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Brief communication

Synthetic blood group antigens for anti-A removal device and their interaction with monoclonal anti-A IgM

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Abstract

Removal of blood group antibodies against the donor organ prior to ABO-incompatible transplantation can prevent episodes of hyperacute rejection. We are developing a specific antibody filter (SAF) device consisting of immobilized synthetic Atrisaccharide antigens conjugated to polyacrylamide (Atri-PAA) to selectively remove anti-A antibodies directly from whole blood. In this study, we evaluated eight anti-A IgM monoclonal antibodies (mAbs) using Enzyme-Linked Immunosorbent Assay (ELISA) to determine their specificity for binding to Atri-PAA. Five of the eight mAbs met our criteria for specificity by binding to Atri-PAA with at least five times greater affinity compared to the negative controls. These selected mAbs will be studied for their binding characteristics to Atri-PAA which will aid in the development of the SAF. The study of kinetics of antibody removal and quantification of antibody removal will be used in our mathematical model to maximize the antibody removal rate and binding capacity of the SAF.

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1. Introduction

Transplantation of solid organs across the ABO blood group barrier is known to cause hyperacute organ rejection, where pre-existing host antibodies cause rapid humoral-mediated graft rejection [1]. Hyperacute rejection associated with ABO-incompatible organ transplantation can be avoided with removal of anti-A and anti-B blood group antibodies, and long-term graft survival from chronic rejection is comparable to ABO compatible transplantation [1–9]. Although blood group antibody levels can rebound after transplantation, humoral rejection of

the organ occurs less frequently due to an accommodation process [2,4]. Alexandre et al. [2] and Bennett et al. [3] first showed that reduction of blood group antibody titer prior to kidney transplantation across the ABO barrier decreased the chance of hyperacute rejection. Tanabe et al. [8] performed a study with a longer term follow-up which showed that graft survival for kidney transplants across the ABO barrier was 79% at 1–4 years, 75% at 5–6 years, and 73% at 7–8 years when antibody titers were reduced to below 1:16. Graft survival in ABO-incompatible transplantation was not statistically different than ABO-compatible transplant after the third year, and patient survival was not different over all 8 years. In liver transplantation, Hanto et al. [4] removed anti-A/B antibodies to titers below 1:8 prior to ABO-incompatible transplantation without immunological graft losses. Similar success with ABO-incompatible liver and kidney transplantations were also reported by Ishida et al. [6], Sonnenday et al. [7], and Tyden et al. [9].

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Blood group antibody removal in the ABO-incompatible organ transplants described above was performed using plasmapheresis, followed by either plasma exchange [2,4,5,7] or immunoadsorption of the separated plasma by perfusion through beaded columns containing immobilized synthetic trisaccharide antigens [3,6,8,9]. The latter approach has the advantages of being more selective of removing blood group antibodies and of avoiding difficulties and complications associated with transfusion of donor plasma including the risk of infectious disease transmission and immunological reactions to non-autologous plasma [10,11]. Anti-A immunoadsorption columns have been developed with the capacity to reduce anti-A antibody titers from 3 to 7 titer units in one pass [12]. Our group is developing a new device to target specific removal of blood group antibodies *directly* from whole blood perfusing the device, i.e. without the need to separate plasma from blood. An early prototype based on the animal-source protein antigen Neutr-AB immobilized on the luminal fiber surfaces of a blood dialysis cartridge was able to reduce the anti-A and anti-B titers of 300–400 ml of type O blood by 75–98%. However, being an animal source derived antigen, Neutr-AB has a significant non-antigenic component that requires purification to maximize antigen utility [13] and the purification of Neutr-AB before immobilization on SAF increased the capacity of the device by a factor of six [13,14].

We are continuing the development of our novel extracorporeal device for whole blood perfusion. This device will obviate the need for plasma separation and plasma exchange as required in the existing clinical devices [12]. The SAF consists of a module of hollow fiber membranes similar to a dialysis device. The current approach involves *synthetic blood group antigens* immobilization on the blood contacting surfaces of the device in place of the Neutr-AB previously used. The synthetic antigen immobilized on the fiber lumen surfaces is an Atri-PAA conjugate consisting of Atrisaccharide multivalently attached to a polyacrylamide (PAA) backbone serving as a hydrophilic spacer [15]. The Atri-PAA synthetic antigen has several advantages over our previous Neutr-AB antigen, including its multivalency, specificity for anti-A (Neutr-AB bound both anti-A and anti-B), potential for tailoring its biocompatibility with other functional groups, and that it is synthesized rather than being animal source derived antigen.

