Neutralization of Blood Group A-Antigen by a Novel Anti-A Antibody: Overcoming ABO-Incompatible Solid-Organ Transplantation

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Background. The major barrier to ABO-incompatible solid-organ transplantation is acute humoral rejection. It is known to be triggered by antidonor blood group A/B antibodies, which might bind to A/B-antigen on the endothelium of the graft. Various strategies to reduce antiblood group antibody by overcoming ABO-incompatible transplantation have been tried. However, antigen-suppressing procedures have not been performed.

Methods. We produced a novel anti-A antibody (K7508) by immunizing mice with salivary mucin of a blood group A individual, thereby clarifying that blood group A-antigen is expressed in endothelial cells of the liver. We investigated whether K7508 can mask A-antigen on the cells in vitro. Next, we immunized mice with A-antigen-expressing cells coated with K7508 or its Fab fragment, and measured anti-A antibody production in the mice.

Results. Blood group A-antigen-expressing cells, such as blood group A-red blood cells (A-RBCs) and A431 cells, coated with K7508 were not recognized by another anti-A antibody in flow cytometry, indicating that A-antigen was masked by K7508 in vitro. The A-antigen on the paraffin-embedded liver tissue was also masked by K7508. Furthermore, the production of anti-A antibody in mice immunized with A-antigen-expressing cells coated with K7508 or its Fab fragment was significantly suppressed compared to that in mice immunized with non-coated cells alone, indicating that A-antigen was neutralized by K7508 in vivo.

Conclusions. The neutralization of blood group antigen by antiblood group antibody and especially its Fab fragment might represent one strategy to overcome ABO-incompatible organ transplantation.

Keywords: ABO-incompatible organ transplantation, Neutralization of blood group antigen, Antiblood group antibody, Fab fragment.

(*Transplantation* 2008;85: 378–385)

Few alternative therapies exist for organ failure except organ transplantation, but immune response makes transplantation difficult (*1*). Rejection of organ grafts by immune response has become a serious problem. Using ABO blood group-identical or compatible grafts is one solution to this problem (*2*, *3*). ABO-incompatible solid-organ transplantation is generally avoided: outcomes generally are unfavorable because of humoral rejection (*4*, *5*). Humoral rejection associated with ABO-incompatible transplantation is triggered by antigen recognition of antidonor blood group A/B antibodies, which bind to blood group A/B-antigen on the endothelium of the graft. Subsequently, the complement and blood clotting cascades initiate (*2*, *5*, *6*). The classical pathway, which is a pathway of complement activation, is induced when the C1q binds to the Fc fragment of the antibody reacted with the antigen (*1*, *7*, *8*). In the graft organ, endothelial damage causes the formation of fibrin thrombi that destroy the microvasculature, leading to acute graft dysfunction and graft failure (*4*, *9*, *10*).

The preoperative anti-A/B antibody titer has been reported to be correlated with long-term graft survival in ABO-incompatible kidney transplantation (*11*). In ABO-incompatible liver transplantation, patients with high titers of antiblood group antibody are also at very high risk of graft failure (*12*, *13*). Various strategies have been developed to overcome ABO-incompatible transplantation, such as plasmapheresis, splenectomy, intensive immunosuppression, anti-CD20 monoclonal antibody, intravenous immunoglobulin, and intraportal infusion therapy, which have improved transplantation outcomes (*14–19*). These strategies, however, present the risks of infection and thrombosis. Furthermore, despite these various therapies, outcomes of ABO-incompatible transplantation are worse than those of ABO-identical or compatible transplantation. Recently, the strategy for overcoming ABO-incompatible transplantation is mainly the reduction of antibodies in plasma or the suppression of antibody pro-

This study was supported in part by a grant (17209044) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to M.K.) by the Osaka Cancer Research Foundation, Japan (M.K.K.), by the YASUDA Medical Foundation, Japan (Y.K.), Inoue Foundation for Science, Japan (Y.K.), and by Mitsubishi Pharma Research Foundation, Japan (Y.K.).

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Received 25 September 2007. Revision requested 16 October 2007.

Accepted 23 October 2007.

ISSN 0041-1337/08/8503-378

DOI: 10.1097/TP.0b013e3181612f84

duction. The outcome of ABO-incompatible transplantation might be more favorable if the blood group antigen on the graft organ was suppressed and the humoral rejection were controlled.

For use in this study, we produced a novel anti-A monoclonal antibody, designated as K7508, and performed A-antigen suppressing procedures. In vitro, A-antigen-expressing cells such as blood group A-red blood cells (A-RBCs) or A431 were coated with K7508 or its Fab fragment. Their reactivity to another anti-A antibody was measured using flow cytometry. In vivo, we immunized mice with A-antigen-expressing cells after coating them with K7508 or Fab fragment, and then measured the production of anti-A antibody in mice sera. This in vivo model using A-antigen-expressing cells might mimic solid organ transplantation.

