J Microbiol Immunol Infect.
2007;40:96-105

Original Article

Glycophorin A is recognized by an antibody population of the rabbit polyclonal antibodies produced against *Citrobacter braakii* O37

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Received: March 20, 2006 Revised: July 6, 2006 Accepted: July 12, 2006

Background and Purpose: Molecular mimicry was found in the case of *Citrobacter braakii* O37, which shares epitopes with human erythrocytes. It is believed that erythrocyte-membrane proteins band 3 and glycophorin A (GPA) have common epitopes. Band 3 was recognized by the anti-*C. braakii* O37 lipopolysaccharide antibodies (LPS-Abs) purified on LPS-affinity columns. This study aimed to investigate the role of GPA in this molecular mimicry.
Methods: Immunochemical methods such as immunoblotting, enzyme-linked immunosorbent assay, inhibition of hemagglutination, and affinity columns were employed.
Results: GPA when immobilized in an affinity column could purify specific GPA antibodies (GPA-Abs) from whole anti-*C. braakii* O37 serum. The purified antibodies, in turn, recognized GPA in immunoblotting tests. Treatment of human erythrocytes with sialidase significantly improved the hemagglutination titer by GPA-Abs. Furthermore, hemagglutination was inhibited to a greater extent by asialo-GPA than by the native form. GPA from blood groups M and N could similarly inhibit hemagglutination, and the most significant inhibition was recorded by GPA from the blood group MN. GPA-Abs could not recognize the LPS from *C. braakii* O37.
Conclusions: Results confirmed that an antibody population in the anti-*C. braakii* O37 serum recognized GPA. However, there was no reactivity with LPS of *C. braakii* O37, indicating that the antibodies may be produced against the outer membrane protein of the bacteria.

Key words: *Citrobacter*; Erythrocytes; Glycophorin; Lipopolysaccharides; Molecular mimicry

Introduction

Molecular mimicry is one of the most important pathogenic factors in microbial infections [1-3]. It is a mechanism by which infectious agents may trigger an immune response against autoantigens [4]. It is also a strategy used by pathogens to enter target cells and to avoid immune responses that inhibit multiplication in the host. Molecular mimicry has been rationalized based on the abundant epidemiological and experimental evidence of an association between infectious agents and the appearance of autoimmune disease, and an observed cross-reactivity of self-antigens with microbial determinants [5].

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Although molecular mimicry is frequently cited as a plausible mechanism to explain the association between an infection and autoimmune disease, there is not enough evidence to support its role in autoimmune diseases [6-8]. Further studies are needed to confirm the role of molecular mimicry in autoimmune diseases.

Autoimmune diseases are often preceded by an infectious illness. Recently, molecular mimicry has been suggested to explain the mechanisms of several autoimmune diseases. For example, Guillain-Barré syndrome (GBS) is a model disease for the study of both microbial and host factors involved in molecular mimicry [9]. The most frequently identified triggering agent of GBS is *Campylobacter jejuni* [10], which shares epitopes with human gangliosides. Therefore, there is strong but indirect evidence for the pathogenic role of molecular mimicry in GBS [11].

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Citrobacter strains are considered to be medically important pathogens, especially in some cases of neural diseases in neonates [12]. Rabbit polyclonal antibodies produced against *Citrobacter braakii* O37 agglutinate human erythrocytes [13]. Investigation of the shared epitopes between *C. braakii* O37 and human erythrocyte membrane may shed light on the mechanisms of pathogenesis of these bacteria. It has been found that N-glycan of band 3 of human erythrocytes and core oligosaccharides of the lipopolysaccharides (LPS) of *C. braakii* O37 are mainly responsible for this molecular mimicry since they share a common epitope with minimal identity [14].

Further investigation, using specific glycophorin A antibodies (GPA-Abs) in the present study, revealed a second different epitope on GPA of the human erythrocyte membrane that was recognized by anti-*C. braakii* O37 serum. This study aimed to investigate the role of GPA in this molecular mimicry.

Methods

Immunization of rabbits

Generation of rabbit polyclonal antibodies against *C. braakii* O37 was performed according to Romanowska et al [15]. Rabbits were immunized with acetone-dried powdered bacterial cell mass suspended in phosphate-buffered saline (PBS). Animals were individually injected with a subcutaneous dose of 100 μg dry cell mass suspended in 1 mL PBS. Subsequently, rabbits were intravenously injected with the bacteria twice a week for 3 weeks. The amount of injected bacteria was doubled gradually (200 μg/mL to 6400 μg/mL). One week after the last injection, rabbits were bled, and sera were collected and decomplemented by heating at 56°C for 30 min. The sera were then stored at –20°C until further use.

