Lectin Array Analysis of Purified Lipooligosaccharide: A Method for the Determination of Molecular Mimicry

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Abstract

Surface glycosylation of bacteria is involved in many critical host-microbe interactions. Lectin arrays consisting of diverse carbohydrate binding proteins have proven to be an important tool for evaluating a wide variety of glycosylation, including that present on whole bacteria. However, assessing glycosylation on whole bacteria using lectin arrays may not reflect bacterial glycosylation, but interactions between bacteria and the glycosylation present on lectins. The lipooligosaccharide of Campylobacter jejuni NCTC1168 and 81-176 are known to mimic the human monosialylated gangliosides. This molecular mimicry by C. jejuni can result in the post infection sequelae Guillain–Barré syndrome. Using C. jejuni as a model system and a discreet lectin and antibody array, a method, applicable to many organisms has been developed and validated by to screening of the purified lipooligosaccharide of C. jejuni for molecular mimicry to monosialylated gangliosides. In case of C. jejuni, knowing whether clinically important bacterial strains are capable of inducing severe autoimmune responses may aid in prevention and/or early diagnosis of debilitating post infection conditions.

Keywords: Lipooligosaccharide; Glycosylation; Lectin arrays

Introduction

Bacterial surface glycosylation is involved in many critical and diverse host-microbe interactions including adherence and immune modulation [1]. The analysis of bacterial surface glycosylation traditionally has been performed using nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS) and blotting based techniques [2-7]. Both NMR spectroscopy and MS are powerful techniques, but require relatively large quantities of highly purified glycan for structural analysis. Blotting methodologies, such as lectin blotting [7], have the advantage of only requiring small amounts of partially purified glycosylated protein or lipid for analysis, however only a single lectin interaction can be assessed per blot and the results can be ambiguous due to the difficulties in ensuring equimolar loading of test compounds into individual wells on the gel. In all these methodologies only provide low-throughput capabilities.

Lectin arrays consisting of diverse carbohydrate binding proteins covalently immobilised on glass microarray slides, have proven to be an important tool for evaluating cell surface glycosylation on whole bacteria and eukaryotic cells [1,8-10], eliminating the need to purify surface glycoproteins and lipids prior to analysis. However, assessing glycosylation on whole bacteria using lectin arrays may not be an accurate reflection of the bacterial glycosylation. Lectins are glycoproteins [11,12], therefore when immobilized on microarray slides they may themselves act as receptors for bacterial carbohydrate binding proteins. That is, bacterial glycan recognising adhesins [13-17] may interact with carbohydrate structures present on immobilised lectins rather than lectins recognising bacterial glycoconjugates. In order to overcome this potentially significant limitation, we report here the analysis of semi-purified bacterial glycan using a discrete lectin array that requires only a small quantity of product for accurate, quick, and reproducible glycan structure determination. As the model systems for lectin array-based glycan structure determination we assessed both purified and crudely isolated C. jejuni lipooligosaccharide (LOS).

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K as described previously [23]. The LOS mini-preparations from single colonies were prepared by collecting and washing cells in 40 µL of sterile water followed by heat lysis. These preparations were diluted 10-fold prior to gel electrophoresis or lectin array analysis.

Purified LOS preparation: *C. jejuni* LOS was purified by subjecting the *C. jejuni* cell biomass to hot phenol-water treatment using 90 % (v/v) aqueous phenol at 65°C for 10 min [24], followed by enzymatic treatment as previously described [25]. The LOS preparations were adjusted to 15 µg/µL with distilled water prior to gel electrophoresis or lectin array analysis.

