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The Sharing of Target-Epitopes between Human Anti-ABO Hemagglutinating and Anti-Pig (Xeno) Endothelium or Anti-Pig Thyroglobulin Antibodies

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To select congenial pairs between donor-pig and recipient-human for the future xenotransplantation, the levels of xeno-IgM natural antibodies (NAb) were analyzed in healthy subjects and hemodialysis patients by ELISA tests, which target swine-derived crude endothelial cells (P16N) or proteins (thyroglobulin; TG).
The total IgM concentration was lower in hemodialysis patients than in healthy subjects, but there was no difference in IgM NAb titer between the two groups. Individuals with non-B blood types (A, O) exibited significantly higher IgM NAb titer compared with those with B blood types (B, AB). A blood type individuals showed higher killing activity against P16N than those with B, AB or O types with a statistical significance.
Sera from A blood type, after being absorbed with red blood cells (RBC) from B blood type, decreased their IgM titer against TG to the level of sera from B blood type. Meanwhile, sera from A blood type significantly decreased hemagglutinin titer against B-RBC after passage through a TG-coated affinity column.
We conclude that human anti-B-RBC and anti-Pig xeno NAb have certain common binding epitopes, which might be a branched B carbohydrate structure.

Key words : Xenotransplantation, Hyperacute rejection, Galα1,3 Galactose, Natural antibodies, ABO blood types

INTRODUCTION

Due to the shortage of human organs for transplantation, the usage of xeno-organs has been advocated, and has already been tried in recent clinical situations [1]. However, the major obstacle for xeno-organ transplantation is hyperacute rejection, which is mainly caused by naturally occurring antibodies (NAb). It was reported that human anti-xeno NAb react with Gal α1,3 Galactose (αGal) moieties (anti-αGal NAb), which are expressed particularly in intravascular endothelium [2]. Pigs have been considered to be the candidates for donors; they also have these carbohydrate antigen on cellular surfaces [3, 4]. It is widely recognized that humans have NAb against sugar chains of red blood cells (RBC) (anti-A or anti-B), and not IgG, but IgM constitutes a major part of those NAb. Quite similarly, anti-xeno NAb is constituted mainly by immunoglobulin M type [5], and is suggested to have a common origin or function with anti-RBC NAb [6]. They are considered to be generated by contact with bacterial wall antigens, which mimic such αGal or human blood moieties [7, 8], and 1 % or 0.25 % of circulating B lymphocytes

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produce anti-Gal NAb or anti-A, B NAb, respectively [9].

Previous studies reported variability in the level of anti αGal NAb among normal volunteers [10-13]. Galili *et al.*, for the first time, demonstrated the crossreactivity between natural anti-Gal and anti-blood B antibodies [10]. McMorrow *et al.* stated that IgG anti-αGal NAb is higher in sera samples from non-B antigen expressing (A, O) compared with B expressing donors (B, AB) [14]. They assumed that the increase was due to the cross-reaction of NAb against αGal and blood B antigens, which differ only in the presence of a fucosyl side chain. Although they did not find any significant difference between B and non-B groups for IgM binding to αGal, the sample number of their study seemed to be too small (12 cases for each RBC type) for a final conclusion.

In the present study, the levels of xeno-IgM NAb of normal subjects and patients undergoing hemodialysis treatment were examined by ELISA, which targeted both swine-derived crude endothelial cells and purified protein antigens (thyroglobulin; TG). The present studies were conducted using a larger scale of samples (121 normal volunteers and 72 hemodialysis patients) than those conducted by McMorrow. Further, the sharing of target epitopes between anti-xeno and anti-blood B type NAb was proved by absorption tests.

MATERIALS AND METHODS

Samples

Human sera samples were collected from normal healthy volunteers (n = 121) and from chronic renal failure patients (n = 72) treated by hemodialysis (HD) at the Kidney Center of Tokai University Hospital after obtaining informed consent. The samples were stored at − 80 ˚C, and heat inactivated (56 ˚C, 40 minutes) for ELISA. Porcine aortic endothelial cells, P16N, were donated by Prof. J. F. Platt (Dept. of Surgery, Mayo Clinic, Minnesota) and maintained in DMEM medium with 10 % FCS. Thyroglobulin was purchased from Sigma Chemical Co (St. Louis, MO).