Hemagglutination and inhibition of hemagglutination

The hemagglutination assay was carried out as described by Steuden et al [16] and McGarey and Allred [17]. Hemagglutination tests were performed in 96-well U-bottomed microtiter plates using 20 μL of serially diluted antibodies in PBS and 20 μL of 2% human erythrocyte suspension. The suspension was incubated for 2 h at 37°C. Inhibition experiments were carried out by pre-incubating 10 μL of antibody with equal volumes of serially diluted (5 mg/mL) solutions of the glycoproteins or glycolipids at room temperature for 1 h before the addition of 20 μL of 2% human red cell suspension. GPA from blood groups M (GPA^M), N (GPA^N) and MN (GPA^{MN}) were applied in their desialylated forms (asialo-GPA) and also in their native forms. However, the native forms of GPA did not show any inhibition. Several carbohydrates including D-fucose (Koch-Light Laboratories, Colnbrook, UK), D-galactose (Merck, Darmstadt, Germany), beta (β)-methyl-galactopyranoside (Gal-β-Me) [Koch Light], β-lactose (Sigma Chemical Co, St Louis, MO, USA), D-glucosamine (Merck), β-methyl-glucopyranoside (Glc-β-Me) [Koch Light], and D-mannose (Merck) were examined for activity as competitive inhibitors. The initial carbohydrate concentration in each was 1.0 M and serial dilutions were prepared. Inhibitors were incubated with the antibodies under the same conditions as described above.

Treatment with enzymes

Treatment of intact erythrocytes with trypsin was performed as described previously [18]. The washed packed red cells were incubated with trypsin at a concentration of 300 μg/mL at 37°C for 90 min. Treatment of erythrocytes with sialidase from *Vibrio cholerae* (1 U/mg; SERVA Electrophoresis GmbH, Heidelberg, Germany) was performed as described by Latron et al [19].

Extraction of sialoglycoproteins from human erythrocytes

Sialoglycoproteins were extracted by phenol-water method at 65°C, as previously described [20]. The washed erythrocytes were hemolyzed by the addition of distilled water, followed by freezing. The membranes were centrifuged at 3000 *g* and washed with acidified water. Finally, the membranes were washed with 0.15 M sodium chloride (NaCl). The suspension of membranes was extracted with an equal volume of 90% phenol at 65°C for 30 min. The mixture was frozen, the water phase was collected, dialyzed against tap water, and then against de-ionized water, centrifuged at 16,000 *g* for 30 min, and lyophilized. The resultant glycophorins were then used for the purification of the specific GPA-Abs.

Extraction and purification of LPS

One gram of dry bacterial cell mass was mixed with 12.5 mL of phenol-water. The mixture was first incubated for 15 min in a water bath (50°C), then at room temperature, followed by centrifugation at 2500 rpm for 45 min. The supernatant was dialyzed against tap and distilled water and then concentrated. The extract

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was incubated at 37°C for 4 h with a solution of 0.8 mg of DNase and 0.4 mg RNase in 0.85 mL of PBS and 0.02 mL of the reagents magnesium chloride and calcium chloride. Purification of LPS was achieved by ultracentrifugation at a speed of 100,000 *g* for 6 h at 10°C.

Isolation of monospecific antibodies by affinity chromatography

Coupling of the soluble LPS O37 or GPA to Sepharose 4B

Sepharose 4B gel was suspended in water after several washes and adjusted to pH 11 by sodium hydroxide. For activation of Sepharose 4B, cyanogen bromide was added after 30 min. The activated Sepharose 4B gel was washed several times with carbonate buffer, pH 7.5. Soluble LPS or GPA protein was added to the activated Sepharose 4B and incubated at room temperature for 3 h with gentle shaking. The unbound sites of Sepharose 4B were blocked with 1 M ethanolamine for 2 h at room temperature. The unbound ethanolamine was washed out. Finally, the mixture was washed with PBS.

Affinity purification of antibodies on the Sepharose 4B affinity columns

Anti-*C. braakii* O37 serum (1:1 in PBS) was loaded on LPS or GPA affinity columns at a slow rate. The column was thoroughly washed with PBS until the proteins could not be detected. Nonspecific antibodies were washed out with 1 M NaCl. The specific antibodies were eluted in 2 mL fractions using 3 M potassium thiocyanate. Fractions were measured at 280 nm and the selected fractions were collected and dialyzed overnight with several changes of PBS. Fractions were concentrated using Amicon 10 plasma membrane (Amicon, Beverly, MA, USA), and then stored at –20°C.

Enzyme-linked immunosorbent assay

Plates coated with erythrocytes

Enzyme-linked immunosorbent assay (ELISA) was performed in flat-bottomed microtiter plates (Nalgene Nunc International, Roskilde, Denmark). The plate was coated with poly-L-lysine (20 μg/mL PBS). After washing, the plate was incubated with erythrocytes or soluble erythrocyte membranes (2 μg/well) for 1 h and then centrifuged at 1000 rpm for 2 min. Erythrocytes were fixed by the addition of glutaraldehyde. The membranes were blocked by incubation with glycine buffer, pH 7 for 1 h. Plates were washed with Tris-buffered saline/Tween 20 (TBS-T). Primary antibodies were incubated for 2 h. Plates were washed with TBS-T. Horseradish peroxidase conjugated with goat anti-rabbit immunoglobulin G (IgG) antibody was added for 1 h. *O*-phenylenediamine (100 μL) was incubated at 37°C for 10 min. Reaction was stopped with sulfuric acid. The optical density was measured at 492 nm using a microplate reader [21].

Plates coated with GPA

ELISA plates were coated with purified GPA (from Dr. Duk, Institute of Immunology and Experimental Therapy, Wroclaw, Poland) [1.5 μg/well] for 2 h. After washing, the plate was blocked with 1% bovine serum albumin (BSA) for 1 h. Antibody and conjugate incubation as well as substrate addition was performed in ELISA plates coated with erythrocytes as described above.