**Electrophoretic analyses**

Equal quantities of either LOS mini-preparations or purified LOS (~15 µg) was resolved on SDS-PAGE (5.5 % (w/v) and 10 % (v/v) stacking and separating acrylamide gels, respectively) containing 6 M urea and 0.3 mM tricine (Tricine-SDS-PAGE) as previously described [26]. Following electrophoresis at 20 V for 1 h to maximize stacking and separating acrylamide gels, respectively)

Electrophoretic analyses

**Lectin and western blotting**

In addition to silver staining, fractionated *C. jejuni* LOS was electrophoresed from Tricine-SDS-PAGE gels to Pall® PVDF membranes using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad) followed by 30 V for 60 min. Membranes were subsequently probed using either horse radish peroxidase (HRP)-conjugated Ctb (Cholera toxin subunit B) (3 µg/mL), HRP-conjugated PNA (Peanut Agglutinin) (5 µg/mL), or HRP-conjugated anti-GM1, ganglioside IgG (diluted 1:3000) in PBS. Membranes were developed using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) according to the manufacturer’s instructions.

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the ProScanArray Microarray 4-Laser Scanner and the ProScanArray imaging software ScanArray Express from PerkinElmer as previously described [30]. Analysis was limited to presence or absence of binding to lectin spots across a 1:2 serial dilution rather than absolute binding levels. All positive reported binding was visible by visual inspection of the array and significantly above background by T-test in Microsoft Excel. Lectin arrays were performed a minimum of twice per LOS sample.

Results

The LOS of C. jejuni strain NCTC 11168 is known to mimic the human ganglioside structure, G\textsubscript{M1}, and was therefore chosen to evaluate the viability of screening both phenol purified and crudely isolated LOS samples using our discrete lectin array. Initial analysis of the two alternatively isolated LOS samples from C. jejuni NCTC 11168 was performed using traditional SDS-PAGE silver staining and lectin blotting analysis (Figure 1). Silver staining revealed no differences in electrophoretic mobility between the phenol purified and crudely isolated LOS preparations (Figure 1A). Lectin blotting using HRP-conjugated PNA and CTB also revealed no differences in binding ability between sample preparations, with as expected [6] significant binding of both lectins (Figure 1B and 1C respectively). Taken together these data show that both LOS preparations have similar glycoconjugate components, specifically LOS that mimics G\textsubscript{M1} (identified through PNA and CTB positive binding), and hence both LOS preparations provide an excellent tool for assessment of lectin array technology. Due to the fact that the C. jejuni LOS structure with molecular mimicry with human gangliosides has been reported [6,32-34], lectins were selected to generate a tailored array that would most effectively identify the individual carbohydrate motifs of structures containing monosialylganglioside mimicry (Table 2). The lectins selected included those known to bind the G\textsubscript{M1} mimic C. jejuni NCTC 11168 LOS (PNA and CTB) [6,34,35], and those assumed to bind based on the published structures of NCTC11168 and 81-176 [6,32].

![Figure 1: Silver-stained SDS-PAGE gel and Lectin blots of Crude and Phenol purified LOS preparations extracted from C. jejuni NCTC 11168. A) Silver stained LOS preparations, B) CTB blotting result for LOS preparations, C) PNA blotting result for LOS preparations. Lane 1 in each pane is Crude LOS preparation, while lane 2 is the phenol purified preparation.](image1)

LFA (Limax flavus agglutinin), MAA (Maackia amurensis agglutinin), VAA (Viscum album agglutinin) and Jacalin (Jackfruit; Artocarpus integrifolia). Both DBA (Dolichos biflorus agglutinin) and ConA (Canavalia ensiformis agglutinin) were used as negative controls due to the absence of terminal GalNAc (recognised by DBA) and Man/ GlcNAc (recognised by ConA) in the published structures [6,32]. In addition, two specific anti-ganglioside antibodies were also included, one anti-G\textsubscript{M1} and one anti-G\textsubscript{M2} (Figure 2).