Our first device targets the specific removal of anti-A because of its greater clinical significance in ABO-incompatible organ transplantation [4,5]. We decided to use monoclonal antibodies for our initial experiments to circumvent the complexities related to the whole blood due to the presence of non-specific proteins. In this study we evaluated the binding of several available monoclonal anti-A IgM antibodies to our Atri-PAA conjugate using ELISA. Our goal was to determine which of these anti-A mAbs had high specificity (binding level in terms of optical density) for our synthetic antigen. The selection criteria were based on mAb binding levels to Atri-PAA exceeding by five-fold or greater than those to Btri-PAA, Glucose-PAA and bovine serum albumin (BSA) negative controls. These selected mAbs will be used in subsequent development work, along with human serum, plasma and blood, to study the anti-

body capture rate and capacity of the SAF devices as we evolve the SAF design and explore changes in its operating parameters. The inclusion of anti-A mAbs into our SAF development and testing program will help us to delineate antibody capture mechanisms and to further develop our design and simulation model of SAF devices [13,14] beyond what could be accomplished by restricting our studies to polyclonal anti-A capture from human blood. Eight mouse monoclonal anti-A IgM antibody candidates were evaluated using ELISA for their specificity for Atri-PAA. Five of these mAbs met our specificity criteria for binding to Atri-PAA and will be used in future studies as we develop the SAF for human clinical use.

2. Materials and methods

The overall methodology involved synthesis of the Atri-PAA (Atrisaccharide-polyacrylamide) conjugates, determination of the starting monoclonal anti-A antibodies as supplied from their manufacturers/sources, and evaluation of the binding specificity of the mAb anti-A candidates to Atri-PAA relative to controls using ELISA.

2.1. Synthesis of antigens conjugated to polyacrylamide

Synthetic blood group antigens, Atrisaccharide (Atri), GalNAc α 1-3(Fuc α 1-2)Gal β , and Btrisaccharide (Btri, as control), Gal α 1-3(Fuc α 1-2)Gal β , were synthesized as described by Korchagina and Bovina [16]. Subsequently the synthesized Atri and Btri, moieties, along with glucose, were covalently coupled to 30 kDa poly *N*-hydroxyethylacrylamide (PAA) using the conjugation technique described by Bovin et al. [17]. Briefly, the polysaccharides, Atri, Btri, and glucose, were coupled to PAA through the condensation of activated polyacrylic acid with amino compounds. Approximately 20% of the activated sites of polyacrylic acid were substituted with polysaccharides resulting in the formation of polysaccharide-PAA conjugates. Atri-PAA, Btri-PAA, and Glucose-PAA were produced and used in our study.

2.2. Preparation of mouse monoclonal anti-A antibodies

Eight mouse monoclonal anti-A IgM antibodies (mAbs) listed in Table 1 with their manufacturer/source were used in this study. ELISA was performed to determine starting (stock) concentrations of the anti-A IgM mAbs. The stock concentration was used to determine the initial dilution required to obtain mAb concentrations of approximately 3000 ng/ml for subsequent ELISA binding studies (see next section). Briefly, the 96-well flat bottom microplates (Nunc Maxisorb, Rochester, New York, USA) were coated with goat anti-mouse IgM (Bethyl Laboratories, Montgomery, TX, USA) in carbonate buffer, incubated for 1 h at 37 °C, and washed. The plates were blocked with BSA (Sigma, St. Louis, MO, USA), incubated for 1 h at 37 °C, and washed. Mouse reference serum (Bethyl Laboratories, Montgomery, TX, USA) or the mAbs diluted in Phosphate buffered saline (PBS, pH 7.4) with 0.1% BSA and 0.02% Tween (Sigma, St.

Table 1
Mouse monoclonal anti-A IgM antibodies, their source, and starting concentration

Company (country)	Clone	Concentration (μ g/ml)
IBGRL (UK)	BRIC 186	891 \pm 82
IBGRL (UK)	BRIC 145	N/A
IBGRL (UK)	BRIC 66	652 \pm 4
Diagast (France)	9113D10	2192 \pm 116
Dominion Biologicals (Canada)	F98 7C6-4	401 \pm 57
Dominion Biologicals (Canada)	F125 7B6-4	654 \pm 12
DAKO (USA)	81 FR 2.2	219 \pm 10
Immucor (USA)	Birma-1	47 \pm 1
Hematolog (Russia)	A-16	37 \pm 3

