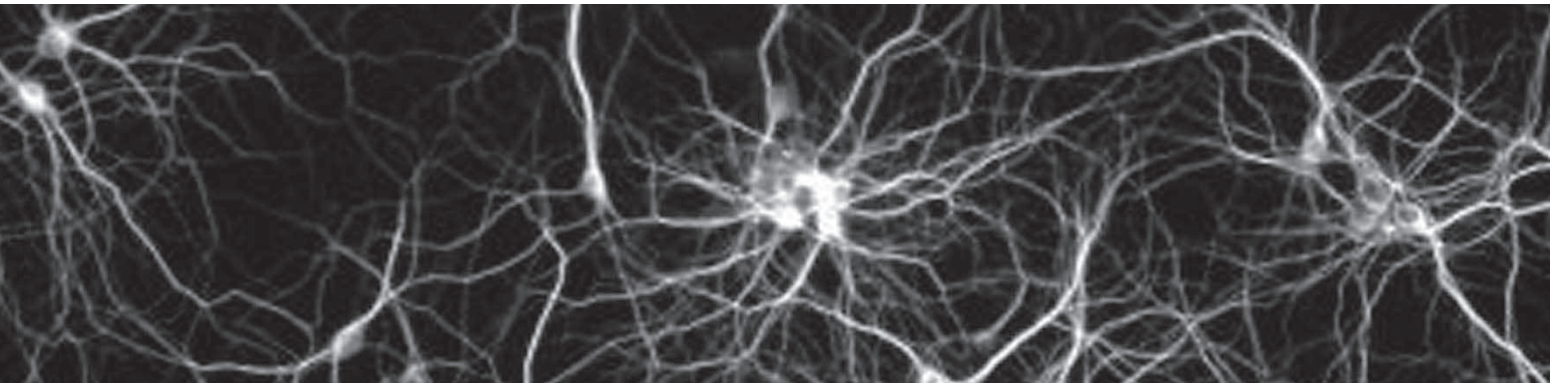

Immunoreactivity and characterization of oligosaccharide determinants in glycoproteins isolated from peripheral nerve and *Campylobacter jejuni* O:19

A. Poceva-Panovska, K. Brezovska, A. Grozdanova[1], S. Apostolski[2], Lj. Suturkova

ABSTRACT – The biology of glycoconjugates and immunopathological responses in reference to the diseases of the nervous system is an area of intensive research. In the last decade, several carbohydrate structures that are target determinants in peripheral nerve diseases have been isolated and partially characterized. Experimental evidence indicate structural similarity between oligosaccharide determinants present in peripheral nerve glycoconjugates and bacterial carbohydrate structures, suggesting that molecular mimicry between bacterial and neural oligosaccharides may have a potential role in the development of autoimmune postinfectious neuropathies. In this study, galactosyl *N*-acetylgalactosamine binding glycoproteins were isolated from human peripheral nerve and bacteria *Campylobacter jejuni* O:19, using peanut agglutinin (PNA) lectin affinity chromatography. Isolated glycoproteins were detected with immunoblot analysis using periodate oxidation and biotinylated peanut agglutinin. Sera from patients with Guillain-Barré syndrome were tested on immunoblot for reactivity to the previously isolated glycoproteins. We detected immunoreactive glycoproteins with similar electrophoretic mobility present in the isolates from peripheral nerve and *Campylobacter jejuni*. N-linked oligosaccharides were released from these immunoreactive glycoproteins, fluorescently labeled and enzymatically sequenced with highly specific exoglycosidases. Further analysis with fluorophore-assisted carbohydrate electrophoresis demonstrated the presence of similar oligosaccharide structures in glycoprotein isolates. Structural similarity and immunoreactivity between human peripheral nerve

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and *Campylobacter jejuni* glycoproteins was detected. This finding indicates the possible role of the isolated bacterial glycoprotein as an antigenic determinant involved in the pathogenesis of Guillain-Barré syndrome.

Key words: *Campylobacter jejuni,* Guillain-Barré syndrome, immunoreactivity, glycoproteins, peripheral nerve

INTRODUCTION

Antibodies involved in the pathogenesis of autoimmune neuropathies usually are directed against carbohydrate epitopes of glycoconjugates. Several carbohydrate-containing structures that are target determinants in peripheral nerve diseases have been identified and characterized. These antigenic structures include molecules of glycoproteins, glycolipids and glycosphingolipids (1).