Plates coated with LPS

ELISA was performed for bacterial LPS, as previously described [22] with some modifications. Plates were coated with LPS suspended in PBS, pH 7.2 (2 μg in 100 μL/well) and incubated at 37°C for 3 h and then overnight at 4°C. Plates were washed 3 times with distilled water and blocked with 0.2% casein in TBS-T for 10 min. After blocking, plates were again washed with water. Primary antibodies diluted in TBS-T were added and incubated for 2 h. Alkaline-phosphatase (AP) conjugated with goat antibody anti-rabbit IgG (Bio-Rad, Richmond, CA, USA) was incubated for 1 h. After washing, 100 μL of AP substrate pH 9.8 was added and incubated until the color reaction developed.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotting

Electrophoresis was carried out at a constant current of 200 V per gel until the dye reached the bottom of the gel [23]. Coomassie brilliant blue, periodic acid-Schiff reagent, and silver stain were applied for staining the erythrocyte proteins, the erythrocyte glycophorins, and the LPS O37, respectively. For immunoblotting, the gel was electrophoretically blotted at 100 mA for 1 h onto an Immobilon P membrane in transfer buffer [24]. The membrane was blocked at 36°C for 1 h with 2% (w/v) BSA-Tween. The blot was then incubated for 2 h with serum diluted 1:200. Membrane was washed twice with TBS buffer for 20 min prior to incubation with goat antibodies anti-rabbit IgG conjugated with AP diluted 1:3000 in TBS-T for 1 h. The blot was stained with 5-bromo-4-chloro-3'-indolyl phosphate *p*-toluidine salt and nitroblue tetrazolium chloride.

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Dot blotting

A nitrocellulose membrane was washed in water and then left to dry for 1 h. Subsequently, 1 to 2 μL of the soluble antigen was applied to the membrane and incubated for 8 min. The membrane was washed 3 times with PBS and then incubated in 1% BSA for 15 min. After blocking, the membrane was incubated for 5 min with the specific anti-*C. braakii* O37 antibodies. After washing, the membrane was incubated with goat anti-rabbit IgG antibodies conjugated with horseradish peroxidase for 20 min. Finally, the membrane was washed 5 times with PBS and the reaction was developed by adding substrate. All the steps were carried out at room temperature.

Statistical analysis

Statistical analysis was undertaken using MINITAB software (Version 13.1, 2002; MINITAB Inc., State College, PA, USA). Data from hemagglutination inhibition were first tested for normality using the Anderson-Darling test and for variances homogeneity prior to any further statistical analysis. Data were normally distributed, and variances were homogeneous; thus, one-way analysis of variance was used to determine overall effects of treatments followed by individual comparisons using Tukey's pairwise comparison.

Results

Reactivity of the whole anti-*C. braakii* O37 serum in hemagglutination

Hemagglutination test showed that anti-*C. braakii* O37 serum reacted specifically with human and horse erythrocytes but not with sheep and chicken erythrocytes [13]. Therefore, the anti-*C. braakii* O37 serum could recognize epitopes on the external surfaces of both human and horse erythrocytes. Treatment of human erythrocytes with trypsin, which cleaves GPA, strongly improved hemagglutination because the anti-*C. braakii* O37 serum could easily recognize its epitope (band 3), which became more accessible on the surface of the erythrocytes. It was previously proven that anti-*C. braakii* O37 serum has an antibody population that could recognize band 3 [14].

Also, desialylation of human erythrocytes by sialidase from *Vibrio cholerae* resulted in a significant increase in the hemagglutination titer with anti-*C. braakii* O37 serum (Table 1). This enzyme selectively removes sialic acids which cover the galactose residues. Therefore, galactose residues, especially those on the O-glycan domain of GPA (GPA accounts for most of the carbohydrate content in human erythrocytes), became more available after sialidase treatment. These results suggested that a second population of antibodies is present in the anti-*C. braakii* O37 serum.

Table 1. Reactivity of anti-*Citrobacter braakii* O37 serum, lipopolysaccharide antibodies (LPS-Abs) and glycophorin A antibodies (GPA-Abs) with untreated, sialidase-treated and trypsin-treated human erythrocytes in hemagglutination

Antibodies	Human erythrocytes		
	Untreated	Sialidase	Trypsin
Whole serum	1/128	1/1024	1/2048
LPS-Abs	1/128	1/128	1/256
GPA-Abs	1/16	1/128	1/8

Purification of LPS-Abs and GPA-Abs from the anti-*C. braakii* O37 serum

Specific antibodies were purified to prove that anti-*C. braakii* O37 serum contains 2 different populations of antibodies and to investigate the second epitope on human erythrocytes. Immunoglobulins were purified from the whole anti-*C. braakii* O37 serum by immunoaffinity purification. Immunoglobulins were loaded onto a GPA affinity column. The specific antibodies were eluted and concentrated. The specific antibodies produced in this manner were named GPA-Abs, while those purified on an LPS column were named LPS-Abs. Thus, the GPA affinity column could specifically purify an antibody population from the anti-*C. braakii* O37 serum. This might confirm that the second population of antibodies in anti-*C. braakii* O37 recognized GPA. Although LPS-Abs were previously studied [14], we used both LPS-Abs and GPA-Abs in this study to compare the difference in their reactivities against human erythrocyte epitopes.