TG-coated column was made as follows; 10 mg of TG was dissolved in 3 ml of 0.1 M borate buffer. After packing in cellulose tube (SANKO Co. Ltd. Tokyo, Japan), it was dialyzed in 0.1 M borate buffer at 4 ˚C overnight. CNBr-Sepharose 4B (Pharmacid Biotech, Uppsala, Sweden) gels, after activation in 0.1 M borate buffer and washing with 1 mM HCl, were packed in a plastic column, to which the dialyzed TG was applied and incubated for 2 hours at room temperature.

Quantitation of xeno-NAb to P16N by ELISA

The binding of IgM to P16N was analyzed by cellular ELISA as previously described [5]. Briefly, 96-well flat-bottom plates (Flow Lab. Inc., McLean, Virginia) were coated with P16N, fixed with 0.1 % glutaraldehyde, and then frozen at − 80 ˚C. After thawing, the plates were blocked with 3 % BSA/PBS (-) for 1 hour, and serially diluted (1:2~1:4) human sera samples were applied. After 3 hours of incubation at 37 ˚C, the plates were washed 3 times with PBS (-), and 1:5000 diluted alkaline phosphatase-conjugated goat Ab specific for human IgM (Sigma) was added. After incubation for 1 hour at room temperature, the cells were washed 5 times with PBS (-), and 100 μl of a developing solution consisting of p-nitrophenyl phosphate in a 100 mM diethanolamine buffer was added. The absorbance at 405 nm was read with a microplate reader (Molecular Devices, Sunnyvale, CA). The titer of a randomly selected serum sample from healthy control was arbitarily determined as 100 U/ml.

Quantitation of xeno-NAb to thyroglobulin by ELISA

The amount of xeno-reactive IgM in sera was also quantitated by ELISA using immobilized porcine TG as a substitute for endothelial cells. Porcine TG was diluted at a concentration of 0.25 mg/ml with carbonate-bicarbonate buffer, and coated onto a Nunc plate. Thereafter the protocol was exactly the same as that of cellular ELISA, as described above.

Cytotoxic assay

The cytotoxic assay against P16N was performed by a standard ^{51}Cr-release assay. Briefly, P16N cells were harvested, and labelled with ^{51}Cr for 1 hour at 37 ˚C. After washing 3 times, they were seeded on 96-well round-bottom plates (IWAKIGLASS Co., Ltd., Chiba, Japan). Human sera from healthy volunteers or patients were added at a dilution of 1:4 without heat inactivation. The wells were incubated at 37 ˚C for 4 hours, and centrifuged at 1500 rpm for 5

minutes, and from each well one half of the medium was collected, and then counted by a gamma-counter (Beckman, Irvine, CA.).

Hemagglutination

Type A or B blood RBCs were centrifuged at 1500 rpm for 5 minutes. The sedimented RBCs were washed twice and then diluted in the saline buffer to make a 0.3 % solution. Each sample (50 μl) was seeded on a 96-well round-bottom plate, on which serially diluted heat-inactivated sera were added. The reaction was continued at 37 ˚C for 60 minutes, and the formation of agglutination was judged using a microscope.

Absorption tests

The results of the following absorption tests were evaluated:

1. Type A or B blood sera (each, n = 10) were mixed with the same volume of 1:10 diluted type B or A RBCs, respectively. The incubation was conducted for 12 hours at 4 ˚C, then absorbed sera were harvested after centrifugation (1500 rpm, 5 minutes) and examined by both for hemagglutination and anti-TG ELISA tests.
2. Type A or B blood sera were applied to TG-column and incubated for 2 hours at room temperature. the absorbed sera were examined by both for hemagglutination and anti-TG ELISA tests.

Statistical analysis

Statistical significance of differences in the mean values among groups was determined using the Mann-Whitney U test. Linear regression analysis was performed using the Stat View-4.02 for Macintosh software.