MATERIALS AND METHODS

Animals

Female BALB/c mice were obtained (Charles River Japan, Inc., Kanagawa, Japan); they were used and maintained in a temperature-controlled room with alternating 12-hour light/dark cycles. The Animal Care and Use Committee of the National Institute of Advanced Industrial Science and Technology approved all mouse manipulations.

Cell Lines, Antibodies, and Tissue Microarrays

An epidermoid carcinoma cell line, A431, which is known to express blood group A-antigen, was obtained from Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan) (*20*). The A431 cells were cultured at 37°C in a

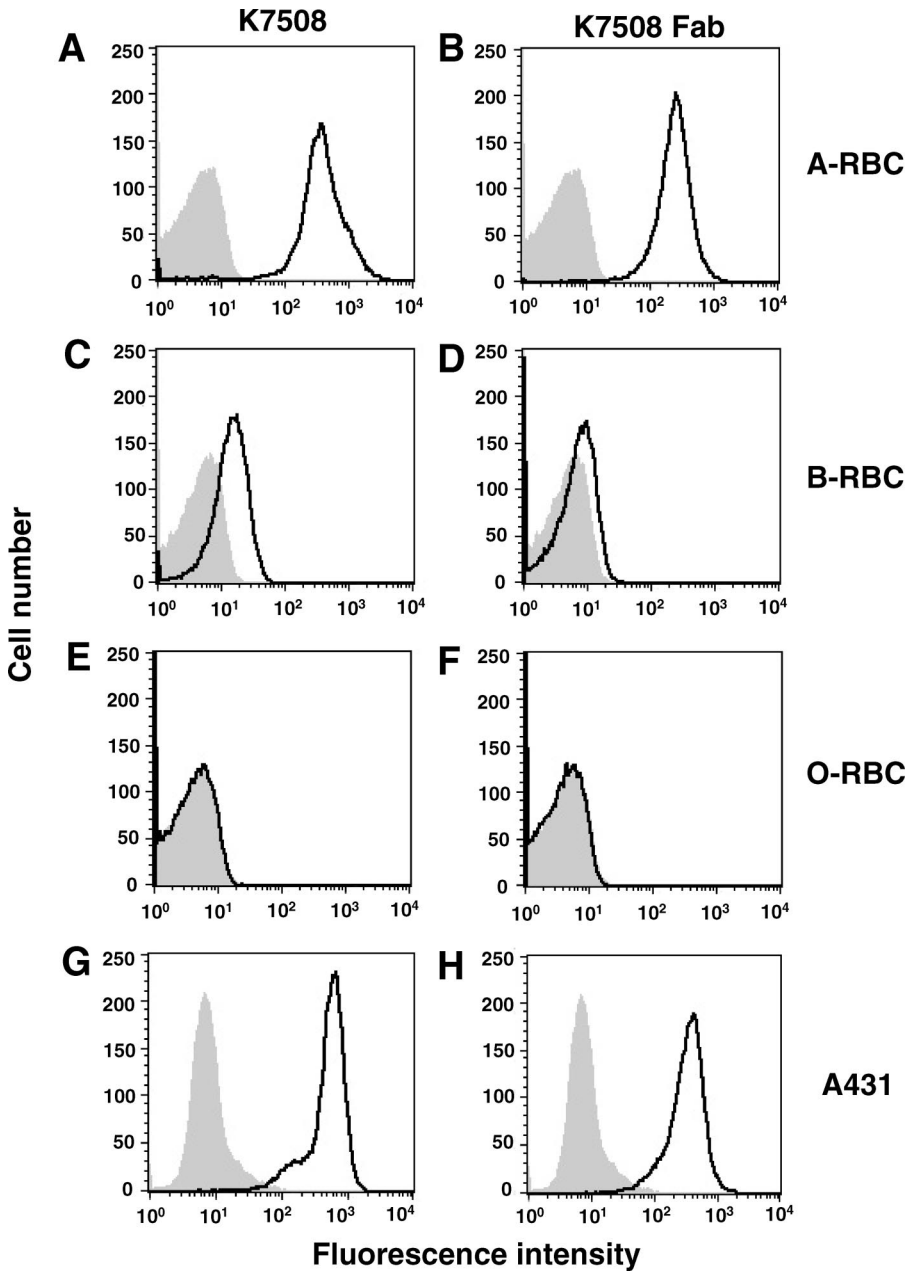

FIGURE 1. Flow cytometric analyses to measure the binding activity of the anti-blood group A antibody, K7508, or its Fab fragment (K7508Fab). The A, B, O-RBCs or A431 was reacted with 1 μg/mL K7508 or K7508Fab as the first antibody, and then reacted with 1/1000 diluted anti-mouse IgG Oregon Green as the secondary antibody. Bold lines show K7508 or K7508Fab. Shaded areas show PBS. A-RBCs were stained strongly using K7508 (A) and K7508 Fab (B). B-RBCs were stained slightly by K7508 (C) and K7508Fab (D). O-RBCs were not stained by K7508 (E) or K7508Fab (F). A431 were strongly stained by K7508 (G) and K7508Fab (H).

