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Performance characteristics of the limulus amebocyte lysate assay and gas chromatography–mass spectrum analysis of lipopolysaccharides relative to nitric oxide production by peritoneal exudates of cells

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Abstract

Limulus amebocyte lysate (LAL) assay and gas chromatography–mass spectrum (GC–MS) analysis are usually used for the quantification of lipopolysaccharides (LPS) from the environment. The LAL assay measures endotoxin units to represent the LPS biological function but GC–MS analysis measures decanoic ($C_{10:0}$) and dodecanoic ($C_{12:0}$) 3-hydorxy fatty acids (3-OH FA) concentration to represent the LPS chemical composition. A study was carried out using these methods to evaluate their degree of correlation. Using the culture supernatants of 30 independent strains of *Pseudomonas aeruginosa*, the bacterial supernatants gave of 0.53 ± 0.45 and 2.49 ± 1.75 mg/l of $C_{10:0}$ and $C_{12:0}$ 3-OH FA, respectively, compared to 17.96 ± 13.28 mg/l of LPS with the LAL assay (1 ng/ml of LPS $\cong$ 0.78 EU/ml). The 3-OH FA concentration relative to the endotoxin unit dose in the supernatants exhibited a positive correlation ($r^2 = 0.5182$, $C_{10:0}$; $r^2 = 0.4359$, $C_{12:0}$). When supernatants having a high level of endotoxin were used to treat peritoneal exudates cells, nitric oxide (NO) was generated in a dose-dependent manner ($r^2 = 0.6174$). To determine if either $C_{10:0}$ or $C_{12:0}$ 3-OH FA can act as an indictor of LPS quantity was correlated with this immunostimulatory effect, the correlation of these 3-OH FA concentrations against the produced NO levels was evaluated. This also exhibited a positive correlation; however, the two indicators of 3-OH FA gave different dose-responsible performances ($r^2 = 0.3211$, $C_{10:0}$; $r^2 = 0.4527$, $C_{12:0}$).

Keywords: Lipopolysaccharides (LPS); Limulus amebocyte lysate (LAL) assay; 3-Hydorxy fatty acids (3-OH FA); Nitric oxide (NO) production

1. Introduction

Environments contaminated with lipopolysaccharide (LPS) are a major clinical problem due to the risk of an allergic response [1]. It is well established that environmental LPS content is associated with asthma severity in humans; however, the actual amount of LPS in a particular environment is still very difficult to evaluate. The limulus amebocyte lysate (LAL) assay has been traditionally used to test LPS biological activity in an environment and to represent the level of exposure to LPS of the target. Fatty acid composition, particularly shorter chain 3-hydroxy fatty acids (3-OH FA; decanoic, $C_{10:0}$; dodecanoic, $C_{12:0}$; tetradecanoic, $C_{14:0}$), has been recently developed as a quantitative indicator of LPS [2–5]. However, the concentration of LPS determined by the biological assay does not seem to perfectly correlate with the LPS chemical composition in a dose-dependent manner [2,3]. This could be because, importantly, that the agglutination of the lysate may not rely on the structure of the 3-OH FA in the LPS. Similarly, the immunostimulatory response caused by the LPS is certainly not restricted to structures that strongly activate agglutination of the lysate, nor the level of 3-OH FA making up the lipid A component.

Pseudomonas aeruginosa is widely distributed in various environments and its LPS structure consists of two major forms,

Abbreviations: LAL, limulus amebocyte lysate; GC–MS, gas chromatography–mass spectrum; $C_{10:0}$, decanoic; $C_{12:0}$, dodecanoic; $C_{14:0}$, tetradecanoic; 3-OH FA, 3-hydorxy fatty acid; MSTFA, *N*-trimethylsilyl-*N*-methyl trifluoroacetamide; LOD, low limit of detection; S.D., standard deviation; RAPD, randomly amplified polymorphic DNA; PEC, peritoneal exudates cells; FCS, fetal calf serum; TNF, tumor necrosis factor

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heap-acylated or penta-acylated FA linked to the polymers of a repeating disaccharide units on lipid A. The short chain $C_{10:0}$ or $C_{12:0}$ 3-OH FA are mainly composed of 3-OH FA linked to polymers [6]. Using *P. aeruginosa* as an environmental bacteria model, the objective of this study was to determine, using both chemical and biological assays, the quantity and analytical characteristics of the LPS released from the bacterial soma relative to their immunostimulatory effect.

