Identification of Blood Group A and B Antigens in Human Glycophorin

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Abstract. Glycophorin A (GPA), the major sialoglycoprotein of human erythrocyte membranes, was isolated separately from blood group A and B erythrocytes using phenol-water extraction. After purification, performed as gel filtration in the presence of SDS, two glycophorin samples GPA-A and GPA-B were run, in duplicate, in SDS-PAGE and electrophoretically onto Immobilon P. After staining with 1) anti-glycophorin antibody and 2) with relevant anti-blood group (A or B) antibody it was shown that the band pattern of the samples in each duplicate was the same. GPA-A and GPA-B samples were also degraded using Carlson degradation (β-elimination in mild alkaline/strong reducing conditions) and from reaction products the fractions of O-glycans and N-glycans were isolated; they were used in hemagglutination inhibition test. It was shown that both sugar fractions derived from GPA-A did inhibit agglutination of blood group A erythrocytes by anti-A antibody, whereas oligosaccharide fractions derived from GPA-B inhibited agglutination of blood group B erythrocytes by anti-B antibody. These results, obtained using immunochemical methods, confirm the presence of blood group A and B determinants in the carbohydrate moiety of human glycophorin, derived from the blood group A or B erythrocytes, respectively.

Key words: human glycophorin; blood group A and B antigens; protein glycosylation; O-glycans; N-glycans.

Introduction

Glycophorin A (GPA) is the major sialoglycoprotein of human erythrocyte membranes. This is integral, type I glycoprotein with heavily glycosylated N-terminal domain. Because GPA is relatively easy to isolate, for many years it has served as a model membrane protein in various immunochemical and biochemical investigations19. GPA is a glycoprotein having more than 50% carbohydrates by mass. It contains one N-glycan and ca 15 O-glycans, of which most abundant structures were already elucidated. The N-linked oligosaccharide was described as biantennary chain sialylated with Neu5Ac linked α2-6 to galactose residues, having bisecting GlcNAc and fucose bound to the innermost N-acetylglucosamine11, 32. The most abundant O-glycan in GPA molecule is a tetrasaccharide, i.e. Neu5Acα2-3Galβ1-3[Neu5Acα2-6]GalNAc. This tetrasaccharide is accompanied by minor amounts of shorter structures, which are in fact its truncated forms, i.e. linear and branched monosialo trisaccharides, disaccharide Galβ1-3GalNAc (antigen T) and single GalNAc

Abbreviations used: GPA - glycophorin A from human erythrocyte membranes, GPA-A glycophorin A from human erythrocytes of blood group A, GPA-B - glycophorin A from human erythrocytes of blood group B, TFA – trifluoroacetic acid, TIC – total ion current, GLC-MS – gas liquid chromatography with mass spectrometric detection, mAb – monoclonal antibody.

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GalNAc-A and therefore, the ABH antigens are still considered a potential carrier of the blood group ABH antigens. The blood group ABO system was discovered by Landsteiner in 1900. It is the longest known blood group system and most important one from practical point of view. As a result of numerous studies it was shown that ABH antigens are carbohydrate, non-reducing terminal structures, residing in glycosphingolipids and glycoproteins, which are components of erythrocyte membranes (Table 1). The glycoproteins having ABO blood group activity (so called blood group substances) are also found in body fluids and secretions, such as saliva, gastric juice, semen and ovarian cyst fluid. Moreover, it was found that natural antibodies against blood group antigens exist in circulation in human beings. Despite the fact that the history of ABO blood group studies is already as long as one century, there are no definitive ideas as to the role of this blood group system in human organism; therefore, the ABH antigens are still considered as the “molecules seeking a function”. Therefore, more and more experimental data regarding this biological phenomenon are available. During past years we have learned a great deal about the biological role of natural compounds bearing ABH antigens. For many years it has been known that the ABO blood group system is an important factor in transplantation, because ABH antigens are expressed on the majority of epithelial cells and because natural anti-A and anti-B antibodies exist. The general tendency, found in clinical practice, is not to transplant across the ABO barrier. ABO blood groups are routinely defined by serological methods using anti-A and anti-B antibodies; the most common methods serve to determine the phenotype of the erythrocytes. The modern procedures, based on PCR-SSP molecular biology method, are also developed to genotype ABO blood groups for organ transplants. In the recent years the tendency is seen to look for the correlation between ABO blood group status and some physiological features, for example the published data show that Helicobacter pylori colonisation in human stomach may be influenced, in part, by host expression of ABH and Le antigens. It has also been established, in glycopathology, that some carbohydrate structures are characteristic for tumor cells; they were given the name “tumor associated carbohydrate antigens” (TACAs). According to this, some preliminary attempts were made to establish the probable involvement of blood group ABH antigens in this phenomenon. During widely performed studies it was found that ABH antigens may be influenced in human cancer in different way: this regards, among others, deletion of A and B structures, phenotypic conversion O→A or B→A, expression of A-like antigen in tumors from O or B individuals. It is worth to mention that Le" and Le" antigens, closely related to ABH structures, are also structurally modified in cancer.