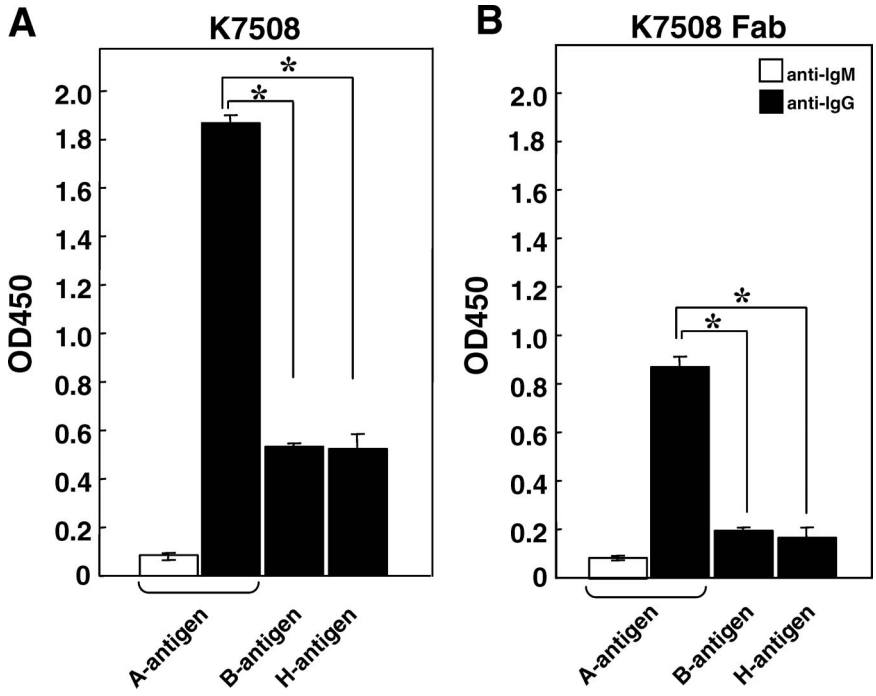

FIGURE 2. Binding assay of K7508 or its Fab fragment (K7508Fab) in ELISA. The ELISA plate wells were coated with 1 μg/mL A, B-trisaccharides, or H-disaccharides. Either K7508 (A) or K7508Fab (B) at a concentration of 0.1 μg/mL was used as the first antibody, followed by 1/1000 diluted anti-mouse IgG-HRP or anti-mouse IgM-HRP. The enzymatic reaction was conducted with a substrate solution containing TMB. After the reaction was stopped with 1 M H_2SO_4, the optical density was measured at 450 nm using an auto-plate reader. Data are the mean ± SD of three independent experiments. *$P<0.01$.

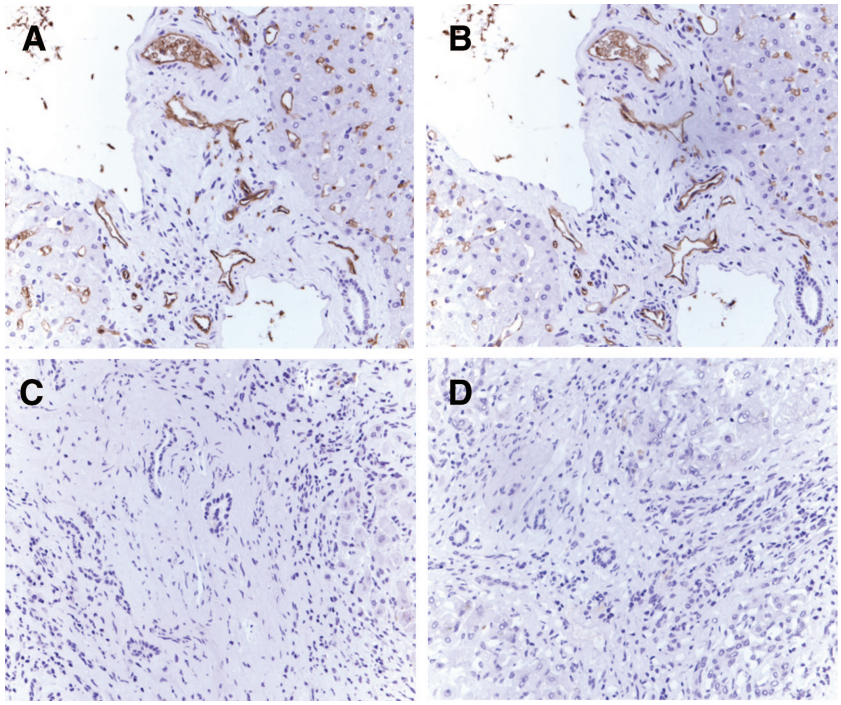

FIGURE 3. Immunohistochemical analysis of liver tissues using K7508 and its Fab fragment (K7508Fab). The K7508 (A, C, and D) and K7508Fab (B) were added to the liver sections, blood group A individual (A and B), blood group B individual (C), and blood group O individual (D), at a concentration of 0.05 μg/ml for 30 min at room temperature. Biotinylated secondary anti-mouse IgG was incubated for 30 min at room temperature followed by the peroxidase-conjugated biotin-streptavidin complex (Vectastain ABC Kit) for 30 min at room temperature. Color was developed using 3,3-diaminobenzidine tetrahydrochloride for 1 min. The sections were counterstained with Mayer's hematoxylin.