2. Materials and methods

2.1. Stimulon preparation

Thirty strains of *P. aeruginosa* that had been previously demonstrated to be genetically independent strains with distinct RAPD (randomly amplified polymorphic DNA) profiles (data not shown) were used. These strains (10^4 CFU/ml) were each incubated in 10 ml of Vogel–Bonner medium (3.3 mM $MgSO_4$, 10 mM citric acid, 28 mM NaH_4PO_4, 37 mM K_2HPO_4, 214 mM D-gluconic acid; pH 7.4) at 37 °C for 7 days. After culture, the supernatants were filtered using a 0.45 μm filter, lyophilized and resuspended into 3 ml of distilled water.

2.2. LAL assay

The LPS contents of the stimulons from the 30 independent strains were estimated by a chromogenic endotoxin-specific assay with LAL coagulation enzyme (ES test; Seikagaku Corp., Tokyo, Japan). Briefly, the sample (50 μl) and LAL (50 μl) were mixed and incubated at 37 °C. After 10 min, the mixtures were added into the 37 °C pre-incubated 96-well microtitre plate and then incubated with substrate solution (100 μl) at 37 °C for an additional 6 min. The reaction was stop with 25% (v/v) glacial acetic acid. The absorbance of the developed yellow color was determined using an Anthos 2010 microplate reader (Anthos Labtec Instruments GmbH, Wals, Salzburg, Austria) at 405 nm by the endpoint approach method. The stimulons were serially diluted with LPS-free water (Sigma, The Sigma Chemical Co., St. Louis, MO, USA) until their endotoxin levels were in the range of 0.1–1 endotoxin units [EU]/ml relative to the reference endotoxin (*E. coli* O111:B4) calibration system provided. The LPS concentration (pg/ml) or units (EU/ml) of the stimulons was determined using the LAL coagulation data based on the reference endotoxin by linear regression analysis.

2.3. Determination of 3-OH FA concentration

The 3-OH FA concentrations were determined by GC–MS (gas chromatography–mass spectrum) analysis. To prepare GC–MS ready extracts from each of the 30 independent strains, the lyophilized stimulons were dissolved in 1 ml of 15% methanolic NaOH in combination with 30 μM deuterated $C_{14:0}$ FA as an internal standard. The samples were then sealed into a glass tube and heated at 100 °C. After 30 min, the tubes were vigorously vortexed for 10 s and returned to 100 °C for another 16 h. Then 2 ml of acid metholic solution (1083 μl of 6N HC1 and 917 μl of methanol) was added to the tube and the tube heated at 80 °C for 1 h. Hexane (1.25 ml) was added into the tube next and the tube gently tumbled on a rotator for 10 min. As the next step, 3 ml of methanol and 3 ml of 0.24 M NaOH were added to the tube and the organic aqueous phase (the top layer) was pipetted into a new glass tube. The organic aqueous phase was then dried using nitrogen gas. The final step consisted of adding 50 μl of MSTFA (*n*-trimethylsilyl-*n*-methyl trifluoroacetamide; Sigma) to form derivatives and this was done by heating at 80 °C for 15 min.

An Agilent 6890 gas chromatograph/5973N mass selective detector (GC–MS) system was used for this study. The GC was equipped with a 30 m Hewlett-Packard (Andover, MA, USA) 5MS fused silica capillary column (0.25 mm i.d.; 0.25 μm film thickness). The injector and interface temperature were maintained at 260 and 280 °C, respectively. Oven temperature was held at 85 °C for 5 min, then programmed to increase to 285 °C at 7 °C/min, and was then held at the final temperature for 1 min. The following parameters were used for injecting samples into the GC/MSD system: sample size, 1 μl; injection mode, splitless; injector purge-off duration, 1 min.

The mass range adopted for collecting the full-scan mass spectra was *m*/*z* 50–550. Based on the ion intensity data from the full-scan mass spectra, the differentially fragmented ions derived from the trimethylsilyl derivatives of the hydroxylated FA were used for identification and quantification. The amounts of 3-OH FA after adjustment relative to the recovered internal standard concentration were plotted against the ratio between the areas of the tested FAs and the areas of the standards of derivatives of FA. Each step of the preparation process for GC–MS analysis was carried out carefully to avoid loss of extracted FA.

