

Modification of Anti-Glycan IgG and IgM Profiles in Allergic Inflammation¹

V. I. Butvilovskaya^{a,*}, O. V. Smoldovskaya^a, G. U. Feyzkhanova^a, M. A. Filippova^a,
L.V. Pavlushkina^b, S. A. Voloshin^a, and A. Yu. Rubina^a

^aEngelhardt Institute of Molecular Biology, Russian Academy of Sciences, Moscow, 119991 Russia

^bFilatov Moscow Pediatric Clinic no. 13, Healthcare Department of Moscow, Moscow, 123001 Russia

*e-mail: v.butvilovskaya@gmail.com

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Abstract—Glycans and anti-glycan antibodies (AGAs) are essential for infiltration of inflammatory cells in various allergies. The glycocalyx structure of the cells is modified during disease progression, and this modification is possible to evaluate by assessment of AGAs. A printed glycan array with 55 immobilized glycans and immobilized antibodies to IgG, IgA, and IgM was used to study the changes in AGA profiles in bronchial asthma (BA). Levels of antibodies to certain glycans in BA patients statistically differed from levels in healthy donors ($p < 0.0007$ by the Mann–Whitney test); the glycan set included 6Su-6'-SiaLec, SiaLeX, Sia6Htype2; T $\alpha\alpha$, Man β 1-4GlcNAc, and Man α 1-4Man β . The obtained results help to better understand the mechanisms of the cell-mediated immune response in bronchial asthma and other types of allergic reactions.

Keywords: allergy, bronchial asthma, anti-glycan antibodies, glycan array, immune response, siglecs, galectins

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INTRODUCTION

Bronchial asthma (BA) is a chronic inflammatory disease of the airways. Apart from epithelial cells, many cells of the innate and adaptive immunity system are involved in BA, causing its main clinical symptoms of bronchial obstruction and asphyxia. BA affects both adults and children throughout the global population.

Nonallergic and allergic BA forms are recognized. According to European Academy of Allergology and Clinical Immunology, allergic BA is diagnosed in more than 7% of pediatric BA patients in Russia and 20% in the United Kingdom [1]. A variety of allergens may contribute to BA, including pollen, epidermal structures, house dust mites, and mold spores. Clinically significant sensitization to certain allergens is observed in severe allergic BA. For instance, an increase in clinical severity of BA has been associated with sensitization to molds (*Cladosporium* or *Alternaria*) or mites (*Dermatophagoides pteronyssinus*) [2].

Abbreviations: GBP, glycan-binding protein; AGA, anti-glycan antibody; AGA-IgM and AGA-IgG, class M and class G AGAs, respectively; BA, bronchial asthma; PBS, phosphate-buffered saline; PBST, PBS supplemented with Tween 20 (washing buffer); PVA, polyvinyl alcohol; PVP, polyvinylpyrrolidone; PBSP, PBS supplemented with PVA (blocking buffer); DB, dilution buffer; BS, background signal; Cy5 and Cy3, cyanine dyes 5 and 3, respectively; Su, sulfate; Sia, sialic acid; LN, lactosamine; DAMP, damage-associated molecular pattern.

Basic therapy in BA is aimed at controlling BA symptoms and progression. This is achieved with anti-inflammatory drugs, including inhalation and systemic corticosteroids, leukotriene receptor antagonists, hormones, β_2 agonists, long-acting theophyllines, and anti-IgE agents [3]. However, BA control is still problematic in a substantial proportion of cases in spite of the broad range of approaches to anti-inflammatory basic therapy [4]. It is therefore of particular interest both theoretically and clinically to study the molecular mechanisms of BA [1, 5, 6].

Glycan structures and their interactions with glycan-binding proteins (GBPs) receive increasing attention in studies of the regulatory mechanisms of inflammatory reactions. Interactions of GBPs with glycans trigger a cascade of biological reactions [7]. Glycans and GBPs are an essential component of pathogen recognition by the immune system and the development of an inflammatory response [8, 9].