humidified atmosphere of 5% CO_2 and 95% air in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma, St. Louis, MO), 2 mM L-glutamine (Invitrogen Corp., Carlsbad, CA), 100 units/mL of penicillin, and 100 μg/mL of streptomycin (Invitrogen Corp.). From healthy volunteers, RBCs of blood groups A, B, and O were extracted. An anti-A monoclonal antibody (IgM) and rabbit anti-A polyclonal antibody were purchased (Wako Pure Chemical Industries Ltd., Tokyo, Japan), as were normal liver tissue microarrays (Cybrdi Inc., Frederick, MD).

Hybridoma Production

Female BALB/c mice (4 weeks old) were immunized by neck subcutaneous injections of the salivary mucin of the blood group A-individual with Adjuvant Complete Freund (Difco Laboratories, Detroit, MI) to obtain anti-A-antigen, which is attached to protein. One week later, secondary intraperitoneal immunization was performed. The booster injection was given intraperitoneally 2 days before spleen cells were recovered. The spleen cells were fused with

mouse myeloma P3U1 cells using polyethylene glycol (Mr 4,000); the hybridomas were grown in RPMI 1640 medium with hypoxanthine, aminopterin and thymidine selection medium supplement (Sigma). The culture supernatants were screened using ELISA for binding to the synthetic A-antigen.

Preparation of Fab Fragment

Papain digestion was performed to obtain the Fab fragment using Immobilized Papain (Pierce Biotechnology Inc., Rockford, IL). The 10 mg of anti-A antibody was added to a final concentration of 20 mM cystein·HCl and 10 mM EDTA. The 1.0 mL of sample was mixed with the 0.25 mL of Immobilized Papain and incubated overnight in a shaker at 37°C. After separating the immobilized enzyme from the product by 0.22-μm filter, the whole IgG that was not disassembled by the enzyme was removed using Amicon Ultra 100 K (Millipore Corp., Tokyo, Japan).

Flow Cytometry

The RBCs or A431 cells were incubated with purified monoclonal antibodies or mice sera (diluted in phosphate-buffered saline [PBS]) for 30 min at 4°C. After washing with PBS, diluted secondary antibodies (1:1000): fluorescein-conjugated goat IgG fraction to mouse IgM; μ chain-specific (MP Biomedicals, Solon, OH) or Oregon Green 488 goat anti-mouse IgG(H+L) (Invitrogen Corp.) were reacted for 30 min at 4°C. Fluorescence data were collected using a flow cytometer (FACS Caliber; BD Biosciences, Braintree, MA).

Enzyme-Linked Immunosorbent Assay (ELISA)

The A-trisaccharides conjugated to bovine serum albumin (BSA) (Calbiochem Novabiochem Corp., San Diego, CA), B-trisaccharides conjugated to BSA (Dextra Laboratories, Reading, UK), and H-disaccharides conjugated to BSA (Dextra Laboratories) were independently coated onto Nunc-immuno plates (Nunc, Roskilde, Denmark) at 1 μg/mL for 1 hr. After blocking with 1% BSA, the plates were incubated with purified monoclonal antibodies or mice sera for 1 hr. After washing with 0.05% Tween 20 in PBS, the plates were incubated with HRP anti-mouse IgG (Dako, Glostrup, Denmark) or peroxidase-conjugated affinipure donkey anti-mouse IgM, μ chain specific (Jackson Immuno Research Laboratories Inc., Suffolk, UK) for 1 hr. After subsequent washing, the enzymatic reaction was conducted with 1-Step Ultra TMB-ELISA (Pierce). After the reaction was stopped with 1 M H_2SO_4, the optical density was measured at 450 nm using an auto-plate reader. These reactions were performed with a volume of 50 μL at room temperature.

Immunohistochemistry

Immunohistochemical staining was performed using the avidin-biotinylated immunoperoxidase method. In brief, 5-μm sections were deparaffinized and rehydrated. All tissues were then exposed to 3% hydrogen peroxidase for 5 min. Then, K7508, its Fab fragment (K7508Fab), or rabbit anti-A polyclonal antibody (Wako Pure Chemical Industries Ltd.) was added to the sections for 30 min at room temperature. Biotinylated secondary anti-mouse IgG (Dako) or anti-rabbit IgG (Dako) was incubated for 30 min at room temperature followed by the peroxidase-conjugated biotin-streptavidin complex (Vectastain ABC Kit; Vector Laboratories Inc., Burlingame, CA) for 30 min at room temperature. Color was developed using 3,3-diaminobenzidine tetrahydrochloride tablet sets (Dako) for 1 min. The sections were counterstained with Mayer's hematoxylin.