![Figure 2: Published structures of the LOS of C. jejuni NCTC 11168, 81-176 and human G\textsubscript{M1} and G\textsubscript{M2}. A) C. jejuni NCTC 11168 [8] and the expected binding for lectins/antibodies present on the array. B) C. jejuni 81-176 [32] and the expected binding for lectins/antibodies present on the array. N.B. Expected lectin binding is predicted from specificities listed in Table 2. Lower specificity interactions are also possible. Presented structures are the published majority structure for each strain [6,32]. C) Structure of G\textsubscript{M1} (bolded sugar residues are identical to published C. jejuni 11168 structure shown in A). D) Structure of G\textsubscript{M2} (bolded sugar residues are identical to published 81-176 structure shown in B).](image2)

Figure 3: Analysis of live C. jejuni 11168 compared to purified LOS. A) Lectin array analysis of crude LOS extract prepared from C. jejuni 11168. B) Lectin array analysis of phenol purified LOS extract prepared from C. jejuni 11168. C) Lectin array analysis of live C. jejuni 11168. D) Graphical representation of the array analysis. Units are fluorescence above background. Error bars equal one standard error of the mean. Any data not significantly different from background has been set to zero units. Lectins are listed in same order as Table 2.

Table 2 summarises the binding results obtained using our lectin array for both purified (11168 [P]) and crudely isolated (11168 [C]) LOS preparations. Even though binding of BODIPY labelled LOS to all lectins/antibodies with the exception of DBA, ConA and CTB was observed, some differences with respect to the level of binding was...
observed (Table 2; Figure 3). That is, three lectins were found to bind both LOS preparations down to 125 μg/mL (lowest concentration printed), the β-Gal recognising lectins PNA and VAA, and the α-Gal recognising Jacalin (structures recognised shown in Figure 2). The lack of LOS binding to DBA and ConA was anticipated, however the inability of either LOS preparation to binding immobilised CTB was surprising, particularly given that lectin blot analysis using HRP-conjugated CTB showed significant binding (Figure 1B). Both anti-ganglioside antibodies were bound by both LOS preparations, however binding to anti-G\(_{\text{M1}}\) was only observed at the highest concentration printed, while anti-GM1 was bound down to the 1:1000 dilution (Table 2). This is not surprising given that the published C. jejuni LOS structure is known to be exclusively G\(_{\text{M1}}\) under specified growth conditions [35]. The low binding therefore observed to anti-G\(_{\text{M1}}\) may simply reflect some cross-specificity to the underlying G\(_{\text{M1}}\) structure within the G\(_{\text{M1}}\) mimic. In comparison, live C. jejuni 11168-O bound to all printed spots that contained protein including CTB (Figure 1B).

Further analysis was performed on LOS isolated using the crude method from other C. jejuni strains, one with a published LOS structure 81-176 (Structure shown in Figure 2) [32] and a strain without a published structure, C. jejuni 224. C. jejuni 224 was chosen for analysis as it has a LOS biosynthesis class (Class R; Table 3A) capable of producing molecular mimicry (Class A, B, C, R and M; Table 3B) [28,29].

Lectin array analysis confirmed C. jejuni 81-176 produces LOS with G\(_{\text{M1}}\) mimicry rather than G\(_{\text{M1}}\). Binding was observed for the two highest concentrations of the Anti-G\(_{\text{M1}}\) antibody, but no binding was observed for G\(_{\text{M1}}\). The LOS from 81-176 also had decreased binding for PNA, Jacalin and VAA when compared to NCTC11168 further confirming the absence of the terminal galactose from the structure (Figure 2). Binding of the 81-176 LOS to LFA and MAA confirmed the presence of sialylation on the LOS.

Analysis of C. jejuni strain 224 revealed binding to the anti-G\(_{\text{M1}}\) antibody but other binding to structures lectins such as PNA, Jacalin and VAA were only equal in binding to those observed for 81-176 rather than NCTC11168. The LOS isolated from C. jejuni 224 was confirmed to be sialylated due to positive binding by LFA and MAA. No binding was observed for the anti-G\(_{\text{M1}}\) antibody.

Discussion

The use of lectin arrays to determine surface glycan structures on whole bacteria has been previously described [1, 8]; however, to our knowledge, our approach of analysing fluorescence labelled isolated LOS on lectin array is completely novel, and offers a powerful analytical technique. This is particular the case, because the use of whole bacteria on lectin arrays may not accurately reveal the nature of the glycosylation present on the cell. As previously stated, lectins and antibodies are glycoproteins [11,12], therefore carbohydrate recognising adhesins present on bacteria [13-17] may bind these structures, rather than immobilised lectins binding to bacterial surface glycoconjugates. Testing of whole C. jejuni found binding to all lectins printed including CTB which had failed to bind in all other experiments (Figure 3; Table 2). Our novel approach of utilising isolated LOS overcomes this potentially significant shortcoming. Using the C. jejuni 11168 LOS as a model our discrete lectin array identified all carbohydrate components (Table 2) as predicted from the published structure (Figure 2).