Glycan structures exposed on the eosinophil surface have recently been associated with the development of an inflammatory response in BA [10, 11]. Pro-inflammatory influences on human lung endothelial cells have been found to affect the molecular architecture of glycoproteins and glycolipids, leading to synthesis of hypoglycosylated carbohydrate structures [12]. Structural changes that arise in the cell membrane during inflammation lead to the formation of the so-called damage-associated molecular patterns

(DAMPs) [13, 14], which act as neo-antigens and may cause autoantibody development [15]. At the same time, natural anti-glycan antibodies (AGAs) are found in the human blood, occur at much the same level throughout life, and arise without any association with previous immunization [16, 17]. The biological role of these antibodies is yet not fully understood.

Aberrant glycosylation and subsequent changes in serum AGA levels are characteristic of various disorders, including dysfunction of the female reproductive system [18, 19], autoimmune disorders, and oncology diseases [20–22]. Changes in the levels of antibodies to glycan structures became possible to assay after various platforms were presented for constructing glycan arrays [23–25].

A 3D glycan array as one form of glycan arrays has been designed in the Engelhardt Institute of Molecular Biology [25, 26] to detect changes in antibody levels with high accuracy and to allow testing one serum sample for dozens of glycans in one experiment. A homogeneous glycan distribution through the hydrogel volume is a unique feature of the 3D glycan array. In addition, proteins and various glycosylated molecules are possible to immobilize in the 3D glycan array in similar conditions and with similar efficiencies, the range including oligosaccharides, polysaccharides, glycoproteins, glycosaminoglycans, and neoglycoconjugates. It should be noted that changes in AGA repertoire has not been studied in BA so far.

The objective of this work was to study the changes in the repertoire of AGA-IgM and AGA-IgG to 55 glycans of various structures, including selectin and galectin ligands characteristic of eosinophilic inflammation [27, 28], in sera of patients with allergic BA exacerbation caused by house-dust mites, molds, pollen, and food allergens [6, 29].

EXPERIMENTAL

Reagents and buffers. Corning 2947 micro slides (United States) were used in the study. Antibody conjugates were purified chromatographically on Micro Bio-Spin Laboratories columns (Bio-Rad, United States). We used methacrylamide, N,N-methacrylamide bis-methacrylamide, PBS tablets, polyvinyl alcohol (PVA) 50000 Da, polyvinylpyrrolidone (PVP) 360000 Da, biotin N-hydroxysuccinimide ester, Tween 20, Sephadex G-25 coarse (Sigma-Aldrich, United States), Bind Silane (Amersham Pharmacia Biosciences, United States), and fluorescently labeled Cy5-streptavidin (Healthcare, United Kingdom). To obtain antibody conjugates with fluorescent dyes, we used mouse monoclonal antibodies against human IgG (anti-IgG) (Southern Biotech, United States) and human IgM (anti-IgM) (Bethyl, United States). Anti-human IgG (anti-IgG), anti-human IgM (anti-IgM), anti-human IgA (anti-IgA) antibodies and goat polyclonal antibodies against a mixture of human IgA,

IgM, and IgG (GAH; (Pierce, Thermo Fisher, United States) were immobilized in the glycan array.

The following buffer solutions were used: PBS (0.01 M phosphate buffer, pH 7.2, 0.15 M NaCl), array washing buffer PBST (PBS, 0.01% Tween 20), blocking buffer PBSP (PBS, 1% PVA), and dilution buffer DB (PBS, 0.14% PVA, 0.14% PVP). All solutions were prepared using Milli-Q deionized water (Millipore, France).

Clinical samples. Serum samples from 35 patients with exacerbated BA and 60 healthy donors were obtained at Filatov Moscow Pediatric Clinic no. 13 (Moscow, Russia). Venous blood (5 mL) was collected in Vacuette vacuum tubes, stored at 4°C for no more than 24 h, and centrifuged at 2000 g at 4°C for 15 min. Serum samples were stored at –20°C until use.