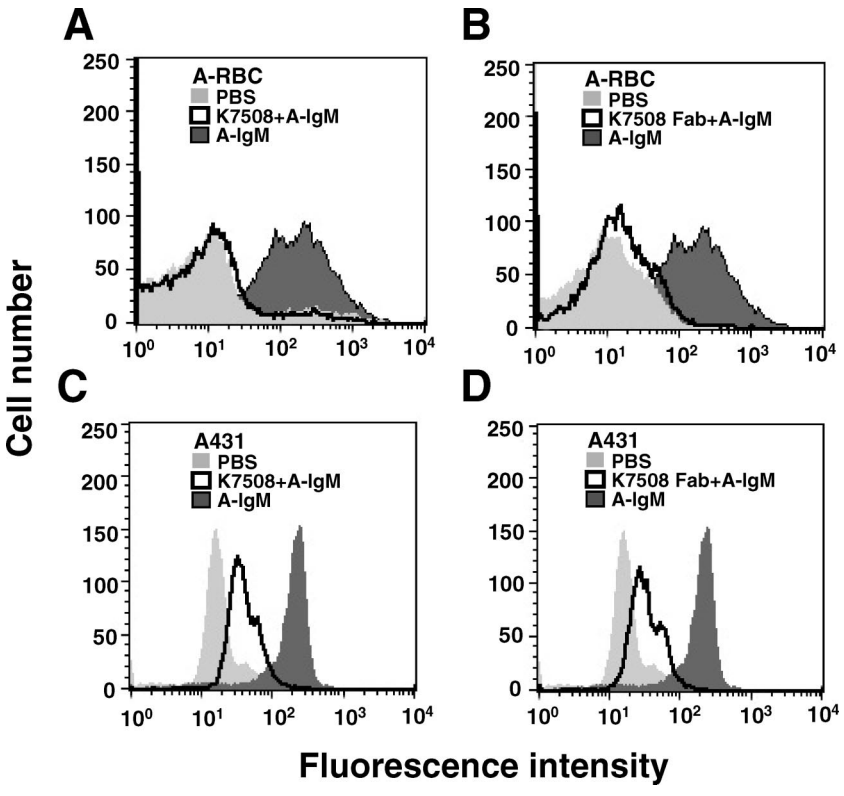

FIGURE 4. Blood group A-antigen was masked using K7508 or its Fab fragment (K7508Fab) in flow cytometry. First, 1×10^6 cells of A-RBCs were coated with 3 μg of K7508 (A) or 10 μg of K7508Fab (B); alternatively, 1×10^5 cells of A431 were coated with 100 μg of K7508 (C) or K7508Fab (D). Then, another anti-A antibody (A-IgM) diluted 1/100 was used as the first antibody, followed by anti-mouse IgM-FITC, diluted 1/1000, as the secondary antibody. Bold lines show cells coated with K7508 or K7508Fab, followed by A-IgM. Dark shaded areas show A-IgM. Light shaded areas show PBS as a negative control.