2.4. The preparation of peritoneal exudates cells

Mouse (Balb/c; 8 months old) peritoneal exudates cells (PEC) were isolated by peritoneal lavage at 4 days after intraperitoneal injection of 2 ml of sterile 3% Brewer thioglycolate medium (Difco Laboratories, Detroit Mich, USA). The cells were washed and then resuspended in RPMI 1640 medium containing 10% fetal calf serum (FCS), penicillin and streptomycin (100 μg/ml).

2.5. Nitric oxide (NO) production

The stimulons were respectively prepared from the 30 independent strains. The stimulons were serially diluted relative to their LPS concentration as determined for either LAL units or by 3-OH FA concentration, until the end of the calibrated range (10–100 μM). PEC samples (10^5 cells/well) were treated with the indicated LPS quantities in a 96-well microtitre plates for 2 h. The supernatants (50 μl) were mixed with Griess Reagent (Calbiochem-Novabiochem Co., San Diego, USA) following as: 50 μl of sulfanilamide solution for 5 min and then 50 μl of *n*-1-napthylethylenediamine dihydrochloride for 5 min, at room temperature. NO formation was measured as the stable end product nitrite (NO_2) in the culture supernatants using an Anthos

Table 1
The differentiated ion fragments of hydroxy fatty acids with trimethylsilyl derivatives

	Fatty acids								
	Denterated $C_{14:0}$ FA	2-Hydroxy				3-Hydroxy			
		$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$	$C_{10:0}$	$C_{12:0}$	$C_{14:0}$	$C_{16:0}$
Ion fragments (*m/z*)	343	215	243	271	299	259	287	315	343
Retention time (min)	20.06	17.26	20.41	23.26	26.11	17.26	20.37	23.24	26.10

2010 microplate reader (Anthos Labtec Instruments GmbH) at $OD_{540\,nm}$.

2.6. Statistical methods

The mean and the standard deviation (mean ± S.D.) derived from duplicated or triplicated tests, and linear regression analysis were used and carried out using the statistical functions of Excel software.

3. Results and discussion

3.1. Analysis of LPS extracts with GC–MS

The extracted major unique ion fragments at specific retention times were used to differentiate the hydroxylated FA trimethylsilyl derivatives (Table 1). A typical GC–MS profile of a *P. aeruginosa* LPS extract gave concentrations of $C_{10:0}$ and $C_{12:0}$ 3-OH FA that could be readily detected by the GC–MS; however, $C_{14:0}$ 3-OH FA was not detectable (Fig. 1). The quantification of the $C_{10:0}$ or $C_{12:0}$ 3-OH FAs relative to the standard in a range of 0.4–2 μM was determined by a linear regression analysis using a calibration curve ($C_{10:0}$, $y = 29.897x + 1.8288$, $r^2 = 0.9677$ and $C_{12:0}$, $y = 31.012x - 2.6968$, $r^2 = 0.9595$). The low limit of detection (LOD) for both $C_{10:0}$ and $C_{12:0}$ 3-OH FAs was 0.2 μM, which was defined as 3 S.D. (standard deviation) above the measured blank average.

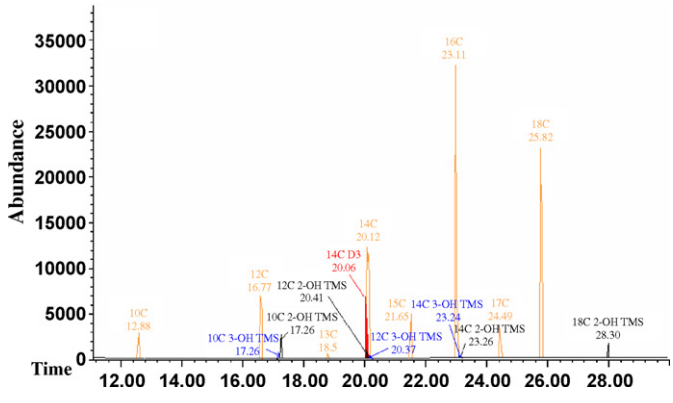

Fig. 1. A GC–MS typical profile from the analysis of *Pseudomonas aeruginosa* LPS. GC–MS analysis was performed using selected ion monitoring (SIM) for saturated FA, 2-OH FA and 3-OH FA. This profile shows the overlapping of the three SIM profiles, namely saturated FA (orange color), 2-OH FA (black color) and 3-OH FA (red color).