The study included 95 children of three age groups: 4–7 (12 BA patients and 17 healthy donors), 8–13 (12 BA patients and 23 healthy donors), and 14–17 (11 BA patients and 20 healthy donors) years.

Allergic BA was diagnosed on the basis of disease history and the levels of allergen-specific IgE against the most common inhaled and food allergens; the IgE levels were assayed using an IMMULITE 2000 Immunoassay System (Siemens Healthineers, Germany). BA exacerbation was inferred from a higher (>35 ng/mL) level of the eosinophil cationic protein.

Preparation of 3D hydrogel arrays followed a copolymerization immobilization technique [25, 26]. The polymerization mixture contained gel-forming methacrylamide-based monomers with the proteins and oligosaccharides that were intended for immobilization and were modified with proper spacers. Microdroplets (0.1 nL) of the mixture were applied on Bind Silane-activated glass slides with a Qarray pin robot (Genetix, United Kingdom). To allow polymerization of gel cells, arrays were exposed to UV light with a maximum at 350 nm from a Sylvania GTE lamp (F15T8/350BI, United Kingdom), which was 8 cm away from the arrays, in a nitrogen flow at 20°C for 50 min. After polymerization, the arrays were washed with PBST for 40 min and rinsed with distilled water. To prevent nonspecific interactions, the arrays were treated with PBSP for 1 h and washed with distilled water. The resulting hemispherical 3D gel elements were 120 µm in diameter.

Quality of arrays was checked by their appearance in transmitted light, using TestChip and QualityControl software. When gel elements showed deviations in radius of more than 5% within an array or 8% within an array lot, the array was rejected. Accepted arrays were covered with 120-µL plastic incubation chambers and stored at 2–8°C.

Glycan arrays contained glycans immobilized via spacers at a working concentration of 3.3 mM (3.3×10^{-13} moles per cell). The glycans immobilized are listed in Table 1S (see Supplementary Materials at <http://www.molecbio.ru/downloads/2018/4/>

supp_Butvilovskaya_rus.pdf). In addition, the arrays contained anti-IgG, anti-IgA, and anti-IgM monoclonal antibodies and polyclonal goat antibodies against a mixture of IgG, IgA, and IgM; the antibodies were used at 0.16 mg/mL (1.6×10^{-11} moles per cell). Gel elements that did not contain any protein were included in each array as a control for nonspecific binding. Each gel element was used in quadruplicate.

Antibody conjugation with fluorescent dyes. Cyanine N-hydroxysuccinimide ester (Cy5 or Cy3; Healthcare, United Kingdom) (2 μ L, 2 mg/mL in a dimethylformamide solution) was added to 75 μ L of an anti-IgG or anti-IgM solution (1 mg/mL in 0.01 M bicarbonate buffer, pH 9.5). The reaction was carried out in the dark at 22°C for 1 h with continuous agitation (550 rpm). Excess dye was removed by gel filtration on a Micro Bio-Spin column filled with Sephadex G-25 and equilibrated with PBS. A staining protocol was selected so that the protein–dye molar ratio was 1 : 3. The working concentrations of the resulting anti-IgG-Cy5 and anti-IgM-Cy3 secondary antibodies in the mixture applied on glycan arrays were 0.005 mg/mL.

Immunoassays on glycan arrays. Serum samples from BA patients and healthy donors (control) were diluted 15-fold with DB [30] and incubated with glycan arrays at 37°C for 2 h. The arrays were washed with PBST for 30 min, rinsed with water, dried in an air flow, and incubated with 120 μ L of a secondary antibody mixture at 37°C for 1 h. After final washing (PBST, 30 min), the arrays were rinsed with water and dried in an air flow.

Fluorescence measurement and data processing. Glycan arrays were examined using a Genepix 4200A scanner (Molecular Devices, United States) at 10 μ m resolution. The fluorescent signal intensity was calculated for each cell with Genepix Pro 6.0 software (Molecular Devices), using fluorescence intensities at $\lambda = 635$ nm (column F635 median in Genepix Pro 6.0) for AGA-IgG and $\lambda = 532$ nm (column F532 median in Genepix Pro 6.0) for AGA-IgM.