RESULTS

Distribution of xeno-reactive NAb against porcine endothelium or protein

The titers of anti-pig, xeno NAb were widely distributed both in healthy individuals (n = 121) and patients (n = 72). The titer against endothelial cells, P16N, showed 40.3 ± 4.8 U/ml of mean (min. 1.60, median 20.90, max. 306.50) in the healthy group, but only 32.6 ± 4.2 U/ml (min. 1.10, median 15.75, max. 157.80) in the patient group; the titer against TG showed 63.3 ± 4.0 U/ml (min. 3.80, median 48.90, max. 183.20) in the healthy, whereas it was 66.1 ± 8.6 U/ml (min. 1.60, median 27.15, max. 348.00) in the patient group (Fig. 1, a-d). Both groups were combined and plotted as shown in Figure 2. There was a significant corelation between the two parameters ($n = 193$, $r = 0.63$, $p < 0.01$).

Although there was a satistically significant difference in the total IgM concentration between the two groups (healthy 211.1 ± 7.7 mg/dl; patient 154.8 ± 10.6 mg/dl, $p = 0.002 < 0.01$), no difference in the anti-xeno NAb titer was observed.

Difference in NAb titer among different RBC types.

Next, the titer was plotted separately for each blood type group, as shown in Figure 3. In P16N ELISA, non B blood types, A and O individuals, from both healthy (3a) and patient groups (3b), showed significantly higher titer than B blood types, B and AB. The same result was also found in TG ELISA (healthy = 3c, patients = 3d).

Difference in cytotoxic NAb against porcine endothelial cells

The correlation between xeno-NAb titer and cytotoxic reactivity against P16N was studied in 95 (50 = healthy, 45 = patients) samples with a sufficient volume for the assay (Fig. 4). The r-value (0.594) showed a statistical significance ($p < 0.01$). RBC A type showed the highest killing activity, which was significantly higher compared to the other RBC types (Fig. 5; healthy = a, patients = b).

Absorption tests

Ten representative sera from blood type A and B healthy groups were mixed with B and A RBCs, respectively, in order to absorb hemagglutinating NAb; these were then assayed for xeno-NAb by TG-ELISA. In all blood type A sera, the xeno-NAb titer was diminished (mean reduction of 62.4 %), to the comparable level as shown by type B sera. However, type B sera did not change the xeno-NAb titer after absorption (Fig. 6a).

In contrast, when sera were firstly absorbed for xeno-NAb on a TG-coated column, only blood type A sera showed a decrease in anti-B titer (mean reduction of 86 %), whereas the titer in type B sera did not change at all (Fig. 6b).