In Guillain-Barré syndrome (GBS), a prototype of postinfectious autoimmune disease, the gram-negative bacterium *Campylobacter jejuni* (*C. jejuni*) is a frequent antecedent pathogen with overall prevalence of 32% (2). Numerous *C. jejuni* serotypes have been reported in association with GBS. A strong association between *C. jejuni* serotype and GBS has been reported by Kuroki *et al.* (3), with a predominance of serotype O:19, present in 81% of GBS population (4).

Analysis of *C. jejuni* lipopolysaccharide (LPS) showed that the terminal structure (Gal(β1-3)GalNAc β1-4 [NeuAc α1-3]Galβ) is identical to the terminal tetrasaccharide of the GM1 ganglioside, a glycosphingolipid that is mostly abundant in the human peripheral nervous system. These were the first findings that demonstrated the existence of molecular mimicry between neural tissue gangliosides and the infectious agent isolated from patients with GBS (5,6). Further animal studies supported the hypothesis that expression of ganglioside-mimicking structures of *C. jejuni* LPS is a triggering factor for the induction of antiganglioside antibodies and development of GBS (7-10).

Several studies have reported reactivity of GM1 positive GBS patient sera to Gal(β1-3)GalNAc glycoproteins from human peripheral nerve (11) and glycoproteins isolated from *C. jejuni* (12,13). Flagellar glycoprotein was isolated and purified from the reference strain of *C. jejuni* O:19 and the results obtained support the hypothesis that in GBS patients, antiflagellar antibodies are induced during *C. jejuni* infection (14). In addition, the glycosyl modifications that are surface exposed in the flagellar filament appear to be highly immunogenic (15). These findings suggest that the presence of non LPS antigens may also be involved in the development of the disease. The association of glycosylated flagellin with the development of GBS remains speculative, but the possibility of molecular mimicry between flagellar glycoproteins and eukaryotic glycoproteins exists (16).

Since most of the research in this field is oriented toward the lipopolysaccharide antigens in bacteria and ganglioside structures in the nervous system, we investigated other nonlipopolysaccharide antigens in bacteria and glycoproteins in human peripheral nerve. In this study, we analyzed the carbohydrate composition of two N-linked glycoproteins isolated for human peripheral nerve and *C. jejuni*, which were recognized by GBS patient sera, using a technically simple biochemical method of fluorophore-assisted carbohydrate electrophoresis (FACE).

MATERIAL AND METHODS

Isolation of Gal(β1-3)GalNAc binding glycoproteins from peripheral nerve and C. jejuni serotype O:19

Human sciatic nerve was obtained postmortem from a 45-year-old male patient who died from non-neurological disease (Institute of Forensic Medicine, Faculty of Medicine, Ss. Cyril and Methodius University, Skopje, Macedonia) and kept frozen at -70 °C until use. It was delipidated in chloroform:methanol (1:2) and solubilized in 0.5% Triton X-100, 0.4% SDS with protease inhibitor cocktail.

C. jejuni serotype O:19 (ATCC 700 297) was cultured on Campylosel® (bioMerieux, La Balme, France) in microaerophilic conditions using Campy Gen® (Oxoid, Basingstoke, UK) at 37 °C for 48 hours. The identity of *C. jejuni* was confirmed with microscopic examination, staining according to Gram and biochemical tests, at the Institute of Microbiology and Parasitology, Faculty of Medicine, Skopje. Hot phenol-water method, described by

Westphal and Jann (17) was adapted for extraction of proteins from the phenol phase. Proteins from the peripheral nerve and *C. jejuni* were further purified with affinity chromatography using 2-mL column with agarose bound peanut agglutinin (Sigma-Aldrich, St. Louis, USA) (11).

Identification of isolated glycoproteins

Isolated glycoproteins from the peripheral nerve and *C. jejuni* were separated with SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis) on 7.5% gel and analyzed on Western blot.

Carbohydrate structures were detected using periodate oxidation as prescribed in Bio-Rad immunoblot kit for glycoprotein detection. Detection of PNA-binding glycoproteins was done using lectin from *Arachis hypogaea* (peanut) conjugated with biotin (Sigma-Aldrich, St. Louis, USA). For visualization, avidin-peroxidase in dilution 1:1000 (Sigma-Aldrich, St. Louis, USA) and chromogen diaminobenzidine (SIGMA FAST™, Sigma-Aldrich, St. Louis, USA) were used.