The experimental data on the presence of the blood group ABH antigens in human glycoporin are still obscure. In 1976 Takasaki and Kobata succeeded in enzymatic incorporation of N-acetylgalactosamine into carbohydrate moiety of glycoporin isolated from blood group O erythrocytes, which clearly suggested the presence of H antigens in this glycoprotein. In the next paper the same authors used the fraction of isolated, reduced O-glycans from human glycoporin, similarly originating from erythrocytes of blood group O, as a substrate for enzymatic incorporation of GalNAc. They managed to incorporate N-acetylgalactosamine into neutral O-glycans, using enzyme A from human milk. This experiment revealed that in substrate O-glycans the blood group H antigens were present. In 1993 a communication was presented where the authors investigated a pool of N-glycans obtained from GPA, derived from O erythrocytes. During a mass spectrometric analysis in FAB mode an ion, among others,
of m/z 638 was obtained, together with a sequence ion of m/z 606. These two ions, corresponding to the sequence Fuc-\textasciitilde{}Hex-4HexNAC1 indicated the presence of H antigen in the studied N-glycans, which is equal to the sequence Fuc1-2Gal1-4GlcNAC1.1.

In this investigation we present evidence, obtained by immunocchemical methods, for the presence of blood group A and B antigens in human glycophorin, both in N-linked and O-linked glycans.

Materials and Methods

Reagents. All chemical reagents used were of analytical grade. Water for all solutions was from Milli-Q apparatus (Millipore), working in a reversed-osmose mode. Mouse monoclonal antibodies (mAb): anti-A (A008) and anti-B (B006), in a form of the 10-times concentrated hybridoma culture fluids, were obtained from MonoCarb AB (Lund, Sweden). Mouse monoclonal anti-GPA antibody (PEP80) was from this Institute.1 Immobilon P was from Whatman (USA), two reagents used for immunocchemical staining on the blots: BCIP (5-bromo-4-chloro-3-indolyl phosphate) and NBT (nitro blue tetrazolium) were purchased from Fluka (Switzerland).

Glycophorin. Outdated erythrocytes of blood-group A and B were obtained from the Regional Blood Transfusion Center in Wroclaw (Poland). Glycophorin A crude preparations were obtained from the membranes of erythrocytes by phenol-water extraction.1 They were further purified by gel filtration, using Sephadex G-200 column (2.6 x 80 cm) eluted with 0.05 M pyridine/acetic acid, pH 5.3, containing 1% SDS. The elution profile from the column was monitored for neutral sugars, then the chosen tubes were checked for the presence of GPA using SDS-PAGE16 with concomitant PAS staining.5 After pooling the content of appropriate tubes, SDS was removed by dialysis against 50% ethanol in water and then pure water only. GPA preparations were stored at 4°C after lyophilization.

Mild alkaline degradation (β-elimination). Purified, lyophilized GPA preparations were degraded using modified conditions of Carlson degradation1, i.e. 0.1 M NaOH/1 M NaBH₄ at 50°C for 18 h in the presence of Cd⁺⁺ ions.18 After reaction the solution was neutralized with 50% acetic acid and the reaction products were fractionated by gel filtration on BioGel P-2 (in water), Sephadex G-50 (in 0.05 M acetic acid/pyridine buffer, pH 5.3) and BioGel P-4 (in water) columns. Two types of oligosaccharide fractions were obtained: a fraction of free, reduced O-glycans and a fraction of short glycopeptides, containing N-glycans. Both fractions were used in hemagglutination inhibition tests. β-Elimination procedure was also performed in order to remove O-glycans from glycophorin, immobilized on Immobilon P. This incubation was done under described conditions, i.e. 0.055 M NaOH at 40°C for 16 h.