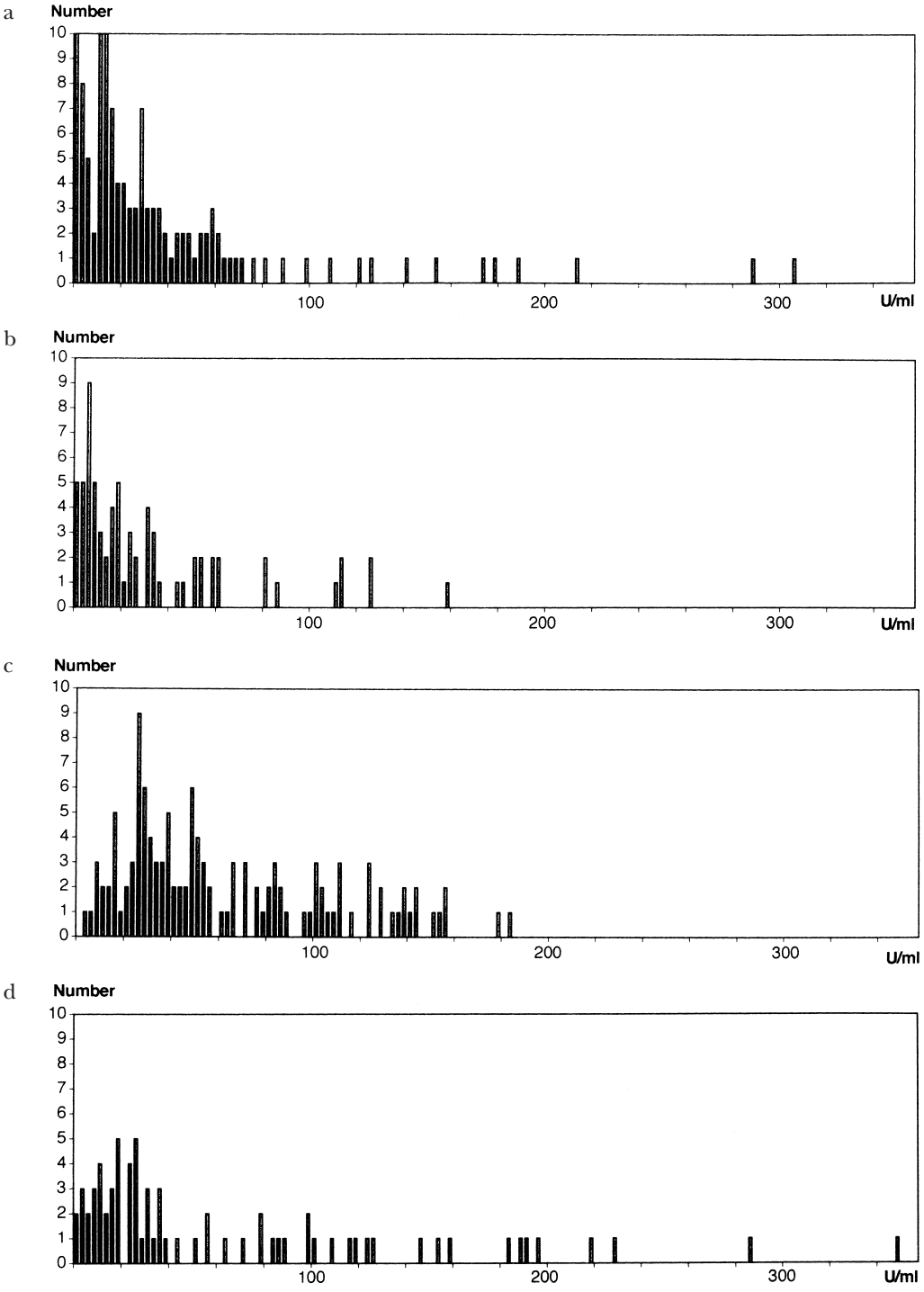

Fig. 1 The levels of anti-pig, xeno-natural IgM antibody (NAb) among healthy (n = 121), and hemodialysis patients (n = 72). Sera were tested for the titer of NAb by ELISA, which used pig-derived endothelial cells (P16N) (healthy = a, patients = b) or proteins (Thyroglobulin; TG) (healthy = c, patients = d), as target antigens. The titer of a randomly selected serum from a healthy control was determined as 100 U/ml. The mean values were as follows; Anti-P16N, 40.3 ± 4.8 and 32.6 ± 4.2 U/ml, in healthy and patient groups, respectively; Anti-TG, 63.3 ± 4.0 and 66.1 ± 8.6 U/ml, in healthy and patient groups, respectively.

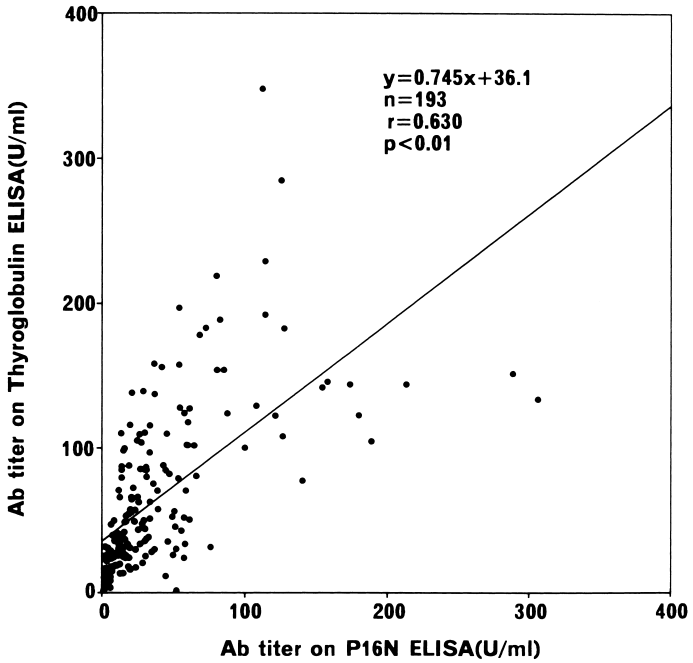

Fig. 2 The correlation between anti-P16N and anti-TG xeno-NAb titer. There was a significant correlation between the two parameters ($n = 193$, $r = 0.63$, $p < 0.01$).