3.2. Endotoxin units relative to $C_{10:0}$ and $C_{12:0}$ 3-OH FA concentration

The LPS quantities in the bacterial cultures were determined by both methods. Firstly, the supernatants of each of the 30 independent strains of *P. aeruginosa* were assayed by GC–MS to give a concentration for $C_{10:0}$ and $C_{12:0}$ 3-OH FAs. Secondly, the concentration of LPS by LAL assay was estimated in endotoxin units (EU). After a 7 days incubation of the bacterial strains, the concentration of $C_{10:0}$ and $C_{12:0}$ 3-OH FA in the supernatant minimal medium averaged 0.53 ± 0.45 and 2.49 ± 1.75 mg/l, respectively. When these supernatants were assayed by the LAL method, there was, on average, 17.96 ± 13.28 mg/l based on the equation 1 ng/ml of LPS ≅ 0.78 EU/ml. Both the $C_{10:0}$ and $C_{12:0}$ 3-OH FA concentrations by GC–MS analysis exhibited a positive correlation ($r^2 = 0.5182$, $C_{10:0}$; $r^2 = 0.4359$, $C_{12:0}$) with number of endotoxin units as measured by the LAL assay when the extracts of each individual stain were compared (Fig. 2). The $C_{10:0}$ and $C_{12:0}$ 3-OH FA concentrations of each strain are listed in Table 2. The molar ratio of $C_{10:0}$ *versus* $C_{12:0}$ 3-OH FA in the supernatants was 1:4.63 ± 1.62. The presence of a high measurement in terms of endotoxin units in the supernatants seemed to rely on the co-presence of $C_{10:0}$ and $C_{12:0}$ 3-OH FA.

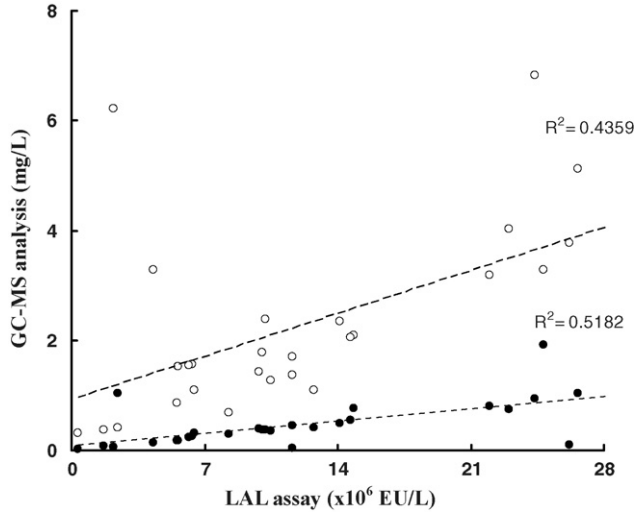

Fig. 2. Correlation of endotoxin units by LAL assay with $C_{10:0}$ and $C_{12:0}$ 3-OH FA concentration. Thirty samples extracted from individual *P. aeruginosa* strains were analyzed in terms of endotoxin units by the LAL method and for $C_{10:0}/C_{12:0}$ 3-OH FA concentration by GC–MS analysis. The correlation between endotoxin units and 3-OH FA concentration was calculated by linear regression analysis. (●) $C_{10:0}$ 3-OH FA; (○) $C_{12:0}$ 3-OH FA.

Table 2
Characteristics of chemical and biological analysis for LPS in each independent strains