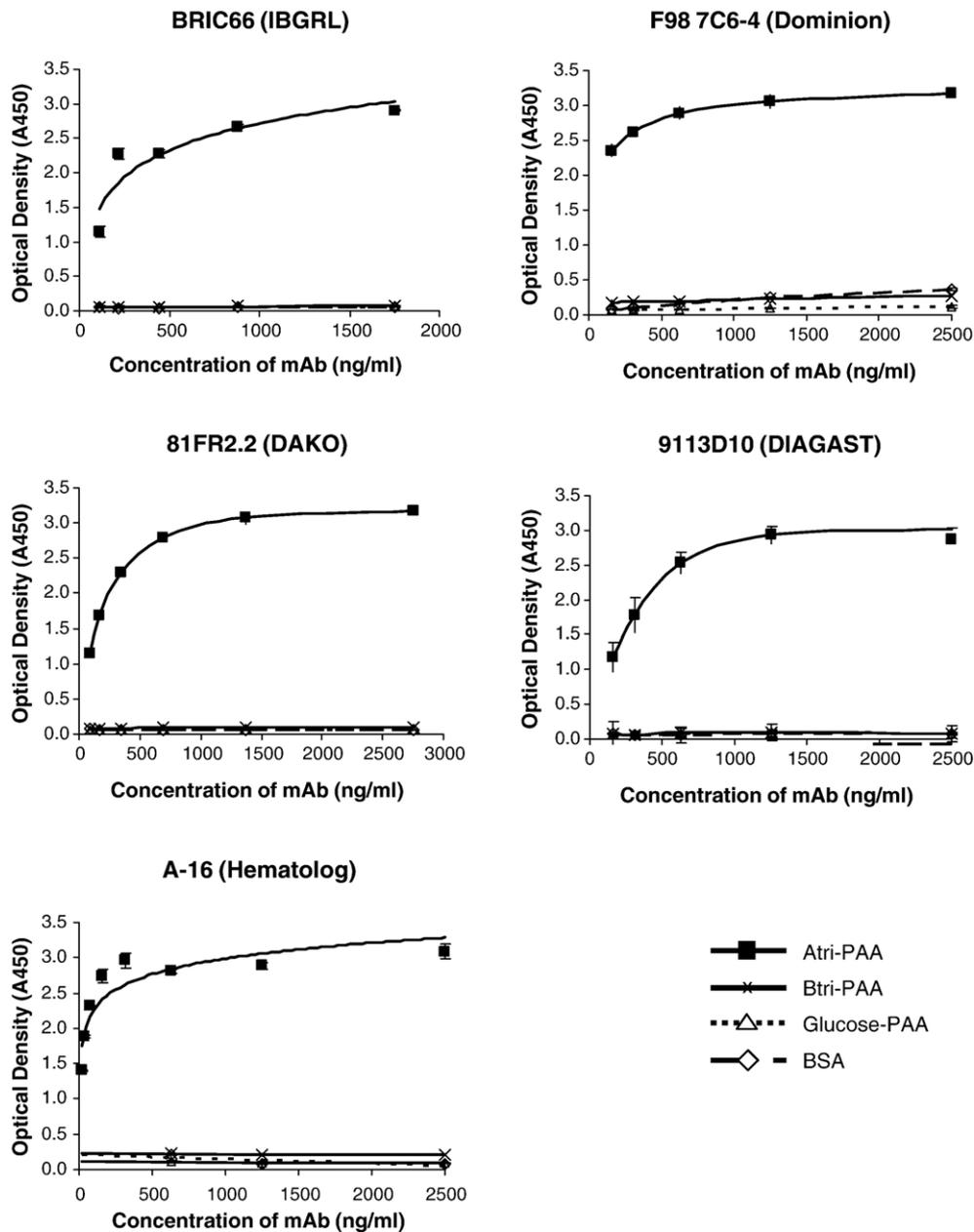


Fig. 1. Binding curves for the five mAbs that were specific to Atri-PAA with an optical density greater than 2.5 for Atri-PAA and less than 0.5 for controls at saturation.

Louis, MO, USA) were added to the wells in duplicate. The plates were incubated for 15 min on a shaker and 45 min at 37 °C and washed. Horseradish peroxidase (HRP) labeled goat anti-mouse IgM antibodies (Bethyl Laboratories, Montgomery, TX, USA) were added to each well. The plates were incubated at room temperature for 15 min on a shaker, for 45 min at 37 °C, and then washed. Tetramethyl benzidine (TMB) substrate (KPL, Gaithersburg, MD, USA) was applied to the plates, and the plates were incubated at room temperature for 10 min and 2 M H₂SO₄ (Sigma, St. Louis, MO, USA) was added to stop the reaction. The optical density was read at 450 nm using a microplate reader (Thermo Max, Molecular Devices, Sunnyvale, CA, USA).

The starting concentrations determined for each of the eight mAbs are listed in Table 1.