Immunoreactivity of Gal-β(1-3)-GalNAc binding glycoproteins from peripheral nerve and C. jejuni

Sera from eight male and two female patients (aged 40-62 years) originating from Serbia, diagnosed with GBS, were provided by the Institute of Neurology, Clinical Center of Serbia in Belgrade. Sera from one female and two male blood donors were used as negative controls. Antibody titer to gangliosides GM1 and GA1 in these patients was determined by ELISA using intra-laboratory protocol standardized according to the method suggested by INCAT (European Inflammatory Neuropathy Cause and Treatment) group (18,19). Sera (1:200) were also tested for reactivity to the isolated glycoproteins on Western blot, following separation of the glycoproteins on 7.5% polyacrylamide gel. Peroxidase conjugated anti-human IgM and IgG (Sigma-Aldrich, St Louis, USA) were used as secondary antibodies (1:500). Visualization was done with diaminobenzidine (SIGMA FAST™, Sigma-Aldrich, St. Louis, USA).

Analysis of oligosaccharides in immunoreactive glycoprotein

The glycoproteins from peripheral nerve and *C. jejuni* that gave signal when incubated with patient sera (~60 kDa) were isolated using preparative SDS PAGE (7.5% gel) and lyophilized. Isolated glycoproteins were characterized by oligosaccharide profiling and sequencing, using the method of fluorophore-assisted carbohydrate electrophoresis (FACE) (20-22).

Enzymatic release of N-linked oligosaccharides

Enzymatic release of asparagine linked (*N*-linked) oligosaccharides from immunoreactive glycoproteins from peripheral nerve and *C. jejuni* was done using Peptide *N*-glycosidase F (PNGase-F) (Glyko® ProZyme Inc., San Leandro, Ca, USA) by a protocol prescribed by Glyko®. After overnight incubation at 37 °C with PNGase F, the *N*-linked oligosaccharides released were labeled with fluorophore (8-aminonaphthalene-1,3,6-trisulfonate, ANTS), followed by the addition of reducing agent (sodium cyanoborhydride, $NaCNBH_3$). The labeled oligosaccharides were separated on 21% polyacrylamide gel using commercial *N*-linked oligosaccharide buffer (Bio-Rad®). Determination of the relative migration of the oligosaccharides was done using a mixture of glucose oligomers ranging from $Glucose_1$ to larger than $Glucose_{20}$ (glucose ladder). Separated oligosaccharides were visualized in UV chamber (long UV) and analyzed using TotalLab® (Nonlinear Dynamic, UK) image software.

Enzymatic sequencing of N-linked oligosaccharides

Oligosaccharide band from the profiling gel was cut out and glycans were extracted and subjected to sequential enzymatic digestion and electrophoresis. Following enzymes purchased from Glyco® were used: neuraminidase (NANase III) specific for all α2-3,6,8,9 linked *N*-acetylneuraminic acid; β-galactosidase (GALase III) specific for β1-4 linked galactose; βN-acetylhexoaminidase (HEXase III) specific for β1-2,3,4,6 linked *N*-acetylglucosamine and α-mannosidase (MANase II) specific for α1-2,3,6 linked mannose. Oligosaccharide sequencing was performed with series of enzyme digests by sequencing protocol obtained from Glyko® *N*-linked oligosaccharide sequencing kit.

The structure of the glycan was determined by comparing the electrophoretic migration patterns of the digestion products with the glucose ladder. Relative mobility shift after releasing of each monosaccharide unit was given in the protocol. No changes in the migration indicated that sugar was not present or not in the correct linkage. The se-

quencing gels were analyzed using TotalLab® image software.

RESULTS

Identification of the isolated glycoproteins

Following the electrophoretic separation and immunoblotting, the isolated glycoproteins from peripheral nerve and *C. jejuni* were visualized with periodate oxidation and with biotin labeled lectin from *Arachis hypogaea* (peanut agglutinin, PNA) that specifically binds to the Gal-β(1-3)-GalNAc determinant. It was shown that isolates from both peripheral nerve and *C. jejuni* contained few PNA-binding glycoproteins with molecular masses of approximately 200 kDa, 120 kDa, 70 kDa and 60 kDa (Fig. 1).