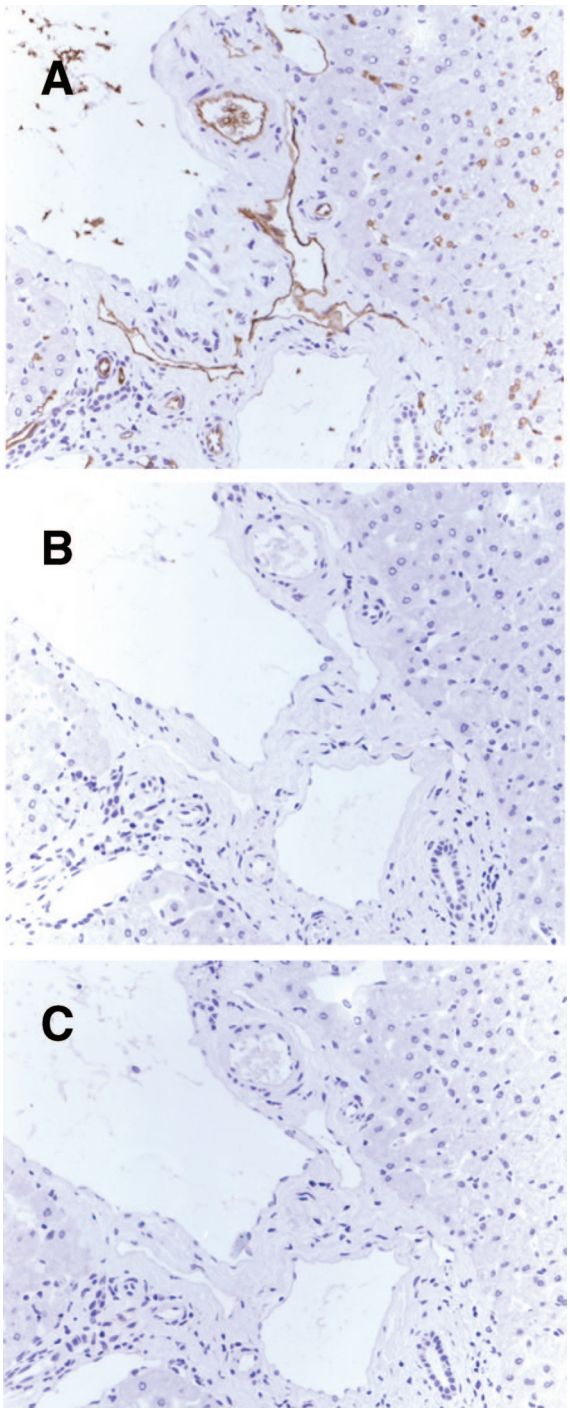

FIGURE 5. Blood group A-antigen was masked using K7508 or its Fab fragment (K7508Fab) in immunohistochemical analyses of liver tissues. Rabbit anti-A antibody stained endothelial cells of liver (A). Either K7508 (B) or K7508Fab (C) was added to the liver sections of a blood group-A individual at a concentration of 100 μg/ml for 30 min at room temperature. Then, 1/1000 diluted rabbit anti-A antibody was reacted for 30 min. Biotinylated secondary anti-rabbit IgG was incubated for 30 min at room temperature, followed by the peroxidase-conjugated biotin-streptavidin complex (Vectastain ABC Kit) for 30 min. Color was developed using 3,3-diaminobenzidine tetrahydrochloride for 1 min. The sections were counterstained with Mayer's hematoxylin.

Immunization of A431 Cells

The A-antigen-expressing cells, A431, were coated with K7508 or its Fab fragment for 30 min at 4°C. Mice were immunized by intraperitoneal injections of A-antigen-expressing cells mixed with the Imject Alum (Pierce).

Statistical Analyses

Results are expressed as the mean ± standard deviation. Student's t test was used to determine significance among the groups. A value of $P<0.05$ was considered significant.

RESULTS

Specificity Definition of a Novel Monoclonal Antibody to the Blood Group A-Antigen

After generating a monoclonal antibody, K7508, to the blood group A-antigen, we first characterized its specificity to the A-antigen by flow cytometric analysis. Both K7508 and its Fab fragment (K7508Fab) demonstrated specificity for the blood group A-antigen by strong binding with A-RBC (Figs. 1A and 1B) as well as A431, a cell line that expresses the blood group A-antigen (Figs. 1G and 1H). Specificity of K7508 or K7508Fab for the A-antigen was shown by the lack of reactivity with O-RBC (Figs. 1E and 1F) and weak reactivity with B-RBC (Figs. 1C and 1D). The specificities of K7508 and K7508Fab were also characterized by ELISA (Fig. 2). The data also show that specificity is directed to the blood group A-antigen (trisaccharide) and not to the B- or O-antigens (tri- or disaccharides, respectively). K7508 is of the IgG1 subclass (data not shown). Immunostaining studies revealed that K7508 reacted strongly with RBCs and endothelia of capillaries and arteries of liver from a blood group A individual but did not react with those tissues from B or O-individuals (Fig. 3).

Blood Group A-Antigen Neutralization by Anti-A Antibody (K7508) in Vitro

We next investigated whether A-antigen on cells were neutralized by K7508 in flow cytometric analyses or immunohistochemistry. Results of flow cytometric analyses indicated that A-RBCs coated with K7508 were not detected by another anti-A antibody (A-IgM): A-antigen on A-RBCs was masked by K7508 in vitro (Fig. 4A). In the same way, the reactivity of A-IgM was diminished after A431 cells were coated with K7508 (Fig. 4C). It was noteworthy that K7508Fab also inhibited A-IgM-binding to A-antigen on A-RBCs and A431 (Figs. 4B and 4D), indicating that K7508Fab had similar binding activity to that of K7508 and masked A-antigen on the cells. In immunohistochemistry, polyclonal anti-A antibodies recognized RBCs and endothelia of capillaries and arteries of blood group-A liver (Fig. 5A). By contrast, the liver tissues coated with K7508 or K7508Fab were not stained by polyclonal rabbit anti-A antibody (Fig. 5B and 5C), indicating that A-antigen on the endothelia of the liver tissues was masked by K7508 or K7508Fab. These results suggest that K7508 and K7508Fab masked A-antigen on tissues and might be useful for therapeutic use in vivo.

Suppression of Anti-A Antibody Production in Mice by Neutralization of A-Antigen With K7508 or K7508Fab

We next investigated whether K7508 or K7508Fab suppress the anti-A antibody production by neutralizing A-antigen in

vivo. We first immunized mice with A431 cells after coating with K7508 by intraperitoneal injections. This in vivo model might mimic organ transplantation because the A-antigen of the donor graft induces the production of anti-A antibodies. Flow cytometry (Fig. 6A) showed that sera from mice immunized by A431 coated with K7508 (S-A431-K7508) reacted to A-RBCs more weakly than sera from mice immunized with non-coated A431 (S-A431), suggesting that S-A431-K7508 contained a lower concentration of anti-A antibodies. These results indicate that K7508 neutralizes A-antigen on A431 cells and that the production of mouse anti-A antibodies is inhibited in vivo. Furthermore, sera from mice immunized by A431 coated with K7508Fab (S-A431-K7508Fab) reacted weakly to A-RBCs (Fig. 6B), indicating that K7508Fab also possesses neutralizing activity against A-antigen.