The median fluorescence intensity I of four identical cells of the glycan array was used in further calculations. A background signal (BS) was obtained as the median signal from four identical cells for each glycan after incubating the array with DB as an antigen sample and development the array with the secondary antibody mixture.

Only I values at least twice as high as BS were used in further calculations of AGA levels and statistical analyses. The results were statistically analyzed to detect regularities.

Statistical analyses and data presentation. Results were statistically analyzed using Microsoft Office 2010 Excel spreadsheets and MedCalc 11.4.2.0 software (Belgium). Between-group differences were examined by the nonparametric Mann–Whitney (MW) and exact Fisher's tests [31, 32]. The Bonferroni correction for multiple testing was used [33], and results were

consequently considered at $p < 0.0007$. Data summarized in Table 1S (Supplementary Materials) are median I values.

Lower limit of detection with the glycan array for AGAs. The analytic sensitivity of detecting AGAs with the glycan array was assayed using anti-LeC glycan antibodies, which were kindly provided by N.V. Bovin (Institute of Bioorganic Chemistry, Moscow).

Anti-LeC antibody solutions in PBS with concentrations of 50, 10, 2, 0.08, 0.016, 0.008, and 0.004 μ g/mL and DB without anti-LeC antibodies were applied on a glycan array, which was preliminarily treated with PBSP. Immunoassay was carried out as above. An anti-IgM-Cy3 and anti-IgG-Cy5 secondary antibody mixture was applied onto the array. The lower limit of detection was obtained using a sample dilution curve, as a point where the fluorescent signal was two standard deviations higher than BS .

Estimation of the total immunoglobulin level in sera of BA patients and healthy donors. The IgM and IgG levels were inferred from the fluorescent signals I from cells in array position (49) with immobilized goat polyclonal antibodies against a mixture of human IgG, IgA, and IgM. Measurements were performed using 532- and 635-nm filters, respectively.

RESULTS AND DISCUSSION

A glycan array including 55 immobilized glycans of various structure (Supplementary Materials, Table 1S) was used to determine the repertoire of AGA-IgG and AGA-IgM in children with exacerbated allergic BA and healthy children. Serum samples were obtained from 35 BA patients aged 4–17 years and 60 healthy children.

The 3D glycan array was manufactured by a technique developed in the Engelhardt Institute of Molecular Biology [25, 26] and was designed to estimate the levels of antibodies against 55 glycans in one experiment. The method has a broad dynamic range and allows quantitation of both low and high antibody titers [25, 26, 34].

Both spacer-containing glycans and O-polysaccharides were immobilized in the array, including those of *Candida albicans* and *Escherichia coli*, laminarin, and zymosan A (Supplementary Materials, Table 1S). In addition, anti-IgG, anti-IgA, and anti-IgM antibodies were immobilized in the array to control the array performance, to check for cross-reactions, and to estimate the total Ig levels in the blood serum.

As soon as internationally certified commercial control materials to evaluate the analytical sensitivity of AGA detection methods are currently unavailable for in vitro diagnosis, anti-LeC AGA-IgM which are detectable in nearly all healthy people were consequently used for the purpose [17].