Strains	Assay for				Linear regression
	3-OH FA (mg/l)		LAL ($\times 10^6$ EU/l)	Griess assay ($\times 10^6$ μM)	
	$C_{10:0}$	$C_{12:0}$			
Group 1: $[C_{12:0}]/[C_{10:0}] = 3.00–6.25$					
1	0.38 ± 0.06	1.77 ± 0.07	10.01 ± 2.21	27.09 ± 3.47	[LAL] *vs.* $[C_{10:0}]$; $r^2 = 0.9707$
2	0.18 ± 0.03	0.85 ± 0.05	5.57 ± 1.75	12.52 ± 4.42	[LAL] *vs.* $[C_{12:0}]$; $r^2 = 0.9208$
3	1.17 ± 0.12	5.36 ± 0.13	37.82 ± 6.84	38.39 ± 6.81	[NO] *vs.* $[C_{10:0}]$; $r^2 = 0.7939$
4	0.08 ± 0.02	0.37 ± 0.03	1.70 ± 0.09	8.23 ± 2.43	[NO] *vs.* $[C_{12:0}]$; $r^2 = 0.8210$
5	0.74 ± 0.02	4.02 ± 0.09	23.02 ± 3.27	25.37 ± 5.59	[LAL] *vs.* [NO]; $r^2 = 0.7746$
6	0.92 ± 0.04	3.70 ± 0.11	29.20 ± 3.81	31.00 ± 2.88	
7	0.35 ± 0.03	1.27 ± 0.07	10.51 ± 1.64	15.33 ± 3.39	
8	0.50 ± 0.05	2.35 ± 0.09	14.12 ± 2.28	21.59 ± 4.35	
9	0.81 ± 0.03	3.20 ± 0.12	22.01 ± 2.98	20.87 ± 7.02	
10	0.46 ± 0.02	1.71 ± 0.08	11.63 ± 2.45	14.15 ± 3.82	
11	1.03 ± 0.06	5.12 ± 0.15	26.63 ± 4.29	36.41 ± 8.25	
12	0.32 ± 0.02	1.10 ± 0.09	6.47 ± 1.35	18.27 ± 4.94	
13	0.39 ± 0.06	1.42 ± 0.04	9.85 ± 2.87	19.15 ± 2.18	
14	0.25 ± 0.05	1.57 ± 0.06	6.33 ± 2.54	14.07 ± 3.76	
15	0.54 ± 0.04	2.05 ± 0.09	14.68 ± 2.66	16.40 ± 3.36	
Group 2: $[C_{12:0}]/[C_{10:0}] > 6.25$					
16	0.13 ± 0.02	3.29 ± 0.15	4.33 ± 2.14	15.49 ± 3.38	[LAL] *vs.* $[C_{10:0}]$; $r^2 = 0.3023$
17	0.09 ± 0.02	3.77 ± 0.09	26.21 ± 4.55	38.18 ± 7.20	[LAL] *vs.* $[C_{12:0}]$; $r^2 = 0.2073$
18	0.04 ± 0.02	1.37 ± 0.12	11.63 ± 3.58	28.43 ± 6.76	[NO] *vs.* $[C_{10:0}]$; $r^2 = 0.1292$
19	0.02 ± 0.01	0.31 ± 0.04	0.33 ± 0.09	17.55 ± 3.18	[NO] *vs.* $[C_{12:0}]$; $r^2 = 0.1950$
20	0.37 ± 0.03	2.38 ± 0.09	10.20 ± 1.96	30.37 ± 5.73	[LAL] *vs.* [NO]; $r^2 = 0.7403$
21	0.06 ± 0.01	6.21 ± 0.06	2.21 ± 0.08	25.16 ± 3.41	
22	0.94 ± 0.02	6.82 ± 0.15	24.35 ± 3.33	32.33 ± 5.01	
23	0.17 ± 0.03	1.53 ± 0.11	5.60 ± 0.97	17.93 ± 2.74	
24	0.23 ± 0.06	1.54 ± 0.08	6.17 ± 1.20	22.85 ± 5.52	
Group 3: $[C_{12:0}]/[C_{10:0}] < 3.01$					
25	1.91 ± 0.05	3.29 ± 0.10	24.82 ± 3.86	35.49 ± 8.82	[LAL] *vs.* $[C_{10:0}]$; $r^2 = 0.3765$
26	1.04 ± 0.07	0.41 ± 0.07	2.45 ± 1.04	21.59 ± 7.12	[LAL] *vs.* $[C_{12:0}]$; $r^2 = 0.9494$
27	1.38 ± 0.06	4.08 ± 0.13	36.50 ± 5.57	38.14 ± 9.04	[NO] *vs.* $[C_{10:0}]$; $r^2 = 0.6515$
28	0.30 ± 0.02	0.69 ± 0.09	8.27 ± 4.66	19.28 ± 6.63	[NO] *vs.* $[C_{12:0}]$; $r^2 = 0.7460$
29	0.77 ± 0.02	2.10 ± 0.05	14.87 ± 2.95	18.40 ± 5.41	[LAL] *vs.* [NO]; $r^2 = 0.7724$
30	0.41 ± 0.03	1.09 ± 0.05	12.78 ± 3.64	23.39 ± 3.71	
Ref.[a]	0.06 ± 0.04	0.59 ± 0.02	25.64 ± 0.05	18.44 ± 6.21	

[a] Refer to *E. coli* O111:B4 LPS.