Reactivity of the specific antibodies

Hemagglutination of the sialidase-treated human erythrocytes was studied using LPS-Abs and GPA-Abs. LPS-Abs reacted with sialidase-treated and untreated human erythrocytes in similar titers. However, the reactivity titer of the GPA-Abs was improved after desialylation of the erythrocytes. In the case of whole serum (mixed LPS-Abs and GPA-Abs), an additive effect was observed with the sialidase-treated erythrocytes (Table 1).

The different behaviors of the LPS-Abs and GPA-Abs in hemagglutination suggested that these specific antibodies were indeed different populations in the anti-*C. braakii* O37 serum. Thus, they should be

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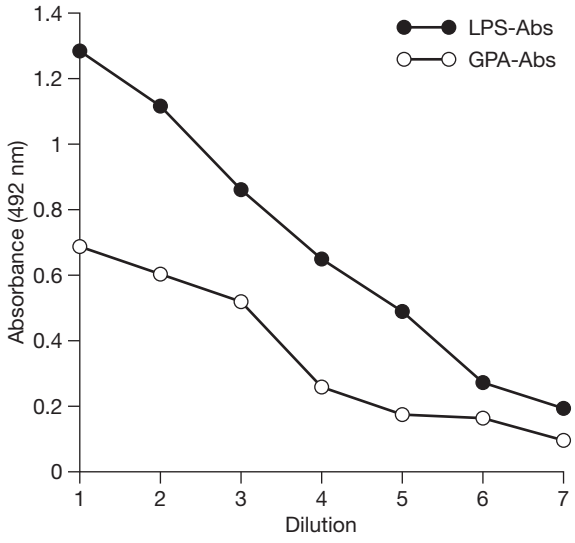

Fig. 1. Reactivity of both glycophorin A antibodies (GPA-Abs) and lipopolysaccharide antibodies (LPS-Abs) with LPS from *Citrobacter braakii* O37 in enzyme-linked immunosorbent assay.

produced in the serum against 2 different epitopes of the bacterial cell wall. Therefore, both of them were applied against LPS O37 to determine their specificities against this active bacterial antigen. LPS-Abs showed specific and strong reactivity with its homologous LPS O37 in ELISA. However, as expected, GPA-Abs showed a weak reactivity with LPS O37 (Fig. 1). On the other hand, the reaction between GPA-Abs and GPA was 2-fold greater than that between LPS-Abs and GPA in ELISA (Fig. 2). The results of the immunoblotting assay confirmed these results. Fig. 3 shows the electrophoretic pattern of LPS O37 which was immunostained with LPS-Abs and GPA-Abs. It shows strong immunostaining

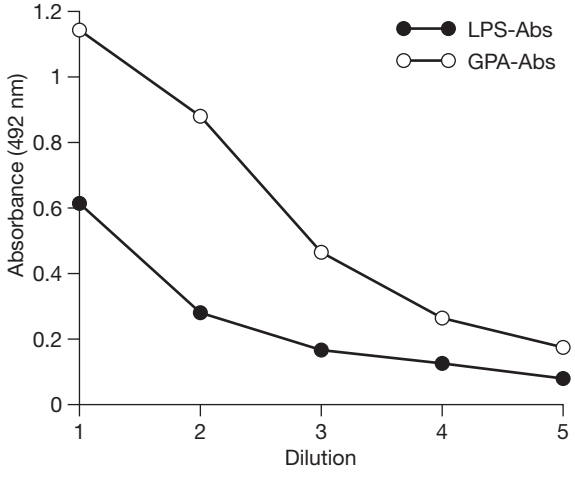

Fig. 2. Reactivity of both glycophorin A antibodies (GPA-Abs) and lipopolysaccharide antibodies (LPS-Abs) with GPA.

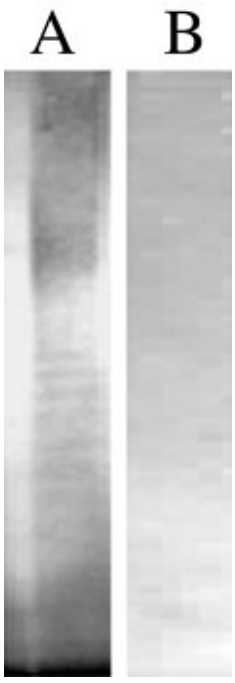

Fig. 3. (A) Immunostaining of the lipopolysaccharide (LPS) from *Citrobacter braakii* O37 with the specific antibodies (LPS-Abs) purified on the LPS-affinity column. (B) Immunostaining of the same LPS with the specific glycophorin A (GPA) antibodies purified on a GPA affinity column.

of LPS O37 by LPS-Abs. However, the GPA-Abs did not recognize LPS in the immunoblotting test. All these results suggest that the GPA-Abs population in rabbit serum was not raised against LPS O37.

Furthermore, reactivities of both LPS-Abs and GPA-Abs were compared against human erythrocytes coated on the ELISA plates. Although both antibodies were adjusted to the same concentration before applying on ELISA plates, the LPS-Abs showed a stronger reactivity with human erythrocytes than GPA-Abs (Fig. 4).