Neutral sugar determination. Neutral sugars were determined using phenol-sulfuric acid method. Reduced volumes of the solutions were used, as compared with original method, i.e. 0.3 ml sample, 0.3 ml 5% phenol in water and 1.5 ml of the concentrated sulfuric acid. In the case of quantitative determinations galactose was used as a standard.

Protein determination. Protein content was determined using bichinonic acid method20 on a micro scale (polystyrene microplates) with bovine serum albumin (BSA) as a standard.

Gas liquid chromatography-mass spectrometry analysis (GLC-MS). This method was used for the total sugar content analysis. The samples of glycophorin were hydrolyzed in 4 M trifluoroacetic acid (TFA) at 100°C for 4 h. After evaporation to dryness they were supplemented with xylose as an internal standard, then were derivatized into alditol acetates22 and analyzed in a gas-liquid chromatograph as described previously14.

Polyacrylamide gel electrophoresis (SDS-PAGE), electroblotting and immunocchemical staining. SDS-PAGE was performed according to Laemmli14. The resolved protein bands were transferred onto Immobilon P using described electroblotting procedure19. The glycophorin bands were visualized on the blots using the following reagents: mouse mAb anti-GPA (PEP80) or mouse mAb anti-blood group A antigen (A008), or mouse mAb anti-blood group B (B006), then a rabbit antibody anti-mouse immunoglobulins conjugated with alkaline phosphatase. Finally, alkaline phosphatase substrates BCIP and NBT were used to develop color reaction, i. e. 35 μl of 5% (w/v) BCIP in dimethylformamide and 45 μl of 7.5% (w/v) NBT in 70% dimethylformamide were dissolved in 10 ml of TBS, pH 9.5, containing 1% MgCl₂ x 6 H₂O.

Hemagglutination inhibition test. The test was performed using polystyrene 96-well u-bottomed microplates. First, agglutination titers of anti-blood group A and anti-blood group B mAbs were adjusted, by diluting the commercial solutions of hybridoma culture with 0.15 M NaCl to such extent that each solution used in the test, after double dilution, still agglutinated erythrocytes (starting solution). To perform the inhibition test, the solution of glycophorin O-glycans or N-glycans (20 μl) was diluted two times with 0.15 M NaCl in exponential way, then the starting solution of
antibody (20 μl), corresponding to the phenotype of erythrocytes, was added to each well and incubation proceeded for 30 min. After that 40 μl of 2% erythrocyte suspension in 0.15 M NaCl was added to each well; hemagglutination was read after 30 min incubation at room temperature and was estimated as positive (+), negative (−) or partial (+/−).

Results

Isolation of glycophорin from blood group A and B erythrocytes

The erythrocytes, separately of blood group A and B phenotype, were pooled into appropriate batches (2–5 l), washed with 0.15 M sodium chloride and centrifuged 3 times. After freezing and thawing the membranes were separated from the cytoplasm components and subjected to phenol-water extraction. The crude glycophорin preparations were obtained as lyophilizates (Table 2), with the yield not completely proportional to the volume of erythrocyte suspension, taken for preparation. This is due to the fact that after first centrifugation of erythrocytes the buffy coat of lymphocytes has to be removed from each tube, which is always accompanied by a loss of erythrocytes.

Table 2. Average yield of crude GPA preparations

<table>
<thead>
<tr>
<th>Erythrocyte phenotype</th>
<th>Volume of erythrocyte suspension (l)</th>
<th>Crude GPA preparation (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.25</td>
<td>236</td>
</tr>
<tr>
<td>A</td>
<td>5.25</td>
<td>530</td>
</tr>
<tr>
<td>B</td>
<td>5.50</td>
<td>532</td>
</tr>
<tr>
<td>B</td>
<td>2.10</td>
<td>419</td>
</tr>
</tbody>
</table>

The GPA preparations were obtained using phenol-water extraction of the membranes, derived from a given volume of erythrocyte suspension.