DISCUSSION

The major epitope on the surface of porcine endothelium recognized by human xeno-NAb is Galα(1-3) Gal (1-4) GlcNAcR [2, 15]. It is expressed not only on endothelial cells but also on tissue-derived glycoproteins, such as thyroglobulin, fibrinogen or immunoglobulin G [16]. Several assays, such as ELISA, flow cytometry, or western-blotting, which detect anti- α Gal NAb have been developed [5, 12, 17]. McMorrow *et al.* stated that αGal-reactive human IgG or IgM constitutes 1.0−2.4 % or 3.9 to 8.0 % of total serum IgG or IgM, respectively [13]. Parker *et al.* reported that not IgG but IgM is the major component of such xeno-NAb [5], and they proposed that such IgM xeno-Nab and isohemagglutinins, anti-A or B RBC antibodies, have a similar origin and function [6]. They are present at a similar concentrarion in human sera and vary widely in the human population [11-13]. In the present study, anti-pig IgM NAb were analyzed by ELISA using porcine endothelial cells and thyroglobulin. Against both targets, anti-xeno NAbs were widely distributed as reported previously, and those from non-B blood types (A, O) have significantly higher values than those from B types (B, AB).

The cross-reactivity between blood B terminal carbohydrate and widely αGal has been suggested as both antigens only differ by the presence of a fucosyl epitope. There are several conflicting data concerning titers of xeno-NAb and blood types. Gautreau et al. described that anti-pig hemaggulutinin is decreased in sera of blood AB group [18]. They also showed that human serum of A blood group containing anti-B hemagglutinin alloantibody was completely absorbed by pig red blood cells. In contrast, anti-pig xeno hemagglutinating NAb was not reduced even after it was absorbed by human AB RBC. First, this indicates that the epitopes which anti-B alloantibody recognizes, are expressed quite similarly on pig RBC as on human B-RBC. A common branched B should be such an epitope based on the structual comparison of oligosaccharide of B-RBC and α Gal. Secondly, anti-pig xeno NAb responds to determinants other than branched B, as is