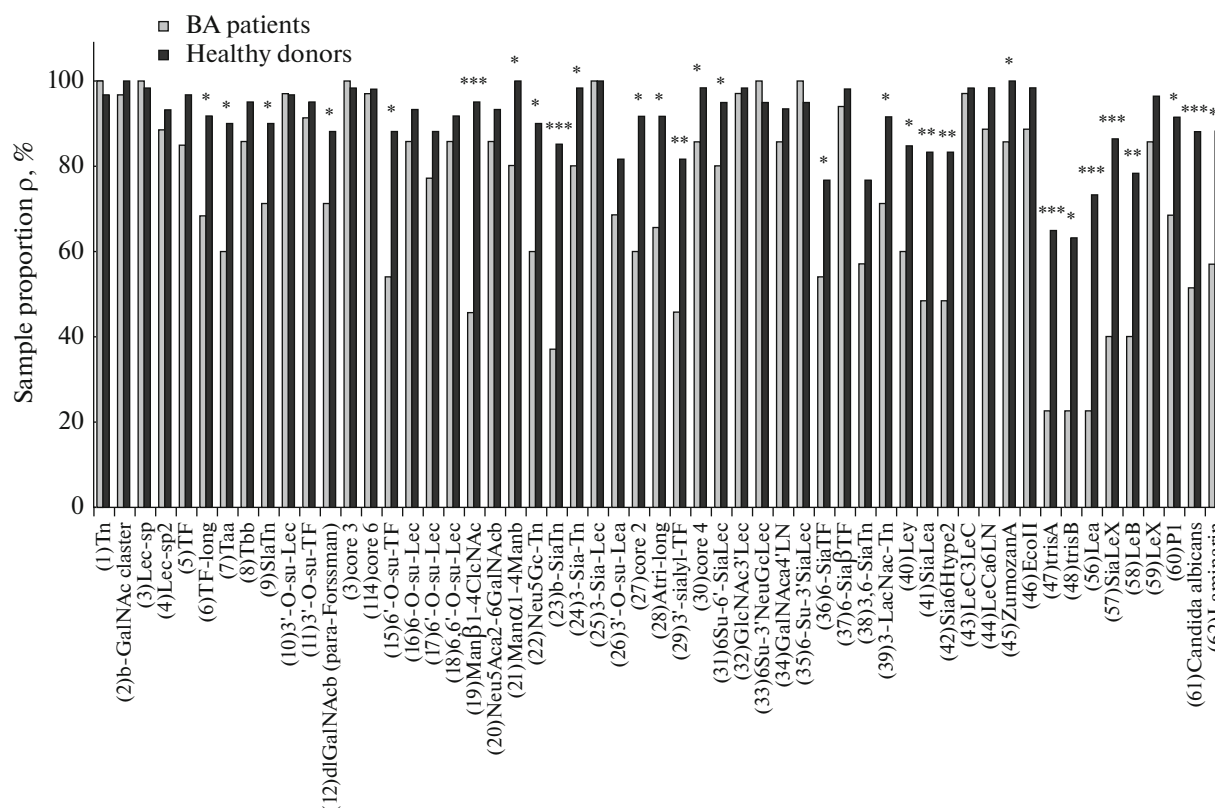


Fig. 1. Proportion (p) of samples where the fluorescent signals (I) from cells with immobilized glycans were at least twice higher than BS after immunofluorescent AGA-IgM detection. BA, bronchial asthma. Differences in AGA-IgM frequencies between the two cohorts were tested for significance by the exact Fisher test. Differences were significant at (*) $p \leq 0.05$, (**) $p \leq 0.001$, or (***) $p \leq 0.0001$; lack of * indicates that the difference was nonsignificant ($p \geq 0.05$).

As is seen from Fig. 1S (Supplementary Materials, http://www.molecbio.ru/downloads/2018/4/supp_Butvilovskaya_rus.pdf), the lowest statistically significant measurable with our method AGA concentration was 0.008 $\mu\text{g/mL}$. This sensitivity is sufficient for assaying IgM and IgG, which occur in the serum at 0.15–1.07 and 1.00–6.81 g/L, respectively, in pediatric BA patients aged 4–17 years [35].

A comparison of the IgM and IgG levels between the BA patients and healthy children was performed by the nonparametric Mann–Whitney test (Supplementary Materials, Fig. 2S) and showed only minor differences for total IgM level. Fluorescent signal intensities (I) were consequently used without normalization to the total IgM level when analyzing the AGA profiles.

When control serum samples were examined with anti-IgG-Cy5, fluorescent signals from gel elements with immobilized glycans did not exceed BS (Supplementary Materials, Fig. 3S). Comparative analysis of changes in AGA profiles in BA was consequently performed only for AGA-IgM.