Moreover, results of immunoblotting of human erythrocyte membranes with LPS-Abs and GPA-Abs confirmed that they are different antibody populations from the anti-*C. braakii* O37 serum. As shown in Fig. 5 (lane 2), LPS-Abs recognized band 3 and its 40-kDa fragment. Weak bands were nonspecifically stained with LPS-Abs similar to band 4.1. GPA-Abs reacted with dimer GPA, mixed GPA and glycophorin B, and monomer GPA of the human erythrocyte membrane (Fig. 5, lane 4). Furthermore, a 60-kDa band was stained with GPA-Abs. However, the interaction of LPS-Abs with band 4.1 and GPA-Abs with the 60-kDa protein appeared to be nonspecific, probably due to the hydrophobic nature of these sodium dodecyl sulfate-denatured

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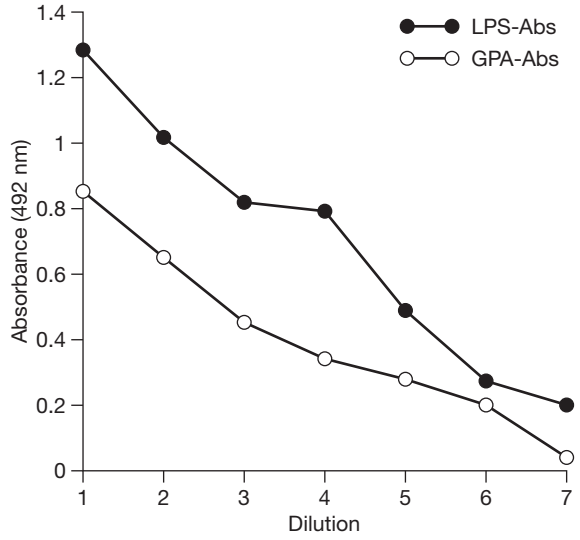

Fig. 4. Reactivity of lipopolysaccharide antibodies (LPS-Abs) and glycophorin A antibodies (GPA-Abs) with human erythrocytes.

and blotted polypeptides. The nonspecific binding of antibodies to band 4.1 and band 4.2 was observed by Ebaid [14] and Lutz et al [18], respectively.

Therefore, results of immunoaffinity purification, hemagglutination, ELISA, and immunoblotting confirmed that, in addition to LPS-Abs, there is another antibody population, GPA-Abs, present in the same rabbit serum. These antibodies recognized an epitope on GPA. Consequently, results demonstrated that, in addition to the role of band 3 (recognized by LPS-Abs), GPA (recognized by GPA-Abs) also played a role in the molecular mimicry between human erythrocytes and *C. braakii* O37. Further experiments were performed to investigate the nature of this epitope on GPA.

Investigation of the nature of the epitope on GPA

Both native and asialo-GPA (desialylated GPA) were separately coated onto the ELISA plates. Results showed that GPA-Abs reacted more strongly with asialo-GPA than native GPA (Fig. 6). This result is in agreement with the results of the hemagglutination of native and sialidase-treated erythrocytes. Also, this result was confirmed with the dot blotting test results. When the soluble human stroma, asialo-GPA, and native GPA were coated onto the nitrocellulose sheet, the GPA-Abs recognized asialo-GPA more strongly than native GPA (Fig. 7). Reactivity of these antibodies with the soluble stroma was very weak. Both ELISA and dot blotting confirmed that the GPA-Abs reacted with GPA, and this reactivity clearly improved after desialylation of this glycoprotein (asialo-GPA).

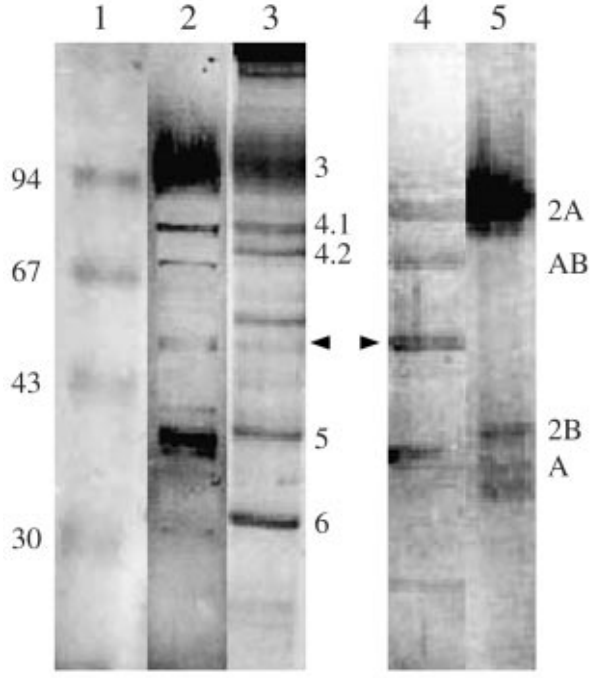

Fig. 5. Coomassie brilliant blue (CBB) staining of the molecular weight marker proteins (lane 1); immunostaining of human erythrocyte membranes bands by specific antibodies (lipopolysaccharide [LPS] antibodies) purified on the LPS-affinity column (lane 2); CBB staining of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) pattern of human erythrocyte membranes (lane 3); immunostaining of human erythrocyte membranes bands by specific antibodies (glycophorin A [GPA] antibodies) purified on the GPA affinity column (lane 4); and periodic acid-Schiff staining of the SDS-PAGE pattern of human erythrocyte membranes (lane 5). Dimmer GPA (2A), mixed GPA and glycophorin B (AB), dimmer glycophorin B (2B) and monomer GPA (A) are shown in lanes 4 and 5. Arrowheads indicate the 60 kDa region in lanes 2, 3 and 4.