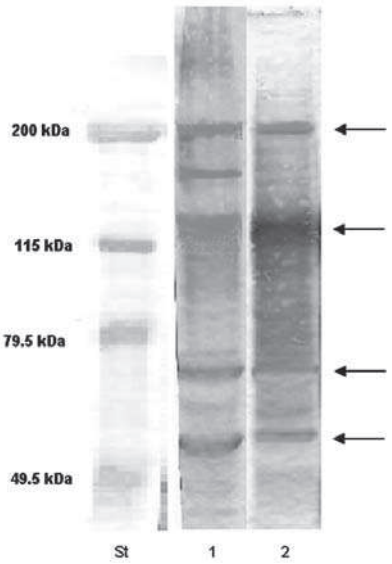

Fig. 1. *Immunoblot detection of (1) peripheral nerve and (2) C. jejuni glycoproteins with biotinylated PNA.*

Immunoreactivity of Gal-β(1-3)-GalNAc binding glycoproteins from peripheral nerve and C. jejuni

In order to test immunoreactivity of the isolated Gal-β(1-3)-GalNAc binding glycoproteins, we used sera from patients with GBS. First, antibody titers of patient sera to GM1 and AG1, the gangliosides that are frequently associated with the syndrome, were determined by indirect ELISA method. Elevated titers of antiganglioside antibodies were found in approximately 70% of tested sera. When immunoreactivity of isolated glycoproteins to GBS patient sera was tested, 50% of the sera were found to have a similar reactivity pattern to glycoproteins with a molecular weight of 60-70 kDa, present in peripheral nerve and *C. jejuni* (Fig. 2). Negative controls showed no reactivity to peripheral nerve and *C. jejuni* glycoproteins. There was no correlation between the anti-ganglioside antibody titer and reactivity to the isolated glycoproteins.

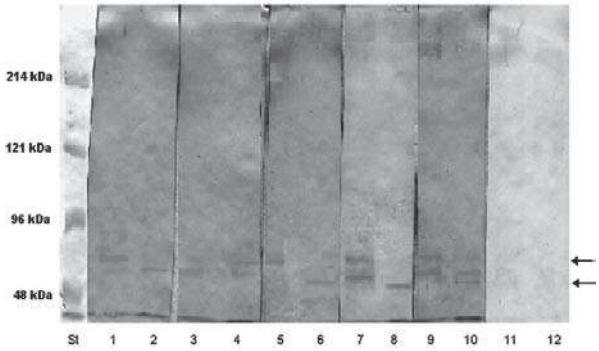

Fig. 2. *Immunoreactivity glycoproteins isolated from peripheral nerve (lanes 2, 3, 6, 8 and 10) and C. jejuni (lanes 1, 4, 5, 7 and 9) with five GBS patient sera. Negative control sera (PN, lane 11; C. jejuni, lane 12).*

Enzymatic release of N-linked oligosaccharides (oligosaccharide profiling)

Oligosaccharide profiles of peripheral nerve and *C. jejuni* N-linked oligosaccharides in immunoreactive glycoproteins, obtained after enzymatic release, labeling and electrophoretic separation are shown in Fig. 3. The migration position is reported in terms of degree of polymerization (DP), which corresponds to the sample migration in relation to the migration of the glucose ladder standards (lane 1).

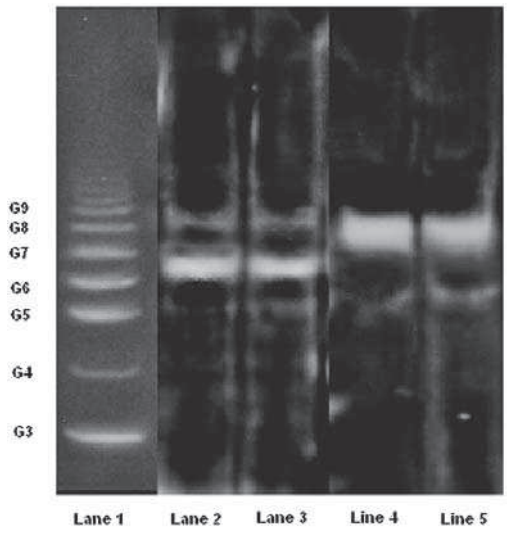

Fig. 3. *Oligosaccharide profiles of N-linked oligosaccharides isolated from peripheral nerve (lanes 2 and 3) and C. jejuni (lanes 4 and 5) immunoreactive glycoprotein; lane 1: glucose ladder (St).*

According to the software analysis, profiles of oligosaccharide released from immunoreactive glycoprotein from peripheral nerve and from *C. jejuni* contained one major band with 6.58 DP and 7.6 DP, respectively. In comparison with the values for the mobility of ANTS labeled *N*-linked oligosaccharides, peripheral nerve oligosaccharide is a monosialylated bi-antennary complex type, while *C. jejuni* has the asialo bi-antennary complex type of *N*-linked oligosaccharide.