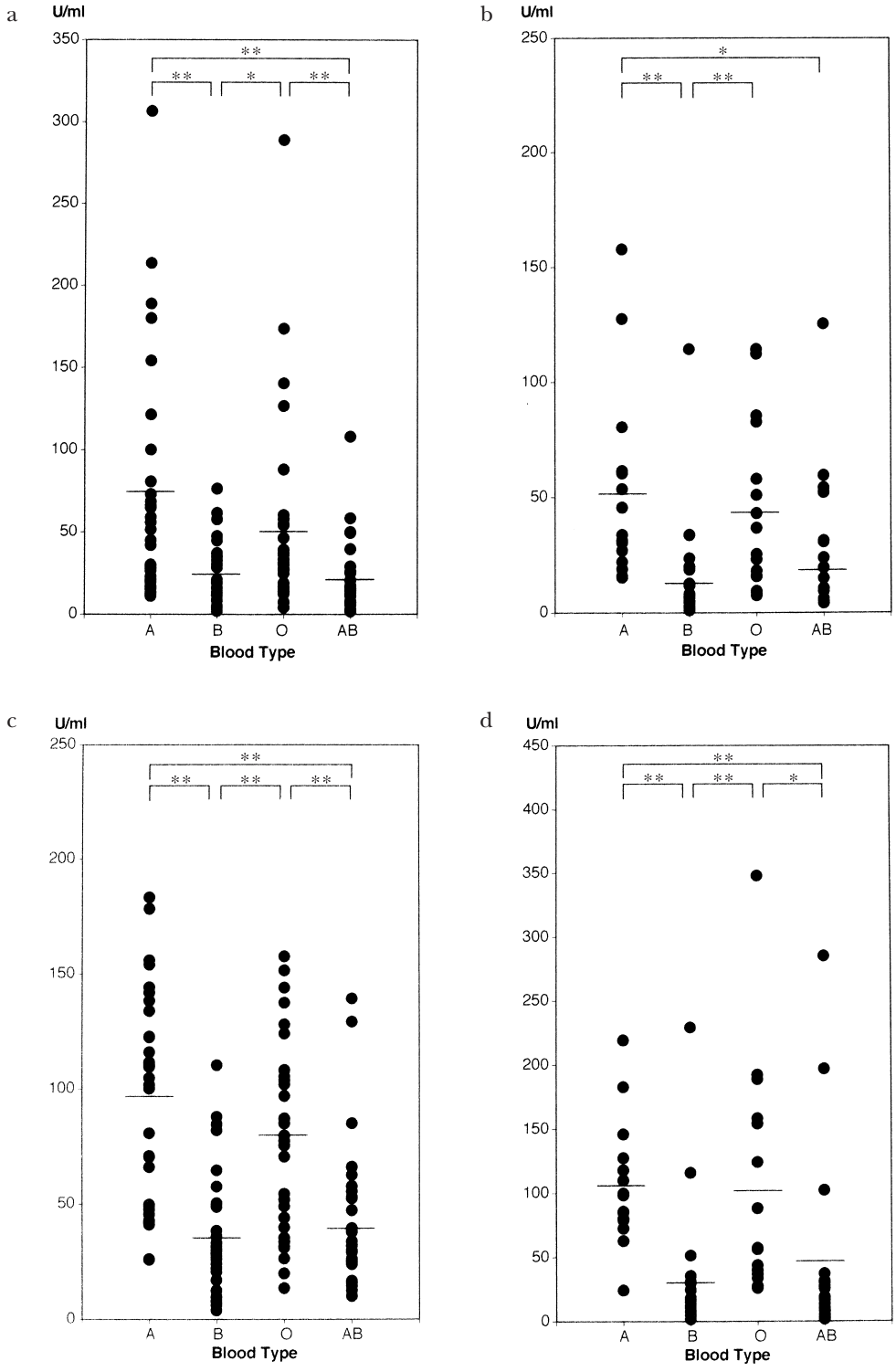

Fig. 3 The difference of NAb titer among different red blood cell (RBC) types (P16N; healthy = a, patients = b, TG; healthy = c, patients = d). Statistically significant differences are indicated by an asterisk (* = $p < 0.05$, ** = $p < 0.01$).

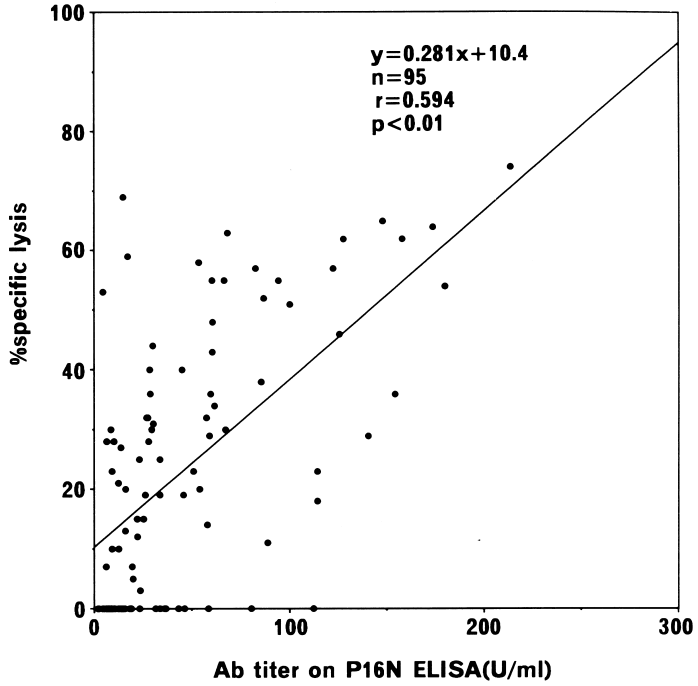

Fig. 4 The correlation between anti-NAb titer and cytotoxic activities against P16N in 95 samples (50 from healthy and 45 from patients). There was significant correlation between the two parameters ($r = 0.594$, $p < 0.01$).