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![Figure 1](image1.png)

Fig. 1. Elution profile of GPA-B (150 mg) chromatography on Sephadex G-200 column. Neutral sugars were estimated (0.3 ml samples) using phenol-sulfuric acid method. The arrows on the chromatogram indicate the tubes, from which the samples (20–40 μl) were taken for SDS-PAGE analysis; inserted picture shows the results. Elution profile of GPA-A chromatography was entirely the same. Purified glycophорin was pooled as indicated.
The crude GPA preparations were further purified by gel filtration (Sephadex G-200 column) in the buffer containing 1% SDS. Figure 1 shows a typical elution profile, obtained by neutral sugar detection. A picture inserted shows SDS-PAGE pattern of the components from the chosen tubes. After pooling a batch of solution from the tubes containing mainly GPA, it was dialyzed in order to remove SDS. Finally, GPA solutions in water were freeze-dried to give purified preparations: GPA-A and GPA-B.

Carbohydrate composition of GPA preparations

Several glycophorin preparations, obtained in this investigation, were analyzed for the total sugar content using GLC-MS procedure. Hydrolyzed samples were derivatized into alditol acetates and were analyzed in a gas chromatograph. Sialic acid was not determined by this method. Two example sugar analyses are presented in the Fig. 2. Table 3 presents the monosugar composition, obtained for GPA-A and GPA-B prepara-
tions. As can be seen, only slight differences in the monosugar content could be detected. These differences do not indicate a phenotype of erythrocytes, from which glycophorin preparations were isolated.

**Immunochemical staining of GPA-A and GPA-B preparations**

GPA-A and GPA-B preparations were run, each in 4 lines, in SDS-PAGE and then were electroblotted onto Immobilon P membrane. The membrane was cut into strips, each containing one resolved GPA sample. Four of these strips: two containing GPA-A and two containing GPA-B were subjected to a mild alkaline degradation, which enables removing the O-glycans from glycoproteins⁴. Then immunostaining of all eight Immobilon P strips was performed using three different mouse mAbs: anti-GPA, anti-blood group A antigen and anti-blood group B antigen (Fig. 3). As can be seen, the staining of the blots for the presence of glycophorin and the presence of blood group A or B antigens gave entirely the same pattern of bands. It can be observed also that the blot with GPA-A, incubated in a weak alkaline solution, gave after staining for the presence of blood group A antigen slightly weaker picture (Fig. 3 line 4).

**Isolation and sugar analysis of O- and N-glycans from glycophorin preparations**

Purified GPA-A and GPA-B preparations were degraded separately, by β-elimination, in 0.1 M sodium hydroxide/1 M sodium borohydride in the presence of Cd⁺⁺ cations. The reaction mixture, after neutralization with acetic acid, was passed through BioGel P-2 column. To estimate the elution profile from the column, showing sugar and salt peaks, the absorbance at 206 nm was determined (Fig. 4-I). A broad peak, containing all carbohydrates, was pooled, concentrated by rotary evaporation and applied on Sephadex G-50 column. Elution profile from this column was estimated by determining neutral sugars (Fig. 4-II). Two resolved sugar-containing peaks were obtained: one smaller peak, eluted in the void volume, containing N-glycans and the other, larger peak containing free, reduced O-glycans. According to the β-elimination method the

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fuc (µg)</th>
<th>Man (µg)</th>
<th>Gal (µg)</th>
<th>GlcNAc (µg)</th>
<th>GalNAc (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPA-A</td>
<td>1.0 (1.6)</td>
<td>2.1 (3.0)</td>
<td>18.0 (25.7)</td>
<td>8.4 (9.8)</td>
<td>14.0 (16.3)</td>
</tr>
<tr>
<td>GPA-B</td>
<td>1.3 (2.1)</td>
<td>2.0 (3.0)</td>
<td>16.6 (24.9)</td>
<td>8.3 (10.1)</td>
<td>14.8 (18.1)</td>
</tr>
</tbody>
</table>

The number of sugar residues re-calculated for 3 mannoses are shown in the brackets.