Enzymatic sequencing of N-linked oligosaccharides

The sequencing gel for peripheral nerve oligosaccharides is shown in Fig. 4. Lane 1 represents the oligosaccharide (OS) profile and lane 2 OS with no enzymatic digestion. The probe in lane 3 contains NANase III, which cleaves terminal sialic acid residues. In Fig. 4, the upwards shift in 1.1 DP relative to the glucose ladder indicates that one sialic acid residue was removed. Digestion with NANase II and GALase III cleaves the glycan further (lane 4) and band shifting of 2.06 DP indicates that two galactose residues were removed. Digestion with HEXase produces band shift of 1.63 DP, which corresponds to removal of two *N*-acetylglucosamine residues (0.75 DP units/*N*-acetylglucosamine removed) (lane 5). Treatment with MANase II gives mobility shift of 1.36 DP units indicating the cleavage of two mannose residues. Digestions with HEXase and MANase determined the number of antennae in the glycan. Since two *N*-acetylgalactosamine residues were linked to trimannosyl core,

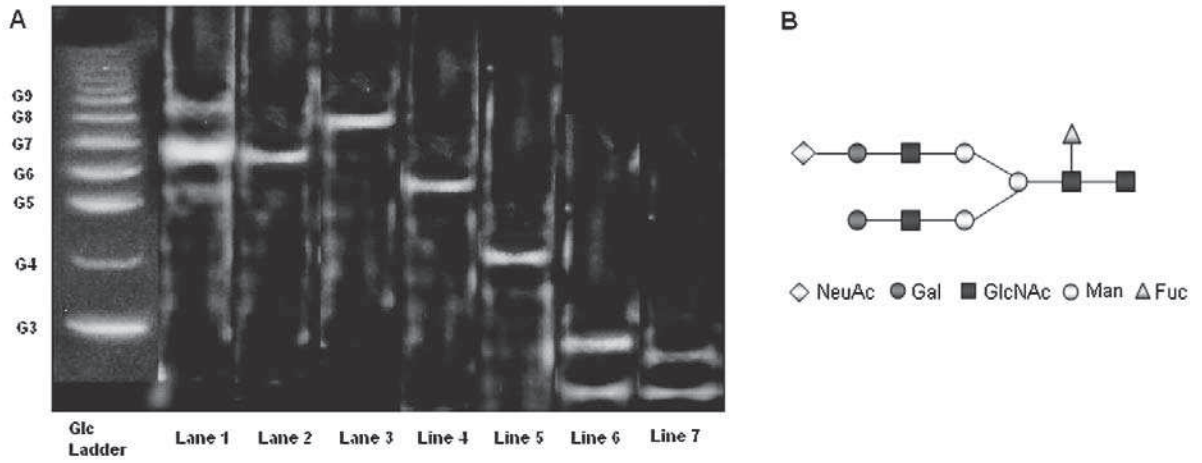

Fig. 4. *(A) Sequenced analysis of N-linked oligosaccharides from peripheral nerve. Lane Glc Ladder: glucose ladder; lane 1: oligosaccharide (OS) profile; lane 2: OS without enzyme; lane 3: OS with NANase III; lane 4: OS with NANase III + GALase III; lane 5: OS with NANase III + GALase III + HEXase III; lane 6: OS with NANase III + GALase III + HEXase III + MANase II; lane 7: core standard; (B) proposed structure based on the analysis.*