We next investigated the concentrations of mouse anti-A antibodies in S-A431-K7508, S-A431-K7508Fab, and S-A431 using ELISA with A-trisaccharides (Fig. 6C). The concentration of anti-A antibodies increased gradually in S-A431 for three weeks. In contrast, that of S-A431-K7508 slightly increased for one week, and then stopped increasing from one week to three weeks. Similarly, the concentration of anti-A antibodies of S-A431-K7508Fab stopped increasing after one week. These results suggest that A-antigen on A431 cells was neutralized by K7508 or K7508Fab.

DISCUSSION

In this study, we produced a novel antiblood group A antibody, K7508, for developing a novel therapeutic strategy for the ABO-incompatible organ transplantation. The major barrier to ABO-incompatible organ transplantation is humoral rejection, which is known to be triggered by antigen-antibody complex reaction (*2*, *5*, *6*). For that reason, the reduction of antibodies in plasma or the suppression of antibody production has been performed to overcome ABO-incompatible transplantation (*15–17*). However, to date, the suppression of blood group antigen has not been performed

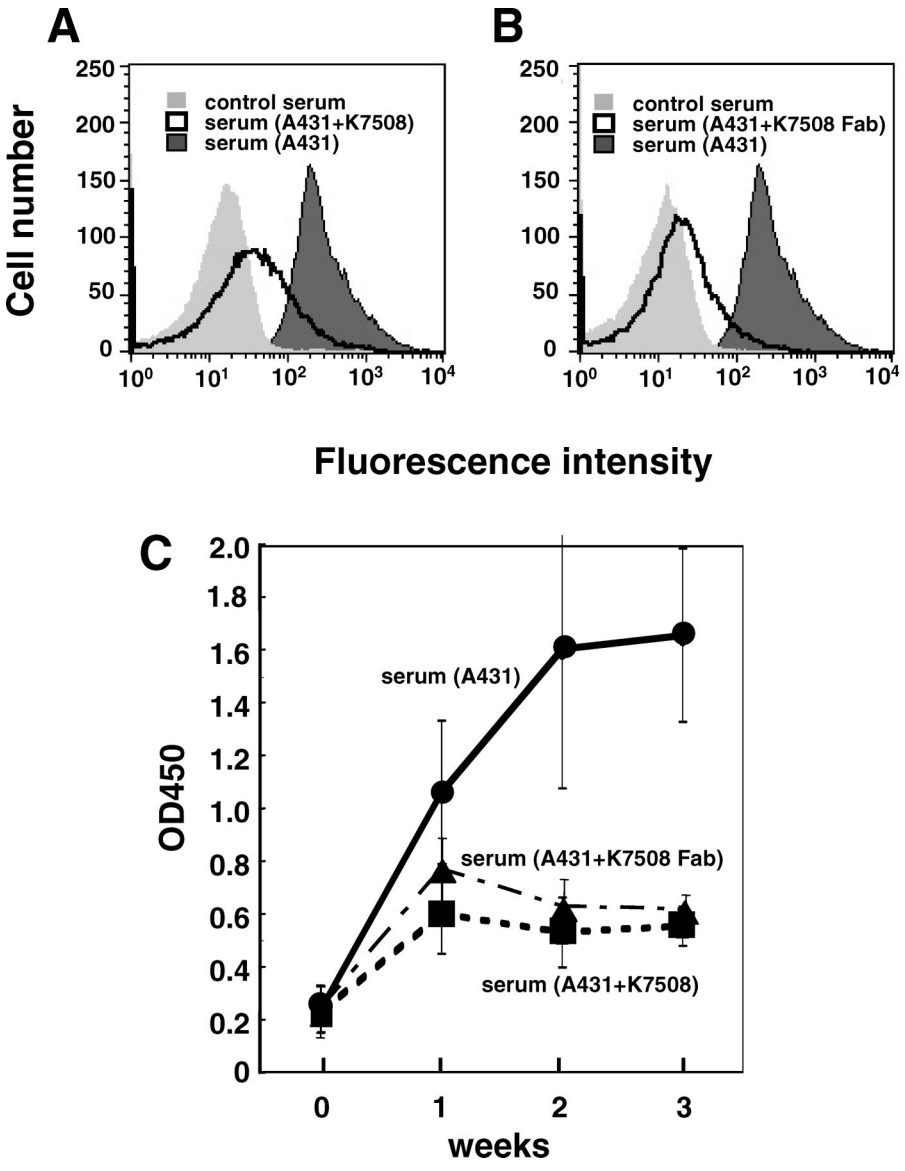

FIGURE 6. Neutralization of blood group A-antigen on A431 cells using K7508 and its Fab fragment (K7508Fab) in vivo. (A, B) Flowcytometric analyses show that mice were immunized with A431 cells coated with K7508 or K7508Fab (bold lines), or noncoated A431 cells (dark shadowed areas). The A-RBCs were reacted with 1/10 diluted sera from immunized mice as the first antibody, followed by 1/1000 diluted anti-mouse IgM-FITC as the secondary antibody. Light shaded areas show sera of mice without immunization as a negative control. (C) Five mice were immunized twice (day 0 and day 7) with A431 cells coated with K7508 (squares), K7508Fab (triangles), or non-coated A431 cells (circles). The ELISA plate wells were coated with 1 μg/ml A-trisaccharides. Then, 1/10 diluted immunized mice sera were used as the first antibody; subsequently, 1/1000 diluted anti-mouse IgM-HRP were used as the secondary antibody. The enzymatic reaction was conducted with a substrate solution containing TMB. After the reaction was stopped with 1 M H_2SO_4, the optical density was measured at 450 nm using an autoplate reader. Data are the mean ± SD of three independent experiments. *$P<0.01$.

clinically, probably because in vivo study about suppression of blood group antigen is very difficult. To the best of our knowledge, this study is the first one to use in vivo model which mimics ABO-incompatible transplantation.

In vitro study, we performed neutralizing experiments by K7508 using both A-antigen-expressing cells such as A-RBC or A431 and human liver tissues. A-antigen-expressing cells are used for application to in vivo study that mimics ABO-incompatible transplantation; on the other hand, human liver tissues were used for discussing the clinical application. A-antigen on A-RBC or A431 were completely masked by anti-A antibody, K7508 (Fig. 4), suggesting that K7508 is also applicable to in vivo study: whether antibodies against A-antigen-possessing graft are suppressed or not. K7508 reacted with A-antigen stronger than other anti-A antibodies in ELISA (data not shown). Furthermore, we speculate that these anti-A antibodies recognize the similar epitope of A-antigen; therefore other anti-A antibodies did not