Several groups of glycans were used as antigens to detect AGA-IgM (Supplementary Materials, Table 1S). The array included 55 structurally different glycans, which were potential ligands for selectins, galectins, and mannose receptors of immune system cells involved in

eosinophilic allergic inflammation [27, 28, 36]. Each group comprised a main core glycan and its derivatives, which carried various substitutes (Su, Sia, LN, etc.) [37, 38].

Frequencies (p) of AGA-IgM in the two subject cohorts are shown in Fig. 1. Between-cohort differences in frequencies were tested for significance by the exact Fisher test (F-test) [31]. In addition, the nonparametric Mann–Whitney (MW) test was used to detect statistically significant between-cohort differences in median signals (Figs. 1, 2; Supplementary Materials, Table 1S). Because the Bonferroni correction for multiple testing was used [33], differences were considered significant by the Mann–Whitney test at $p < 0.0007$.

Between-cohort comparisons showed that the AGA-IgM levels and frequencies in the BA patients were significantly lower than in the healthy donors (Fig. 1; Supplementary Materials, Fig. 4S).

AGA-IgM against Potential Ligands of Siglecs¹

Figures 1 and 2 show the changes in AGA-IgM against the glycan (31) 6Su-6'-SiaLe^c, here (31) is the ordinal number of the glycan in the array (Supplementary Materials, Fig. 1S, Table 1S). A significant (1.7-fold)

