction of LPS antibodies.

As expected from the results of previous studies [2,4,5,13], we found that plasma LPS levels were higher in ART-treated patients than controls. However, in contrast to the findings in ART-naïve patients, plasma LPS levels were not related to serum levels of IgA or IgG anti-LPS or IgA. They were, however, negatively correlated with current CD4⁺ T cell counts (Fig. 2A). These findings suggest that recovery of CD4⁺ T cell counts, and presumably improved gut mucosa integrity, are more important than antibody responses to LPS in determining plasma LPS levels in ART-treated patients. It is unclear why antibodies to LPS show a different association with plasma LPS levels before and after ART but, at least for IgA, it is possible that it reflects differences in regulation of antibody production. Both T cell-dependent and -independent pathways regulate IgA production in the gut [7,18], and it is possible that T cell-independent pathways are more prominent in ART-naïve patients because of the effects of HIV infection on CD4⁺ T cell numbers and/or function.

We found no relationship between serum levels of antibodies to LPS and plasma LPS levels in ART-treated patients but IgA and/or IgG antibodies to LPS correlated inversely with current and nadir CD4⁺ T cell counts and switched memory B cell counts (Fig. 2B-E). We suggest that higher serum levels of LPS-specific antibodies might reflect an immune response against LPS in patients with ongoing microbial translocation resulting from poorer immune reconstitution and, therefore, are an indirect marker of microbial translocation. It is unclear why plasma LPS levels did not correlate with antibodies to LPS in ART-treated patients but did in ART-naive patients. One possible explanation is that sequestration of LPS in immune complexes and/or the clearance of immune complexes was more efficient in ART-treated patients.

The relationships between plasma LPS levels and antibodies to LPS, CD4⁺ T cell counts and switched memory B cell counts were not demonstrated for 16S rDNA and, indeed, plasma LPS and 16S rDNA levels were not correlated. Jiang et al. [5] also demonstrated that 16S rDNA levels did not correlate with CD4⁺ T cell counts but did demonstrate a weak correlation with LPS.
levels ($r = 0.31$). The relationship between 16S rDNA and LPS requires further investigation.

In summary, we have demonstrated that plasma LPS levels correlate inversely with serum levels of IgA and IgG antibodies to LPS in ART-naive HIV patients but are associated with the degree of immune reconstitution in ART-treated patients. Our findings suggest that B cell and antibody responses affect plasma LPS levels in ART-naive patients and raise the possibility that enhancing systemic and/or mucosal antibody responses to LPS might be a means of controlling immune hyperactivation in such patients.

**Acknowledgements**

We thank Dr Zoltán Péterfi (University of Pécs) for his kind gift of LPS from *Shigella sonnei* Re4350 and his expertise in detection of LPS-specific antibodies. This study was funded by Project Grant 404028 and Program Grant 510488 from the National Health and Medical Research Council (NHMRC) of Australia. SRL is an NHMRC Practitioner Fellow.

**Sources of Support:** National Health and Medical Research Council of Australia.

**References**