This is exemplified in Table 2 by the group 1 strains ($r^2 = 0.9707$, $C_{10:0}$; $r^2 = 0.9208$, $C_{12:0}$). If the molar ratio of $C_{10:0}$ *versus* $C_{12:0}$ 3-OH FA in the supernatants were extremely uneven (>1:6.25 or <l:3.01), there was a disruption of the positive correlation between 3-OH FA concentration and endotoxin units in these samples (Table 2).

3.3. LPS quantities relative to NO production

The crude supernatants taken from *P. aeruginosa* cultures were demonstrated to exhibit immunostimulatory ability. This was carried out by showing that they induced NO production in the PEC assay, which has been shown to be dependent on the presence of LPS (endotoxin) in bacterial cultures [7]. All 30 independent strains were also used for this comparative study. We confirmed that NO was induced by adding the bacterial culture supernatants to PEC cultures. The level of NO generation relative to the endotoxin units by LAL assay from the bacterial culture supernatants showed a positive correlation ($r^2 = 0.6174$) (Fig. 3).

Next, using the same strains, we determined whether $C_{10:0}$ or $C_{12:0}$ 3-OH FA, as an indictor for LPS quantity, was correlated with the immunostimulatory effects on PECs. When the 3-OH FA concentrations were evaluated against the amount of NO generation in the PEC assay by linear regression analysis, both the $C_{10:0}$ and $C_{12:0}$ FA indicators showed different but positive correlations in terms of dose–response performance ($r^2 = 0.3211$, $C_{10:0}$; $r^2 = 0.4527$, $C_{12:0}$) (Fig. 4). If the analytical data from groups 2 and 3 are removed (Table 2) and the linear regression is re-calculated, then there is an significant improvement in the degree of correlation ($r^2 = 0.7939$, $C_{10:0}$; $r^2 = 0.8210$, $C_{12:0}$).

4. Discussion

The distinct structures of LPS should give rise to different binding activities to responsive receptors and this will result in

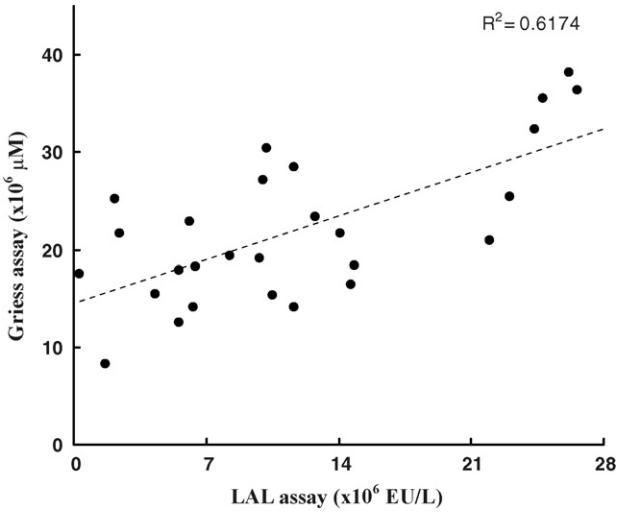

Fig. 3. Correlation of endotoxin units by the LAL assay and NO production. The endotoxin unit level in the bacterial culture supernatants from each of the 30 independent strains was determined by the LAL method. The presence of NO production in treated PEC was measured by a Griess reagent assay kit. The correlation between endotoxin units and NO production was calculated by linear regression analysis.

the induction of different immune responses [8]. For example, structural analysis of the LPS from *P. aeruginosa* reveals a great degree of chemical variability in the position of the O-antigens, and this variation produces a range of immunological characteristics [9]. The LPS from *Burkholderia pseudomallei* consists of a unique unbranched polymer of repeating disaccharide units [10]. This LPS exhibits much weaker murine macrophage activation such as the production of tumor necrosis factor (TNF) and NO than does enterobacterial LPS [11,12]. In this study, the LPS quantified by chemical composition or by biological