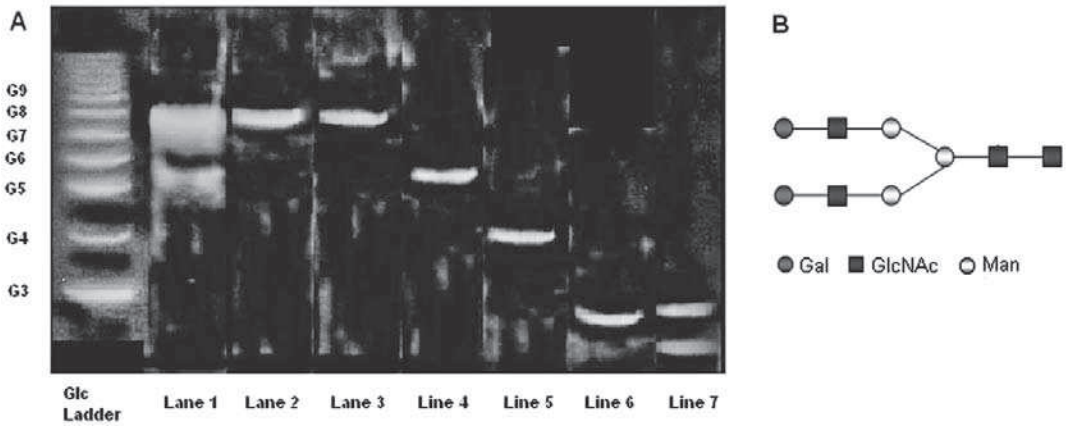

Fig. 5. *(A) Sequence analysis of N-linked oligosaccharides from Campylobacter jejuni: lane Glc Ladder: glucose ladder; lane 1: oligosaccharide (OS) profile; lane 2: OS without enzyme; lane 3:OS with NANase III; lane 4: OS with NANase III + GALase III; Lane 5: OS with NANase III + GALase III + HEXase III; lane 6: OS with NANase III + GALase III + HEXase III + MANase II; lane 7: core standard; and (B) proposed structure based on the analysis.*

the glycan was determined to be of a bi-antennary structure.

To determine the presence or extent of core fucosylation, a mixture of ANTS labeled trimannosyl core oligosaccharides with and without α1-6 fucose was separated in lane 7. The core residue in line 6 co-migrated with fucosylated core standard (upper line). From this analysis, the identity of the glycan derived from peripheral nerve is monosialylated, bi-antennary galactosylated complex type of *N*-linked oligosaccharide with fucosylated core (Fig. 4B).

The sequencing gel for *C. jejuni* oligosaccharides is shown in Fig. 5. In lane 3, there was no shift in mobility, suggesting that sialic acid is not present in oligosaccharide structure. Band shift of 2.15 DP in lane 4 indicates the loss of two galactose residues. *N*-acetylglucosamine (lane 5) produced band shift of 1.64 DP, which corresponds to mobility shifts of two *N*-acetylglucosamine residues. Digestion with MANase II gives band shift in 1.46 DP indicating the presence of 2 mannose residues. The core of the glycan structure is non-fucosylated because it migrates closer to the band of non-fucosylated core in the core standard. The identity of the glycan derived from *C. jejuni* is of asialylated, biantennary galactosylated complex type of *N*-linked oligosaccharide with non-fucosylated core (Fig. 5B).

DISCUSSION

The Guillain-Barré syndrome (GBS) is the most common form of acute neuromuscular paralysis in developed countries (2), but the pathogenesis is still in debate. Research in the field was predominated by the discovery that infections with the gram-negative *Campylobacter jejuni* frequently precede GBS and by the finding of antibodies against various peripheral nerve gangliosides in serum from GBS patients. These anti-ganglioside antibodies recognize the oligosaccharide portion of the gangliosides. This was demonstrated by the cross-reactivity of anti-ganglioside antibodies with gangliosides and other glycoconjugates with homologous oligosaccharide moieties. Anti-GM1 antibodies frequently cross-react with GD1b and asialo-GM1, suggesting that they bind to the Gal(β1-3)GaINAc-structure, which these glycolipids have in common (23). The Gal(β1-3)GaINAc-structure is also widespread in glycoproteins (24), and human monoclonal anti-GM1 antibodies cross-react with glycoproteins in peripheral nerve extracts (25). It is therefore unknown whether anti-ganglioside antibodies bind with gangliosides or other target structures in peripheral nerves. There are studies that report antibodies against the outer membrane proteins to be frequently found in serum from German GBS patients with *C. jejuni* infections (14). The presence of glycosylated proteins in bacteria such as flagellar protein raises the possibility of molecular mimicry to glycosylated moieties on human proteins.