Inhibition of GPA-Abs by GPA and monosaccharides

The human erythrocytes used in the inhibition experiments were first desialylated. The untreated erythrocytes did not show any inhibition. GPA-Abs were used in the inhibition experiments. Results showed that desialylated glycophorins significantly inhibited hemagglutination, while native glycophorins did not show any inhibition (data not shown). Fig. 8 shows that GPA^{MN} recorded the most significant inhibition of hemagglutination. Although GPA^{M} and GPA^{N} showed significant inhibition of agglutination, their effects were approximately one-half that of GPA^{MN}. The whole soluble human stroma showed very weak inhibition of hemagglutination. Neither neutral nor acidic glycolipids showed any inhibition of hemagglutination.

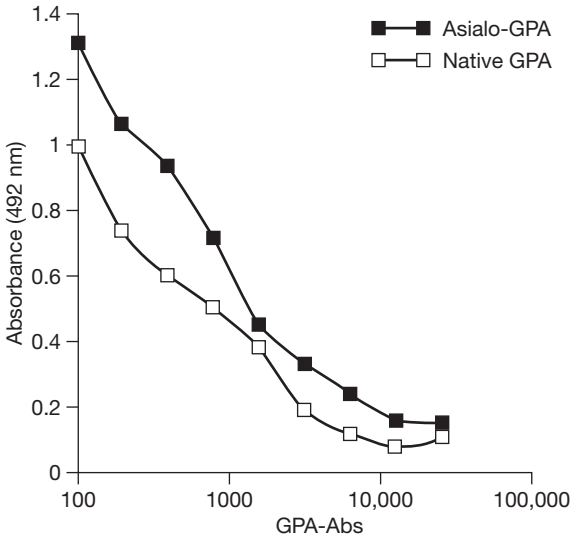

Fig. 6. Reactivity of glycophorin A antibodies (GPA-Abs) with both native and asialo-GPA protein in enzyme-linked immunosorbent assay.

To determine whether the inhibition activity of GPA was specific to GPA-Abs, LPS-Abs were used. GPA did not exhibit any inhibition against LPS-Abs. Although GPA-Abs and LPS-Abs were derived from the same serum, GPA could inhibit GPA-Abs but not LPS-Abs.

Results of ELISA, dot blotting, and inhibition of hemagglutination showed that asialo-GPA was more strongly recognized by the GPA-Abs rather than the native GPA. This indicates that galactose residues should be included for the epitope to be recognized by GPA-Abs. Therefore, inhibition of hemagglutination by galactose and other monosachcharides was performed to compare the inhibitory activity. The most significant inhibition effect was obtained with Gal-β-Me, which is a derivative of galactose (Fig. 9). Galactose showed significantly higher inhibition as compared to some other sugars. Furthermore, fucose, which has a chemical structure similar to that of galactose and lactose (which is obtained from galactose and glucose) significantly inhibited the hemagglutination.

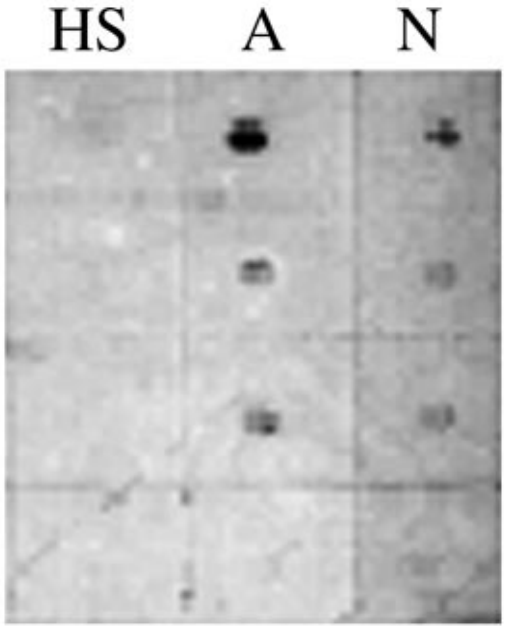

Fig. 7. Immunostaining of the serial dilutions (from top to bottom) of soluble human stroma (HS), asialo- (A) and native (N) glycophorin A (GPA) from blood group MN with specific GPA antibodies purified on a GPA affinity column.

Discussion

Molecular mimicry between *C. braakii* O37 and human erythrocytes is mainly based on the core oligosaccharides from this bacteria and N-glycan of band 3 of human erythrocytes [14]. Further investigation showed that the GPA affinity column could specifically purify an antibody population from the anti-*C. braakii* O37 serum. Comparison between LPS-Abs and GPA-Abs was useful because it showed the difference in their immunological behaviors.