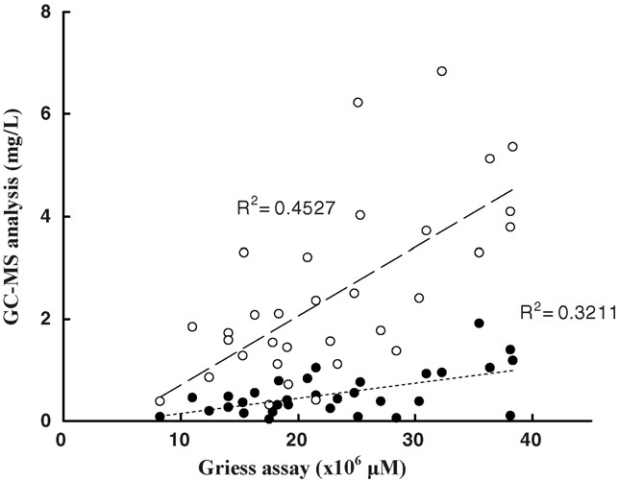

Fig. 4. Correlation of $C_{10:0}$ and $C_{12:0}$ 3-OH FA concentration with NO production. The $C_{10:0}$ and $C_{12:0}$ 3-OH FA concentration was determined by GC–MS analysis. The presence of NO production in treated PEC was measured by a Griess reagent assay kit. The correlation between 3-OH FA concentration and NO production was calculated by linear regression analysis using a total of 30 independent strains. (●) $C_{10:0}$ 3-OH FA; (◯) $C_{12:0}$ 3-OH FA.

function showed different dose–response correlations with NO production. It seems that the immunological modulating effects of LPS were neither fully dependent on the structural response to agglutination of the amoeba lysate nor fully related to the composition of $C_{10:0}$ and $C_{12:0}$ 3-OH FA. The molar ratio of $C_{10:0}$ *versus* $C_{12:0}$ 3-OH FA for the intact LPS of somatic form of *P. aeruginosa* has been reported to usually range from 1:1.5 to 1:4 [6]. In this study, this ratio in supernatants of *P. aeruginosa* cultures was $1:4.63 \pm 1.62$. We found if the ratio between the two types of LPS present in a strain was very much higher or lower than this, then the outcome for the LAL assay was unreliable as a predictor. This implies that the LPS structure containing the relevant molar ratio of 3-OH FA was responsible for the agglutination activity with the amoeba lysate. Thus, the intact structure of the LPS needs to be considered together with whether it is completely released from soma, notwithstanding the fact that the concentration of $C_{10:0}$ or $C_{12:0}$ 3-OH FA is easily measured by GC–MS analysis. In addition, it may be argued that not all detected $C_{10:0}$ or $C_{12:0}$ 3-OH FA originates from the LPS and some may stem from FA metabolites and this may add another confounding factor.

The LAL and NO production assays for LPS are very sensitive and are able to detect LPS down to the nanogram level. However, in particular, the LAL assay can be affected by interference from environmental contaminants such as β-glycan [13]. In a similar manner, the NO production assay may be affected by interfering factors such as the biological bias of living cells and the presence of other active substances in the sample. This may limit the direct application of this assay to the analysis of environmental samples. Even though the LAL and NO production assays for LPS are more closely correlated than either one of them with the 3-OH FA measurements, GC–MS analysis of LPS down to milligram level is still a practical methodology for environmental analysis. Thus, the LPS quantities measured by LAL analysis in combination with the GC–MS assay for shorter chain FA can be used together as a broad evaluation of actually LPS content present in the environment.

5. Conclusion

We determined the relative LPS quantities from bacterial culture supernatants of *P. aeruginosa* using both GC–MS and LAL analysis. The systems were positively correlated with each other. The NO immunostimulatory effect on peritoneal exudates cells from Balb/c mice confirmed that there was a dose-dependent effect ($r^2 = 0.6174$) when the cells were treated with the endotoxic bacterial supernatants. To determine if the concentration of $C_{10:0}$ or $C_{12:0}$ 3-OH FA could be used an indictor for LPS quantity, it was shown that their concentrations were correlated with NO production by the PEC assay; however, the two types of 3-OH FA gave different dose–response correlations ($r^2 = 0.3211$, $C_{10:0}$; $r^2 = 0.4257$, $C_{12:0}$). We suggested that the measurement of environmental LPS quantities needs to use a combination of GC–MS and LAL analysis. Both $C_{10:0}$ and $C_{12:0}$ 3-OH FA can be used as indictors when evaluating the amount of *P. aeruginosa* LPS in an environment.

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