![Fig. 3. Immunochemical staining of GPA-A and GPA-B preparations, run in SDS-PAGE and electroblotted onto Immobilon P membrane. The strips of the membrane were immunostained as follows: lanes 1, 3, 5, 7 – with anti-GPA antibody, lanes 2, 4 – with anti-blood group A antibody, lanes 6, 8 – with anti-blood group B antibody](image-url)
Fig. 4. Elution profiles of three consecutive gel filtrations, run during isolation of O-glycans and N-glycans from β-elimination products of GPA-A or GPA-B preparations. Gel filtration I shows desalting of β-elimination products, chromatogram II presents separation of N- and O-glycans. Gel filtration III presents final desalting of O-glycans; analogous chromatography was performed to desalt N-glycans.
N-glycans were obtained in a form of short glycopeptides. These two peaks were pooled separately and then were desalted on BioGel P-4 column, run in water (Fig. 4-III). O-glycans and N-glycans, obtained from GPA-A and GPA-B, were analyzed for the total carbohydrate content; the GLC-TIC chromatograms are presented in the Fig. 5.

Hemagglutination inhibition test

Three separately obtained preparations of O-glycans and N-glycans, derived from GPA-A and GPA-B glycoporphins, were used to inhibit agglutination of blood group A and B erythrocytes, respectively, by relevant monoclonal antibodies. The results are shown in the Table 4. As can be seen, both types of oligosaccharides inhibited hemagglutination, but N-glycans were much stronger inhibitors; their solutions, having concentration 4 mg/ml, inhibited after at least 32-times dilution (preparation 6), up to 256-times dilution (preparation 4 and 11). On the contrary, O-glycan solutions, which were concentrated 6-7-times more than N-glycans, inhibited hemagglutination after 64-times dilution.
(preparation 7) or less; the least dilution of O-glycans, which still gave agglutination, was 4-times (preparation 8 and 9).

**Discussion**

GPA-A and GPA-B preparations of human glycoporphin were isolated from the pooled erythrocytes of blood group A and B, respectively. The crude preparations were partially purified, using gel filtration in the presence of SDS and then analyzed for sugar content. The gas chromatographic analyses revealed (Fig. 2), that both preparations looked entirely alike. Some differences in the carbohydrate composition could be seen (Table 3), but the range of these differences falls into phenomenon of a well known microheterogeneity of glycoprotein glycosylation. Nevertheless, when these two GPA preparations were subjected to immunochromical staining, after SDS-PAGE and electroblotting, the presence of blood group A antigens in GPA-A and blood group B antigens in GPA-B was clearly evidenced (Fig. 3). This experiment revealed clearly that glycoporphin derived from blood group A erythrocytes contains blood group A antigens and *vice versa* – glycoporphin derived from blood group B erythrocytes contains blood group B antigens. Because GPA contains...
Table 4. Results of hemagglutination inhibition test

<table>
<thead>
<tr>
<th>Row no.</th>
<th>Preparation (no.)</th>
<th>Concentration (mg/ml)</th>
<th>Hemagglutination dilution titer of oligosaccharide preparations</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>mAb anti-A</td>
<td>n.d.</td>
<td>2x 4x 8x 16x 32x 64x 128x 256x 512x 1024x</td>
</tr>
<tr>
<td>2</td>
<td>GPA-A, O-glyc (1)</td>
<td>25</td>
<td>– – – – – – – – –</td>
</tr>
<tr>
<td>3</td>
<td>GPA-A, O-glyc (2)</td>
<td>25</td>
<td>– – – – – – – – +</td>
</tr>
<tr>
<td>4</td>
<td>GPA-A, O-glyc (3)</td>
<td>25</td>
<td>– – – – – + + + +</td>
</tr>
<tr>
<td>5</td>
<td>GPA-A, N-glyc (4)</td>
<td>4</td>
<td>– – – – – – – +</td>
</tr>
<tr>
<td>6</td>
<td>GPA-A, N-glyc (5)</td>
<td>4</td>
<td>– – – – – – – +</td>
</tr>
<tr>
<td>7</td>
<td>GPA-A, N-glyc (6)</td>
<td>4</td>
<td>– – – – – – – +</td>
</tr>
<tr>
<td>8</td>
<td>mAb anti-B</td>
<td>n.d.</td>
<td>2x 4x 8x 16x 32x 64x 128x 256x 512x 1024x</td>
</tr>
<tr>
<td>9</td>
<td>GPA-B, O-glyc (7)</td>
<td>30</td>
<td>– – – – – – – +</td>
</tr>
<tr>
<td>10</td>
<td>GPA-B, O-glyc (8)</td>
<td>30</td>
<td>– – – – – – – – +</td>
</tr>
<tr>
<td>11</td>
<td>GPA-B, O-glyc (9)</td>
<td>30</td>
<td>– – – – – – – – +</td>
</tr>
<tr>
<td>12</td>
<td>GPA-B, N-glyc (10)</td>
<td>4</td>
<td>– – – – – – – – +</td>
</tr>
<tr>
<td>13</td>
<td>GPA-B, N-glyc (11)</td>
<td>4</td>
<td>– – – – – – – – +</td>
</tr>
<tr>
<td>14</td>
<td>GPA-B, N-glyc (12)</td>
<td>4</td>
<td>– – – – – – – – +</td>
</tr>
</tbody>
</table>