The unchanged reactivity of LPS-Abs in hemagglutination and the improved reactivity of GPA-Abs with sialidase-treated human erythrocytes in hemagglutination, ELISA, and dot blotting strongly suggested that they are 2 different antibody populations in the same serum. Therefore, they recognized completely different epitopes on human erythrocytes. This was also evident in the behavior of the whole serum, which contains both LPS-Abs and GPA-Abs, since an additive effect in hemagglutination was observed with the sialidase-treated erythrocytes. This was because one epitope was saturated with LPS-Abs, and after treatment with sialidase, the second epitope was occupied with GPA-Abs (Table 1). Results of ELISA indicated that LPS-Abs showed stronger reactivity with the native human erythrocytes than GPA-Abs. This may indicate that LPS-Abs were more reactive with the native erythrocytes than GPA-Abs. In the native erythrocytes, galactose residues are still covered with sialic acid, and this can explain the lower reactivity of GPA-Abs with erythrocytes than LPS-Abs. This is also in agreement with the above mentioned results of hemagglutination.

GPA-Abs showed slight reactivity in ELISA (Fig. 1) and no reactivity in immunoblotting test with

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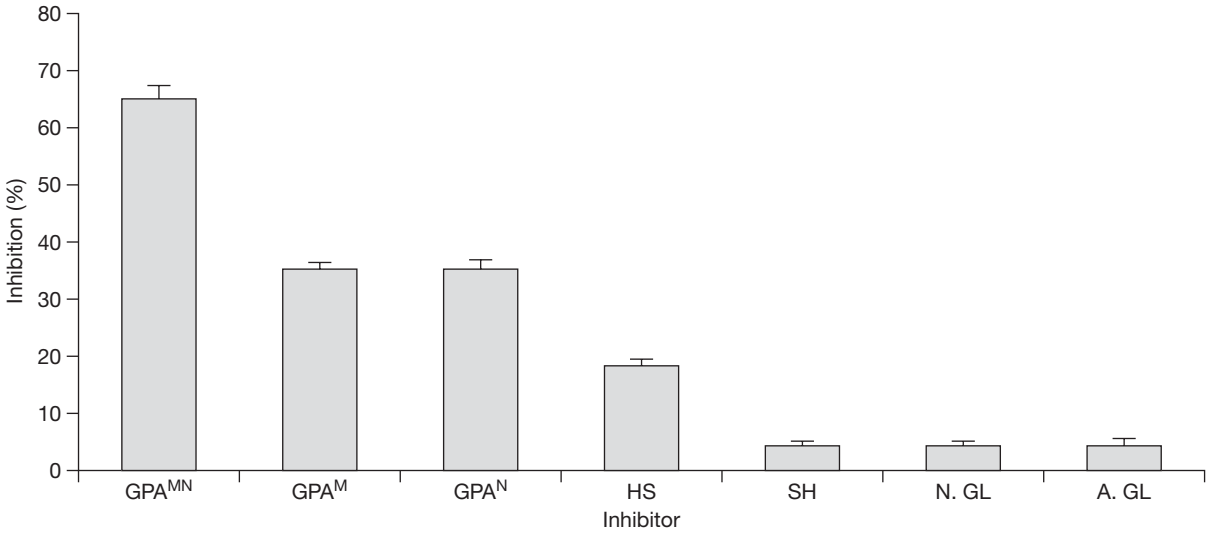

Fig. 8. Inhibition of hemagglutination of desialylated human erythrocytes by glycophorin A (GPA) from blood groups MN (GPA^{MN}), M (GPA^{M}), N (GPA^{N}), soluble human stroma (HS), soluble sheep stroma (SH), neutral glycolipids (N. GL), and acidic glycolipids (A. GL).

LPS O37 (Fig. 3, lane B). On the other hand, weak reactivity was observed for LPS-Abs with GPA in ELISA. These results further confirmed that GPA was specifically recognized by GPA-Abs and not by LPS-Abs. Therefore, LPS-Abs and GPA-Abs should be different antibody populations although both of them were purified from the same anti-*C. braakii* O37 serum. This suggested that the GPA-Abs population may be raised in the rabbit serum against the outer membrane protein (OMP) of *C. braakii* O37 rather than LPS. A surface protein has been identified as a possible virulence factor among strains that cause *Citrobacter* brain abscesses in neonates [12]. OMP was suggested to be one of the immunodominant antigens of many Gram-negative bacteria such as *Salmonella typhimurium* [25], and genes encoding OMPs were used to construct vaccines [26]. Thus, when the bacterial cell mass was injected into rabbits, OMPs, in addition to LPS, could induce antibody responses.