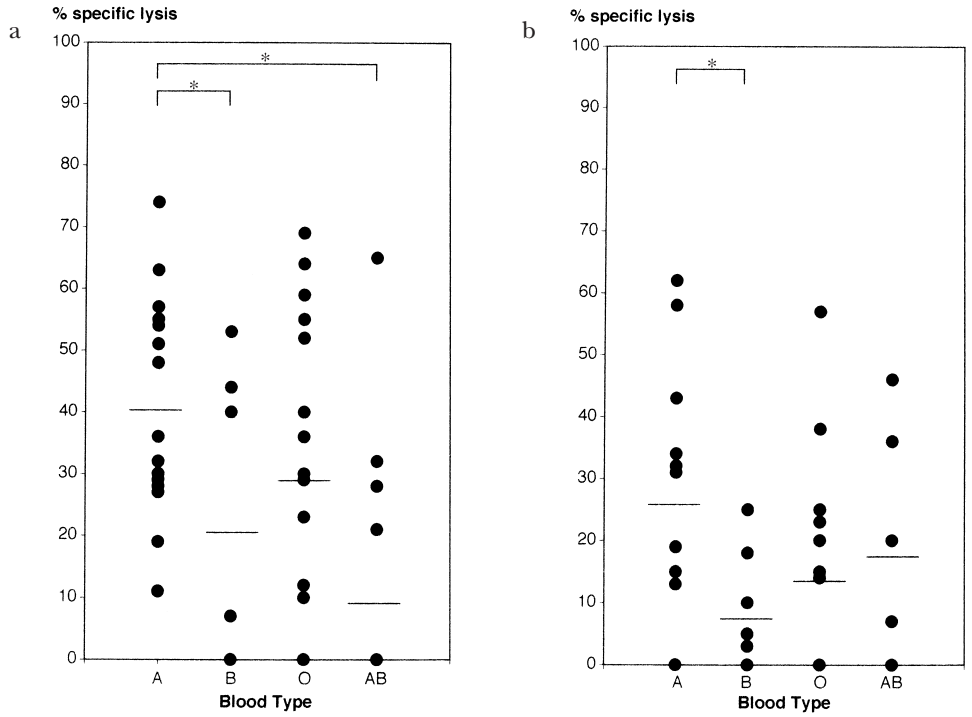

Fig. 5 The difference in cytotoxic activity against P16N among different red blood cell (RBC) types (healthy = a, patients = b). Statistically significant differences are indicated by an asterisk (* = $p < 0.05$, ** = $p < 0.01$).