Agglutination titers of both monoclonal antibodies used in the test, anti-A and anti-B, are included (first well in a row). Concentration of the oligosaccharide solutions is based on dry mass of the preparations. Additional details of the procedure are given in Materials and Methods.

+ indicates full agglutination, +/- indicates partial agglutination, – indicates lack of agglutination, n.d. – not determined.

both types of glycans a question arose in which type of those oligosaccharides the blood group antigens reside. In order to answer this question, mild alkaline degradation of GPA-A and GPA-B preparations was performed on the blots using described procedure and immuno-staining was repeated. As can be seen, in the case of GPA-A the staining was slightly weakened (Fig. 3 lane 4) which suggests that in GPA-A a reasonable amount of A antigens reside in O-glycans, which was further confirmed in hemagglutination inhibition test. In the case of GPA-B this effect was not observed (Fig. 3 lane 8). This experiment revealed also that blood group A and B antigens are present in O-glycans as well as in N-glycans of human glycoporphin.

In order to further investigate the presence of blood group antigens in both types of oligosaccharides of glycoporphin, GPA-A and GPA-B preparations were degraded by β-elimination and relevant oligosaccharide fractions, O-glycans and N-glycans, were isolated. These oligosaccharide fractions were used in hemagglutination inhibition test (Table 4), and it was shown that more active were N-glycans, as compared with O-glycans. When compare GPA-A and GPA-B sugar analyses (Fig. 2) with hemagglutination inhibition test (Table 4) it can be stated that the abundance of oligosaccharides with blood group determinants is quite low. The majority of carbohydrate structures in glycoporphin are common for this protein and do not reflect the blood group of erythrocytes, from which given glycoporphin sample was isolated. Hemagglutination inhibition test confirmed also that in O-glycans from GPA-A there are more blood group antigens than in O-glycans from GPA-B, i.e. GPA-A O-glycan fractions, Table 4 solution no. 1, 2, 3 inhibited better than GPA-B O-glycans, Table 4 solution no. 8, 9. This is in accordance with the result with immuno-staining on Immobilon P blot after degradation in alkaline solution (discussed above), where GPA-A pattern was slightly weakened (Fig. 3 lane 4) when compared with GPA-B pattern (Fig. 3 lane 8).

The O-glycan preparation no. 7 from Table 4 inhibited hemagglutination very well, but most probably it was contaminated with N-glycans. In fact, separation of O-glycans and N-glycans, isolated from GPA-A and GPA-B, was not complete. This assumption was confirmed to some extent by sugar analysis (Fig. 5). As can be seen in the GLC-MS chromatograms, O-glycans contained little amount of mannose, an indication of N-linked chains, and N-glycans contained some amount of GalNAc – a sugar component characteristic for O-glycans.

Taking all above into consideration, the present investigation reveals the presence of blood group A and blood group B antigens in human glycoporphin derived from blood group A and B erythrocytes, respectively. This observation regards both N-linked and O-linked glycoporphin glycans. Determination of the structure of glycoporphin oligosaccharides, bearing blood group A and B determinants, is in progress in our laboratory.

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