GPA is 131 amino acid residues in length and has one N-glycosidic carbohydrate chain and an average

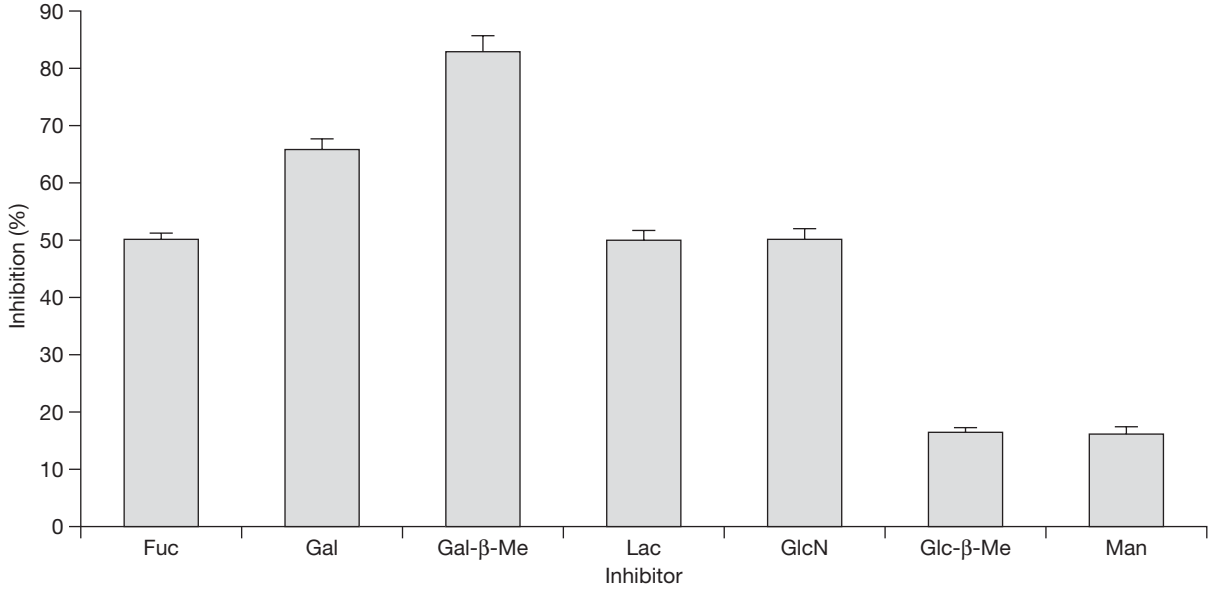

Fig. 9. Inhibition of hemagglutination of desialylated human erythrocytes by D-fucose (Fuc), D-galactose (Gal), beta (β)-methyl-galactopyranoside (Gal-β-Me), β-lactose (Lac), D-glucosamine (GlcN), β-methyl-glucopyranoside (Glc-β-Me), and D-mannose (Man). Two-fold dilutions were prepared starting from 1 M concentration of inhibitor followed by serial dilutions.

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of 15 O-linked oligosaccharide chains [27-29]. Thus, carbohydrates constitute 60% of the GPA. The most abundant O-glycan in human GPA is [Neu5Aca2-3Galb1-3(Neu5Ac2-6)GalNAc] tetrasaccharide [30]. Galactose, which is covered with sialic acid, is expressed in high proportions in human erythrocyte glycophorins [31-33]. Therefore, the hemagglutination ability of desialylated human erythrocytes by GPA-Abs can be explained by the degree of the exposed galactose residues in oligosaccharides.

Results of ELISA (Fig. 6) and dot blotting (Fig. 7) confirmed the significant role of galactose, since GPA-Abs reacted more strongly with the asialo-GPA protein than native GPA. Furthermore, experiments of inhibition by monosacchrides also proved that galactose plays a significant role (Fig. 9). Therefore, the epitope should include the galactose residues. The disaccharide (Galb1-3GalNAc) forms the T antigen which is responsible for erythrocyte panagglutination by naturally occurring serum antibodies after microbial infections [30]. The T antigen may be a domain of the epitope on the GPA recognized by GPA-Abs.

Although the data confirmed the significant role of galactose residues, GPA^{MN} had a higher inhibition effect than GPA^{N} and GPA^{M} (Fig. 8). GPA^{N} and GPA^{M} have different amino acid residues at position 1 (leucine in N and serine in M) and 5 (glutamic acid in N and glycine in M) of the peptide chain [28]. This means that the difference in the polypeptide chain structures in GPA^{N}, GPA^{M}, and GPA^{MN} has an effective role. This conflicts with the results proving the significant role of galactose. However, the location of the exposed galactose residues in the polypeptide chains and the special arrangement on the cell surface should also be considered as factors regulating hemagglutination efficiency [33]. This is also supported by the study of Lisowska [29] which reported that glycophorins are complex antigens carrying peptidic and glycopeptidic epitopes. Peptidic eitopes are variably affected by adjacent O-glycans, which may be required for the proper exposure of an antibody binding site. Therefore, this epitope can be formed by the interaction of amino acids in the polypeptide chain and galactose residues of the O-glycan.

These findings indicate that, beside LPS-Abs, GPA-Abs are another group of antibodies that recognize a glycoprotein epitope on GPA. Therefore, immunization of rabbits with *C. braakii* O37 was primarily controlled by its LPS O37, which produced LPS-Abs (recognized band 3), and may be secondarily influenced by OMP, which may produce GPA-Abs (recognized GPA). Results of this work demonstrated that the molecular mimicry between *C. braakii* O37 and human erythrocyte is not only based on the N-glycan of band 3 but also on the peripheral glycoprotein domain of GPA.

Acknowledgments

The author would like to thank Dr. Andrzei Gamian for providing the immunoblotting materials and Dr. Maria Duk for providing the purified GPA.

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