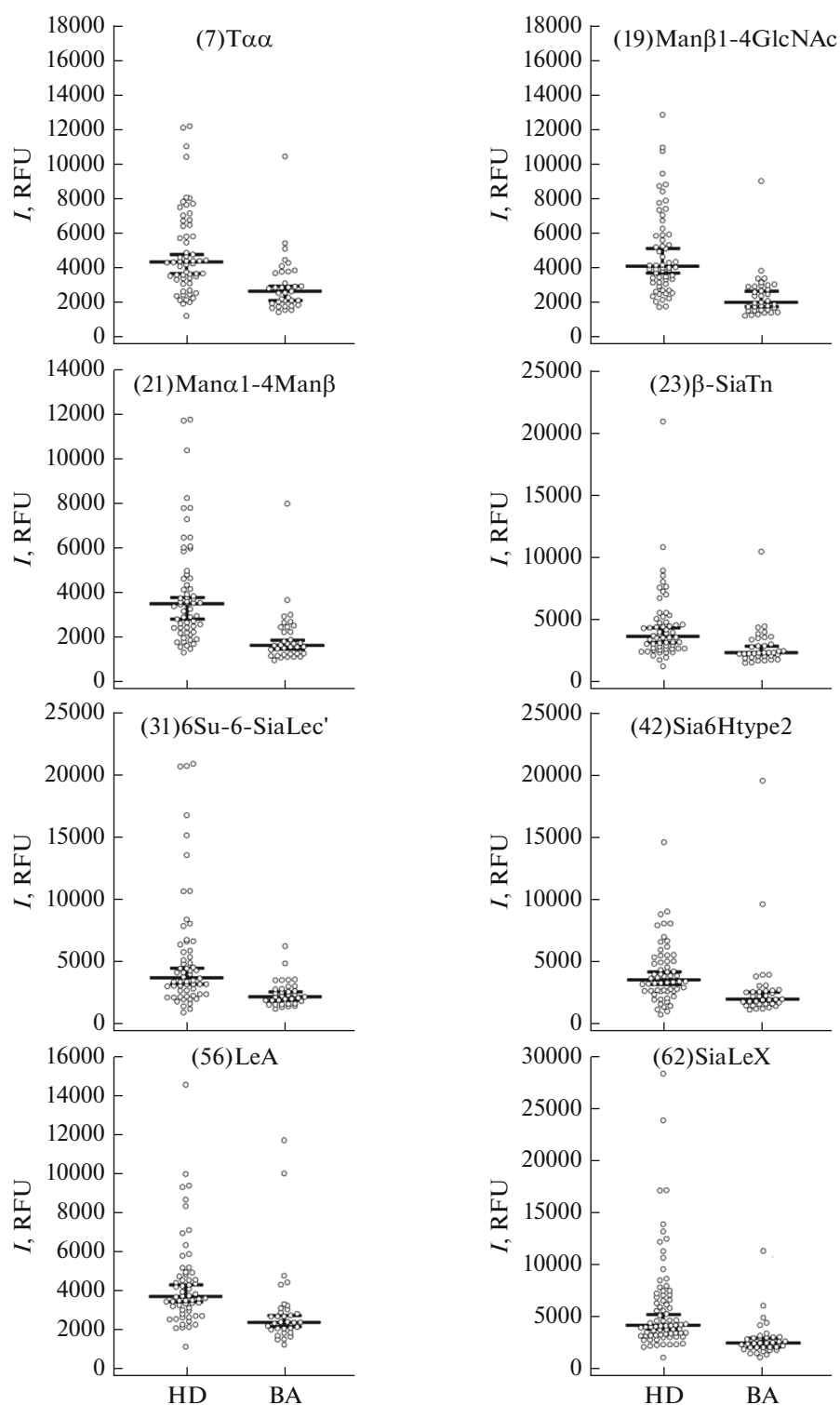


Fig. 2. Diagrams of comparison of the fluorescent signal intensities (I , 532 nm) from cells with immobilized glycans between serum samples from the healthy donors (HD) and BA patients (BA) (see Fluorescence measurement and data processing). I is the median fluorescent signal of four identical cells of the glycan array; RFU, relative fluorescence unit.

decrease in I was observed in the BA patients compared with the controls ($p < 0.0001$ by the MW-test; Fig. 1, $p < 0.034$ by the F-test).

As is seen from Figs. 1 and 2, the BA patients additionally displayed a 1.8-fold decrease in I for AGA-IgM against the glycan (42) Sia6Htype2, which contains

α 2,6-sialic acid ($p < 0.0001$ by the MW-test; Fig. 1, $p = 0.0009$ by the F-test).

In the case of AGA-IgM to the glycan (57) SiaLeX, I in the BA patients was 1.6 times lower than in the control group (Fig. 2, $p < 0.0001$ by the MW test; Fig. 1, $p = 0.0000009$ by the F-test).

The fluorescence intensity decreased in the case of AGA-IgM against the sialylated Tn derivatives (23) β -SiaT_n, (24) 3-SiaT_n, and (9) SiaT_n by factors of 1.4, 1.5, and 1.6, respectively (Fig. 2; Supplementary Materials, Fig. 5S). The MW test results (p -values) are shown in Table 1S (Supplementary Materials); $p < 0.0007$ for each glycan.

Lower fluorescence intensities were observed for the sialylated and sulfated TF derivatives (15) 6'-O-sulfate, (29) 3'-sialyl-TF, and (36) 6-SiaTF; the intensities decreased by factors of 1.4, 1.5, and 1.2, respectively (Fig. 1; Supplementary Materials, Fig. 1S). The MW test results (p -values) are shown in Table 1S (Supplementary Materials); $p < 0.0007$ for each glycan.

Decreases in AGAs against sialylated and sulfated glycans are possible to relate to a potential role of the glycans in binding with siglecs. Siglecs are a family of sialic acid-binding immunoglobulin lectins [39]. Siglec-mediated intercellular interactions have been observed in active alveolar inflammation, which is characteristic of BA [40, 41].

Human Siglec-8 is expressed mostly on eosinophils and mast cells and is structurally similar to mouse Siglec-F, which is exposed on alveolar macrophages [42, 43]. In mouse BA models, 6'-sulfo sialLex has been identified as a ligand of mouse Siglec-F [44].

We observed a decrease in AGA-IgM against the sulfated and sialylated glycan (31) 6Su-6'-SiaLe^c, but not to several other sialylated and sulfated LeC derivatives (Supplementary Materials, Table 1S), which are potential ligands of siglecs.