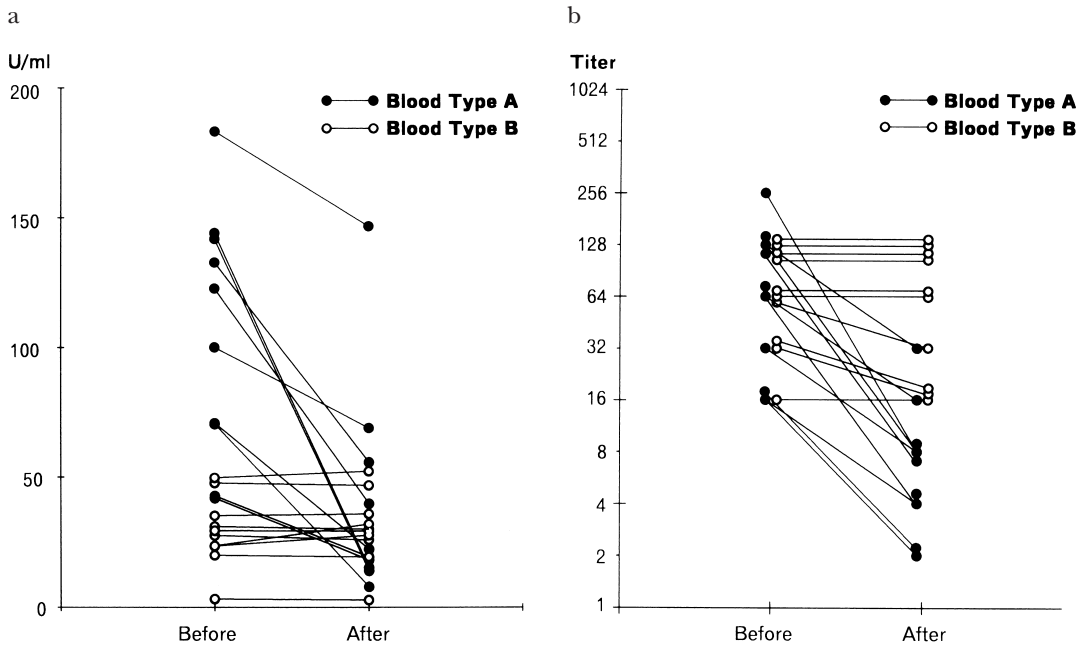

Fig. 6 The change of xeno-NAb titer (a) or hemagglutinating titer (b) after the absorption.
a: Ten each of blood type A or B sera from healthy volunteers were mixed B or A RBCs, respectively, to be absorbed for hemagglutinating NAb, and then assayed again for xeno-NAb by TG-ELISA. All blood type A sera diminished their xeno-NAb titer (mean reduction of 62.4 %), to the comparable level as shown by type B sera. In contrast, type B sera did not change xeno-NAb titers after absorption.
b: Sera were firstly absorbed for xeno-NAb on a TG-coated column, and then analyzed for hemagglutinating AB titer. Only blood type A sera showed a decrease in anti-B titer (mean reduction 86 %), whereas the titer of type B sera did not change.

not significantly absorbed by human B RBC. In the present study, however, in individuals of non-B RBC types, considerable amount of anti-human RBC B antibody cross-reacted with xeno pig antigens, and quite similarly, xeno NAb markedly cross-reacted with human B antigens; thus the epitope which both Ab reacted was assumed to be branched B.

Meanwhile, McMorrow *et al.* reported that α Gal reactive IgG was significantly reduced sera from B antigen expressing donors relative to the non-B antigen donors, but there was no significant difference in α Gal reactive IgM between the two groups [14]. We found that non-B blood individuals had significantly higher titer of anti-pig IgM NAb compared with B blood individuals. The discrepancies might be mainly due to the sample numbers and partially to the differing methods of ELISA in each experiment. We analyzed more samples than in the study of McMorrow *et al.*, and we used whole endothelial cells or proteins as targets, while McMorrow used purified αGal. Determinants other than αGal are also involved in humoral responses of a pig to baboon combination [18, 19], and after treatment by α galactosidase, a specific enzyme to remove α Gal epitopes, human anti-pig antibody binding was lowered by 70 to 80 % but not completely inhibited. Therefore, in our assay, epitopes other than αGal might have contributed to human IgM binding to crude pig cells or antigens.

Pigs can be divided into A- or O-like antigen-positive by specific human hemagglutinin sera [18, 21-23], however only a small fraction of the anti-A alloantibody seems to react with A-like pig RBC. The expression of such antigens on our endothelial cells or TG was tested by mouse monoclonal antibodies or human polyreactive antibodies, which are utilized in routine human blood typing; we found no A-like antigen on their surface (data not shown).

Patients treated by hemodialysis might be candidates for recipient of xeno kidney organs in the future. They showed a significantly lower concentration of total IgM than healthy controls, however the titer of anti-pig xeno NAb was almost equivalent between the two groups. The patients of chronic renal disorders are proved to have decreased immune function but to preserve anti-xeno NAb levels. The antigens such as bacteria within the intestinal tract were thought to constantly provide antigenic stimulation for synthesis of α Gal [7]. An important finding was that anti-xeno NAb titer was higher in non-B blood types than in B blood types also even in the patients.

Clinically, to actually select candidates for xeno organ transplants, the existence of xeno NAb must be analyzed based on their binding and also from their function. Our standard ^{51}Cr release assay using pig endothelial cells showed that human sera from blood A types attacked them more strongly than the other blood types. A significant positive correlation between IgM binding and lysis against the same target, P16N, was observed, however there were several samples that showed negative cell lysis even if they bound to P16N. Another incomprehensive finding was that blood O had lower cytotoxic titers than blood A type. IgG, which bound to the surface of cultured pig cells, was more significantly correlated with cytotoxicity against pig cells than surface-bound IgM [12], and some natural IgG antibodies modulated complement or competed with activation by IgM [24]. Therefore, such anti-pig IgG NAb, although not examined in our cases, might have interfered with interaction between IgM and lysis of P16N.

We conclude that humans with non-B blood types, especially blood A type, should be monitored carefully for the appearance of xeno-hyperacute humoral rejection, and preabsorption of xeno NAb or anti-B RBC alloantibodies must be considered for successful xeno-transplantation.

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