The success of our approach depended largely on the dye used to label the isolated LOS. The lipophilic BODIPY TR methyl ester specifically interacts with the hydrocarbon tail of the LOS. The lack of binding observed to both DBA and ConA on the array indirectly confirms this selective labelling, given that any contaminating glycoconjugates (eg. glycoproteins) co-isolated with the C. jejuni LOS that could have been labelled with BODIPY would be expected to bind DBA immobilised on our array. That is, terminal α-D-GalNAc structures are common in N-linked C. jejuni glycoproteins [36], and if labelled, would have been detected on our lectin array.

Analysis was also performed on two other strains of C. jejuni for LOS molecular mimicry, 81-176 and 224. C. jejuni 81-176 was chosen because, like NCTC1168 [6], 81-176 has a published structure [32]. The results of the lectin array agreed with the known structure produced by 81-176, a G\(_{\text{M1}}\) mimic LOS [32]. C. jejuni 81-176 is known to produce several other structures including G\(_{\text{M1}}\), G\(_{\text{M2}}\) and G\(_{\text{M3}}\), however, these structures are present in smaller amounts than the G\(_{\text{M1}}\) mimicking structures [32]. Therefore it is unlikely these structures would be affecting the outcomes of the array analysis. A wider variety of anti-ganglioside antibodies may prove effective in identifying these less prevalent LOS structures.

C. jejuni 224 LOS analysed by lectin array indicated primarily G\(_{\text{M1}}\) mimicry from the antibody binding but was not 100% confirmed by the binding observed for the other lectins present on the array. Levels of binding for PNA, VAA and Jacalin were lower than those seen for the known G\(_{\text{M1}}\) mimic NCTC11168 (Table 2). However, the LOS structure produced by C. jejuni 224 was definitely sialylated providing further evidence for ganglioside mimicry. A previous study showed that the LOS from C. jejuni 224 was of the same size by electrophoresis as NCTC11168 and bound strongly by CTB [35]. This result together with the lectin array result implicates strongly that the LOS is a G\(_{\text{M1}}\) mimic.

Although our lectin array data correlates well with the known C. jejuni 11168 and 81-176 LOS structures, a discrepancy between observed and expected binding results was noted. Specifically, no binding was observed to CTB immobilised on the lectin array, even though lectin blotting using CTB (Figure 1) showed a strong positive signal. This was a predictable outcome since CTB exists as a pentameric structure [37]. The complex pentameric structure of CTB may therefore be disrupted or constrained when covalently attached to the array through an epoxide-linkage. CTB is likely to be unsuitable for use on lectin arrays and its use restricted to lectin blotting analyses.

We also investigated the suitability of two different LOS preparations (phenol-purified and crudely isolated LOS) for analysis on our lectin array. Apart from a slight increase in non-specific background binding of the crude LOS preparation, compared to the phenol purified LOS (data not shown), no significant difference was observed using either LOS preparations. This suggests that a simple heat lysis and proteinase K digestion of C. jejuni or other bacteria is sufficient to allow rapid and sensitive screening using lectin arrays particularly of strains expressing ganglioside mimicry. The simple methodology reported here can, therefore, be used to rapidly evaluate whether clinical isolates have the potential to produce adverse autoimmune reactions as post infection sequelae, similar to those attributed to C. jejuni (GBS or MFS). Knowing whether clinically important bacterial strains are capable of inducing severe autoimmune responses may aid in prevention and/or early diagnosis of these debilitating post infection conditions.

The method reported here is also applicable, through minor modifications to the lectins/antibody specificities printed on the array, to the screen glycolipids from almost any other species of bacteria for almost any terminal glycoconjugate.
References


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