In our study, we demonstrated that isolated bacterial glycoprotein shows similarity in the carbohydrate structure with human peripheral nerve glycoprotein. Enzymatic sequence analysis of the glycans derived from immunoreactive glycoproteins present in peripheral nerve and *C. jejuni* indicated the presence of two galactose, two *N*-acetylgalactosamine and two mannose residues differing only in the presence of one residue of terminal sialic acid and fucosylated core in peripheral nerve oligosaccharide. An important finding in our research was that 50% of GBS patient sera, when tested on Western blot, showed a similar reactivity pattern to glycoprotein isolates from human peripheral nerve and *C. jejuni*. We also observed that there was no correlation between the anti-GM1 and AG1 ganglioside antibody titer and immunoblot reactivity, to the isolated glycoproteins. This finding supports the hypothesis that the pathology of GBS may be mediated by cross-reactive autoantibodies directed against the GalGalNAc epitope of glycoproteins, and their production may be triggered by homologous antigen in *C. jejuni*.

Structural similarity in the oligosaccharide portion and immunoreactivity of these glycoproteins indicates that they are potentially cross-reactive and may contribute to the disease development. Animal model studies and *in vivo* testing of the isolated glycoproteins will further demonstrate their antigenic potential and possible role in the pathogenesis of GBS.

CONCLUSION

Results from this study revealed structural similarity in oligosaccharide portion and immunoreactivity of the glycoproteins isolated from peripheral nerve and *C. jejuni*, indicating that they are potentially cross-reactive determinants and may contribute to the development of GBS associated with antecedent *C. jejuni* infection. Further structural characterization and *in vivo* analysis of their antigenic potential will elucidate their possible involvement in the development and pathogenesis of GBS.

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Imunoreaktivnost i karakterizacija oligosaharidnih determinanti u glikoproteinima izoliranima iz perifernog živca i *Campylobacter jejuni* O:19

SAŽETAK - Biologija glikokonjugata i imunološki odgovori u odnosu na bolesti živčanog sustava područje su intenzivnog istraživanja. Posljednjih deset godina izolirano je i djelomično karakterizirano nekoliko ugljikohidratnih struktura koji su ciljne determinante kod bolesti perifernog živca. Eksperimentalni nalazi ukazuju na strukturnu sličnost između oligosaharidnih determinanti prisutnih u glikokonjugatima perifernog živca i bakterijskih ugljikohidratnih struktura ukazujući da molekulska mimikrija između bakterijskih i živčanih oligosaharida može imati ulogu u razvoju autoimunih postinfekcijskih neuropatija.

U ovoj smo studiji izolirali glikoproteine koji vežu galaktozil *N*-acetilgalaktozamin iz ljudskog perifernog živca i bakterije *Campylobacter jejuni* O:19 koristeći lektinsku (*peanut agglutinin* - PNA*)* afinitetnu kromatografiju. Izolirani glikoproteini otkriveni su imunoblot analizom uporabom oksidacije periodata i biotinilirani PNA. Serumi pacijenata s Guillain-Barréovim sindromom testirani su imunoblotom na reaktivnost prethodno izoliranih glikoproteina. Otkrili smo da su imunoreaktivni glikoproteini sa sličnom elektroforetskom pokretljivošću prisutni u izolatima iz perifernog živca i *Campylobacter jejuni*. Iz tih imunoreaktivnih glikoproteina oslobodili smo *N*-povezani oligosaharid, fluorescentno obilježili i enzimski sekvencionirali s visoko specifičnim egzoglikozidazama. Daljnja analiza uz pomoć tehnike fluoroforno potpomognute elektroforeze ugljikohidrata pokazala je prisutnost sličnih oligosaharidnih struktura u glikoproteinskim izolatima.

U ovom je radu otkrivena strukturna sličnost i imunoreaktivnost između glikoproteina izoliranih iz ljudskog perifernog živca i bakterije *Campylobacter jejuni* O:19. Taj nalaz ukazuje na moguću ulogu izoliranog bakterijskog glikoproteina kao antigenske determinante uključene u patogenezu Guillain-Barréova sindroma.

Ključne riječi: *Campylobacter jejuni*, Guillain-Barréov sindrom, imunoreaktivnost, glikoproteini, periferni živac