2.3. Evaluation of mAb binding specificity to Atri-PAA

The specificity of the eight mAbs to Atri-PAA was tested over a range of concentrations up to 3000 ng/ml. The stock solutions of mAb were diluted to

different concentration levels (ng/ml) and their affinities to antigen were compared at these concentrations by detecting the bound antibodies using an ELISA technique similar to that of Rieben et al. [18]. Polystyrene 96-well plates were coated with 10 µg/ml Atri-PAA in 0.05 M sodium carbonate buffer (pH 9.6). The plates were incubated overnight at 4 °C and washed once with buffer that consisted of 10 mM phosphate buffered saline (PBS, pH 7.4) containing 0.5% Tween 20. The plates were blocked with 3% BSA in PBS, incubated at 37 °C for 1 h, and then washed once. Mouse anti-A monoclonal antibodies were diluted in buffer consisting of PBS with 0.1% BSA and 0.02% Tween according to the results of the ELISA for quantification of concentration. The mAbs were applied to the plate and titrated by two-fold dilution. The plates were incubated for 15 min on a shaker at room temperature and for 45 min at 37 °C, and then were washed three times. HRP labeled goat anti-mouse Ig antibodies (Southern Biotechnology Associates, Birmingham, AL, USA) were diluted 1/5000 and were applied to each well. The plates were incubated for 15 min on a shaker and for 45 min at 37 °C and washed three times. TMB substrate was added to each well. The plates were incubated for 10 min at room temperature, and then the

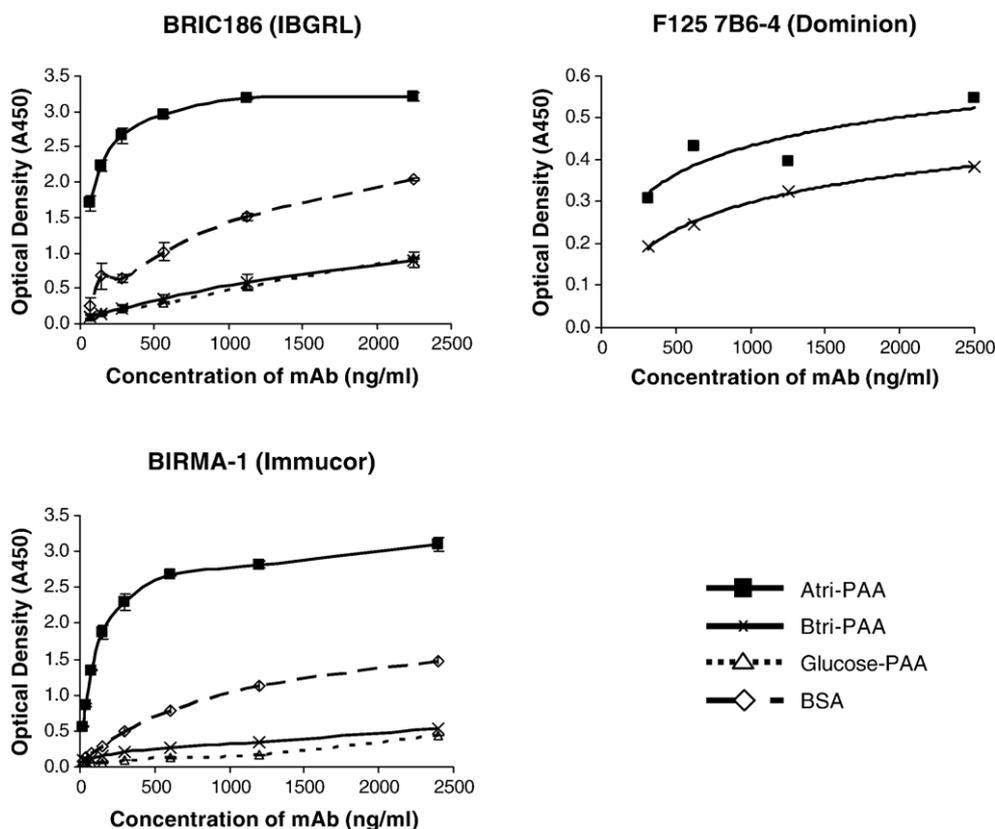


Fig. 2. The binding curves of the mAbs that were not specific to Atri-PAA with an optical density less than 2.5 for Atri-PAA or above 0.5 for controls at saturation.

enzyme reaction was stopped with 2 M H_2SO_4 . The optical density was measured at 450 nm using a microplate reader. Btri-PAA, Glucose-PAA, and BSA were used as negative controls.