A decrease in circulating surveillance IgM antibodies against sialylated and sulfated glycans in BA is possible to explain by the antibody binding with DAMPs arising in eosinophilic inflammation.

AGA-IgM against Potential Ligands of Galectins

A 1.6-fold lower level in BA was observed for AGA-IgM against the glycan (39) 3-LacNAc-T_n (Supplementary Materials, Fig. 5S), which contains N-acetylglucosamine (Gal- β 1-3/4-N-acetyl-D-glucosamine, or LacNAc). The levels of AGA-IgM against the glycans (6) TF-long and (7) T_{αα}, which contained galactose, decreased by factors of 1.3 and 1.6, respectively (Supplementary Materials, Table 1S; $p < 0.0007$ by the MW test; $p < 0.05$ by the F-test).

Changes in the levels of antibodies against glycans containing galactose and lactosamine in the BA patients are possible to explain by glycan binding with GBPs, namely, galectins (Gal-1) and the mannose

receptor [36]. Gal-1 is a component of complex N- and O-glycans of the cell surface [45] and is exposed on human lung cells, as well as macrophages and polymorphonuclear, dendrites, stromal, activated T, endothelial, and epithelial cells [46, 47]. Studies with a mouse model of allergic BA have shown that allergen stimulation leads to a higher eosinophil content and airway hyperreactivity in mice deficient in Gal-1 as compared with wild-type mice [11].

Galectins have recently been implicated in DAMP formation [48]. We assume that BA patients have lower levels of IgM antibodies against potential galectin ligands because circulating surveillance IgM antibodies play their protective role and shield DAMPs, which form in eosinophilic inflammation.

AGA-IgM against Potential Ligands of the C-Type Lectin Mannose Receptor

As is seen from Fig. 2 and Figs. 4S and 5S (Supplementary Materials), the levels of AGA-IgM against the mannose-containing glycans (27) core2, (19) Man β 1-4GlcNAc, and (21) Man α 1-4Man β decreased in the BA patients by factors of 1.7, 2, and 2.14, respectively (Supplementary Materials, Table 1S; $p < 0.0007$ by the MW test; $p < 0.05$ by the F-test).

The levels of AGA-IgM against mannose-containing structures and GlcNAc may change because these glycans are involved in binding with the C-type lectin mannose receptor. Mannose receptors on human dendrite cells serve as receptors of several clinically important allergens, including the Der p1 and Der p2 house-dust mite allergens [49, 50]. Mannose receptors have been shown to play a crucial role in cell differentiation associated with the developing Th₂ response to Der p1 and Fel d1 [51]. A decrease in the levels of mannose- and GlcNAc-binding AGA-IgM in BA patients is possibly due to elimination of the allergens responsible for BA.

Antibodies against Immobilized E. coli O-Polysaccharides, Zymosan A, Laminarin, and Candida albicans Glycans

Differences in the levels and frequencies of AGAs against the O-polysaccharides (45) zymosan A, (61) *Candida albicans*, and (62) laminarin are shown in Figs. 1 and 5S (Supplementary Materials). A decrease in fluorescence intensity from AGAs was 1.6 fold in the case of (45) zymosan A, 1.8 fold in the case of (61) *Candida albicans*, and 1.85 fold in the case of (62) laminarin (Supplementary Materials, Table 1S; $p < 0.0008$ by the MW test; $p < 0.05$ by the F-test).

The role of antibodies against these O-polysaccharides in BA is unclear. It is possible to assume that the O-polysaccharides act as DAMP mimetics and consequently decrease the respective AGA-IgM levels.

Thus, assays for AGA-IgM levels in BA patients and healthy donors revealed a systemic decrease in IgM antibodies to certain glycans in the former, while the total IgM level did not significantly differ between the two cohorts. We assume that eosinophilic inflammation, which accompanies BA, is associated with a lower production of the AGA-IgM that play a role in surveillance over newly forming DAMPs.

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