react with A-antigen after epitope of A-antigen was blocked by K7508. Immunohistochemical analyses showed that A-antigen is highly expressed in endothelia of capillaries and arteries of blood group-A liver (Fig. 3). Importantly, K7508 completely blocked the reactivity of sequentially applied other anti-A antibodies against endothelia of capillaries and arteries (Fig. 5). An in vivo study with the use of human liver tissues is ethically difficult to perform; therefore, we moved on to in vivo study using A-antigen-possessing cells.

The Fc portion of IgG is involved in antibody-dependent cell-mediated cytotoxicity and complement-dependent cell cytotoxicity. The classical pathway, which is one of complement activation, is triggered by the binding of the complement component C1q to the antigen-antibody complex. In the previous study, mouse RBCs coated in vitro with $F(ab')_2$ fragment of mouse RBC-specific antibody, when transfused into mice, survived longer in circulation than RBCs coated with whole IgG of mouse RBC-specific antibody (*21*). Therefore, we prepared the Fab fragment of K7508 (K7508Fab) to prevent the antibody-dependent cell-mediated cytotoxicity or complement-dependent cell cytotoxicity by K7508 for in vivo study. As shown in Figures 1–3, K7508Fab was also useful for flow cytometry, ELISA, and immunohistochemistry. As shown in Figure 6, when A-antigen-expressing cells were coated with K7508Fab, the production of anti-A antibody was also reduced in vivo. These findings suggest that A-antigen was neutralized by K7508Fab, and that A-antigen lost its antigenecity. In vitro study, K7508Fab completely blocked the reactivity of sequentially applied another anti-A antibodies against endothelia of capillaries and arteries (Fig. 5). Taken together, K7508Fab may be clinically applicable in ABO-incompatible organ transplantation.

Using A_2 donors, who have less A-antigen than A_1 donors, has brought success in ABO-incompatible liver transplantation and kidney transplantation (*22–24*). Fishbein et al. reported that transplantation of blood group A_2 livers into blood group O recipients is safe and can be performed without graft loss and without regard to anti-A titer level (*24*). They concluded that the transplantation of A_2 livers into O recipients can partially compensate for the more frequent use of O livers in recipients from other blood groups. Consequently, it is not necessary to neutralize blood group antigen completely, and masking the part of blood group antigen might turn around the outcome of ABO-incompatible transplantation. Moreover, the feature of this method is the specific treatment against blood group antigen. Therefore, broad and intensive immunosuppression, which causes severe infection and posttransplant lymphoproliferative disorder, might not be necessary (*25*, *26*). In this study, we used only anti-A-antigen antibodies; however, we are also planning to produce anti-B-antigen antibodies, which may lead to a clinical use.

In conclusion, we produced a novel anti-A antibody: K7508. Production of anti-A antibody was suppressed significantly in vivo when blood group A-antigen-expressing cells were coated with K7508Fab. In the future, this neutralizing procedure might become an effective strategy to control humoral rejection related to ABO-incompatible solid organ transplantation.

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