3. Results

Figs. 1 and 2 show the affinity of mAbs to antigen and controls as optical densities on *Y*-axis for various mAb concentrations (ng/ml) on *X*-axis. Five monoclonal antibodies showed high specificity for binding to Atri-PAA (Fig. 1) as defined by optical densities at saturation with Atri-PAA being at least 5 times greater than that for the negative controls (i.e. $OD > 2.5$ for Atri-PAA, $OD < 0.5$ for all negative controls). These mAbs which met our specificity criterion were BRIC 66, F98 7C6-4, 81FR 2.2, 9113D10 and A-16. The Hematolog A-16 had the greatest apparent binding affinity to Atri-PAA, reaching binding saturation well below 500 ng/ml. All other candidate mAbs saturated Atri-PAA above 500 ng/ml.

Three monoclonal antibodies did not meet our defined criterion for high binding specificity to Atri-PAA (Fig. 2). Two of these mAbs, BRIC 186 and BIRMA-1, failed our criterion due to binding to one or more controls at an optical density level greater than 0.5 within the concentration range. One mAb, F125 7B6-4, failed our criterion due to binding to Atri-PAA at an optical density level well below 2.5 over the entire concentration range.

4. Discussion

We are developing a specific antibody filter (SAF) that will be used to selectively remove anti-A/B antibodies directly from whole blood prior to ABO-incompatible transplantation. The

SAF incorporates immobilized synthetic blood group antigens to selectively remove anti-A or anti-B antibodies using whole blood perfusion, eliminating the need for plasma separation and plasma exchange required in the existent clinical studies. Anti-A is being studied in our lab because the removal of these antibodies will have the largest clinical impact [4,5]. Atrisaccharide attached to a polyacrylamide (PAA) conjugate was selected as a synthetic antigen to remove anti-A because of its multivalent nature, in that multiple antibodies can bind to one molecule of the antigen conjugate. We initially chose the trisaccharide structure for our device since it is the simplest carbohydrate form recognized by blood group antibodies [18].

The goal of this study was to evaluate eight anti-A IgM mAbs to determine the specificity of each mAb for Atri-PAA using ELISA. Natural blood group antibodies are polyclonal; however, by utilizing mAbs specific to Atrisaccharide, we can study the binding of pure antibodies with the same affinity for Atri-PAA with less complexities compared to the whole blood antibodies. IgM anti-A antibodies were chosen since blood group antibodies are predominantly of the IgM isotype, and these antibodies are generally thought to play the central role in hyperacute rejection [1,19,20]. However, we will also consider whether IgG isotype in human blood are playing a role and if IgG Abs interfere with the capture of IgM Abs. The specificity of mAb was defined as the antibody binding level to antigen by measuring optical density and to satisfy the specificity criteria mAb binding to Atri-PAA was required to be at least five times greater at saturation than that of the controls. Five mAbs met

these criteria for specificity, while three mAbs did not meet these standards. Two of the mAbs we found to be specific, BRIC186 and 9113D10, were tested previously using a similar ELISA technique and also found to have specificity for the Atrisaccharide structure [18].

The mAbs with high specificity for Atri-PAA will aid in the SAF design through studies altering key operating parameters to optimize the antibody removal and binding capacity of the device. These mAbs will be used to determine the immobilization of antigen coverage that removes the highest level of antibody. The mAbs will also be used in scaled-down SAF prototypes and information using the mAb reactions in dynamic conditions will be used to continue development of the mathematical model that will predict antibody binding capacities of the SAF. Further evaluation of the interactions between the mAbs and synthetic antigens will give us knowledge of the kinetics of antibody removal in SAF system, and quantification of antibody removal in SAF prototypes will be evaluated and used in our mathematical model to maximize the antibody clearance.

At present, we have evaluated the mAbs with only the Atrisaccharide antigen attached to the fiber lumen surfaces; however, other blood group mAbs exist that have specificity for Atetrasaccharide. The specificity of these mAbs for the tetrasaccharide structure implies that the fourth sugar in the core composition may influence binding of a population of blood group antibodies [18]. We plan to perform experiments using human plasma, and the antibody removal in terms of titer reduction by hemagglutination assay using the optimized SAF device with Atrisaccharide will be evaluated. To improve antibody removal capacity, Atetrasaccharide will be evaluated as a possible antigen source, as well as mixtures of Atrisaccharide and Atetrasaccharide in order to remove antibodies that have specificity to either structure. Future work will also focus on evaluating the specific binding affinities and reaction rates between the mAbs and Atrisaccharide antigens conjugated to varying polyacrylamide spacer lengths to determine which antigen candidate has the highest antibody binding capacity.

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