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Ex Vivo Spleen and Kidney Absorption of Xenoreactive Natural Antibodies Decreases Severity of Hyperacute Rejection in Pig-to-dog Renal Xenotransplantation

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ABSTRACT

The severe hyperacute rejection in pig-to-dog renal xenotransplantation is mainly caused by xenoreactive natural antibodies (NAb). Organ absorption (ex vivo perfusion of spleen and kidney of donor species) was performed to remove xenoreactive NAb. A pig-to-dog renal transplantation model was used for discordant combination xenografting. The experimental animals were divided into 4 groups: group 1, control; group 2, recipients splenectomized prior to renal xenografting; group 3, splenectomy along with ex vivo spleen xenoreactive NAb absorption; and group 4, splenectomy along with ex vivo spleen and kidney xenoreactive NAb absorption. After the pretransplant treatment of the recipients, the serum titer of anti-pig lymphocytotoxic antibodies, hemagglutination antibodies, IgG and IgM were determined. Postoperative assessment was made of urine output and the rejected kidneys examined histopathologically. The serum titer of all measured antibodies markedly decreased after sequential pig spleen/kidney absorption. Total urine output was significantly larger in group 4 than in group 1. The histopathological findings revealed that the severity of hyperacute rejection was markedly decreased in groups 3 and 4 compared to groups 1 and 2. These results indicate that ex vivo absorption using donor spleen and kidney is useful in preoperative removal of xenoreactive NAb and prolongs renal xenograft survival.

Key words: *Renal Xenotransplantation, Organ Absorption, Hyperacute Rejection*

Hyperacute rejection of organ xenotransplantation is initiated by xenoreactive natural antibodies (NAb) directed against donor antigens[5]. This antibody-mediated response activates an inflammatory reaction and coagulation cascades that ultimately result in ischemic necrosis of the xenograft[5]. Therefore, it is important to define appropriate therapeutic interventions aimed at removing the NAb. To date, the two most common methods used to deplete xenoreactive NAb from a prospective recipient are plasmapheresis and organ absorption[2]. Absorption is believed to be a very effective technique for removing limited amounts of xenoreactive NAb from the endothelial cells lining the vessels of an organ of the donor species[2]. However, little is known of the exact efficiency of organ NAb absorption. In the present study, the effect of organ absorption (ex vivo perfusion of spleen and kidney of donor species) was examined using a pig-to-dog renal xenotransplantation model. This model offers one of the harshest examples of hyperacute rejection[5].

MATERIALS AND METHODS

1. Experimental animals

Male or female outbred dogs weighing 5–13 kg (8.5 ± 1.9 kg) were used as recipients. Male or female domestic pigs weighing 10–29 kg were used as donors.

2. Surgical procedures

(1) Anesthesia

The pigs and dogs were given 10 mg/kg of ketamine intramuscularly and then pentobarbital intravenously: 10 mg/kg initially, followed by additional smaller doses as needed during the course of the operation.

(2) Donor operation

The pigs were anticoagulated with heparin sodium intravenously. After in situ cooling with cold (4°C) Ringer's lactate through an aortic cannula, the kidneys and spleen were removed. These organs were perfused with cold heparinized Ringer's lactate solution at a pressure of 130 cm H_2O.

(3) Ex vivo organ absorption and renal transplantation

The femoral artery and vein of the animal were exposed and cannulated with 9 Fr catheters. The artery and vein of the isolated donor pig spleen or kidney were attached to the catheters of the recipient vessels for ex vivo perfusion. Then, organ absorption was performed using the donor

spleen (for 1 hr) or sequential spleen (for 1 hr) /kidney (for 2 hr). After the removal of the perfused spleen or kidney, the fresh pig kidney was transplanted by attaching the graft vessels to the catheters of the recipient vessels. The transplanted kidney was observed for 2 hr after reperfusion.

3. Experimental design

Allotransplantation:

Dog renal allotransplantation was performed as a control (n=23). Hyperacute rejection does not occur in this model.

Xenotransplantation:

Pig-to-dog renal xenotransplantation was performed.

Group 1 (n=12): the recipient dogs received no treatment.

Group 2 (n=8): the recipient dogs were splenectomized prior to pig renal xenografting.

Group 3 (n=19): the recipient dogs were splenectomized, and ex vivo perfusion of the pig spleen was performed for absorption of NAb prior to pig renal xenografting.

Group 4 (n=19): The recipient dogs were splenectomized, and sequential ex vivo perfusion of the pig spleen/kidney was performed prior to pig renal xenografting.

4. Measurement of urine volume

The ureter was cannulated with polyethylene tubing. The urine output was measured every 30 min for 2 hr after reperfusion to monitor graft function as previously described[15].

5. Blood biochemistry and serum antibodies

Blood samples were collected via the catheter after the pretransplant treatment and 2 hr after renal transplantation in all groups. The following routine blood tests were performed: white blood cell (WBC) count, red blood cell (RBC) count, platelet count, hematocrit, hemoglobin (Hgb), prothrombin time (PT), and fibrinogen. After serum separation from the collected blood, the serum titer of anti-pig lymphocytotoxic antibodies[7], hemagglutination antibodies[7], IgG[12], and IgM[12] were determined.

6. Pathological studies

The xenotransplanted pig kidneys were surgically removed at 2 hr after transplantation. All specimens were fixed in 10% formalin for 3 to 5 days and embedded in paraffin. Serial, 4 μm thick sections were stained with hematoxylin and eosin (HE) or phosphotungstic acid-hematoxylin (PTAH).

For the detection of dog IgM antibody, the avidin-biotin-peroxidase complex (ABC) method of Hsu et al[6] was used. Briefly, the sections were first deparaffinized and incubated with 0.025% trypsin for 120 min at 37°C. Incubation with anti-dog IgM antibody and rinsing in PBS at each step were performed for at least 30 min at room temperature. Endogenous peroxidase activity was inactivated by immersing the specimens in 0.03% hydrogen peroxide in absolute methanol for 20 min. The sections were counterstained with 3% methylgreen. Biotinylated anti-rabbit IgG and avidin-biotinylated horse radish peroxidase complex (ABC) were purchased from Vector Laboratories, Inc, Burlingame, CA, U.S.A..

Anti-dog IgM rabbit polyclonal antibody, kindly supplied by Dr. Toshinori Furukawa (Research Institute for Laboratory Animal Science, Hiroshima University School of Medicine), was diluted 1:2000 with saline.

The specificity of immunostaining was examined as follows; (1) normal rabbit IgG was used as the first layer, (2) 3,3'-diaminobenzidintetrahydrochorides or H_2O_2 was omitted from the incubation medium. The control slides were invariably negative for immunostaining.

RESULTS

1. Gross appearance of the graft after reperfusion

The grafts (group 1) became pink in color and soft in consistency immediately after revascularization. Thereafter, in about 20 min, the color of the grafts turned first pale and then violet and dark. Coincidentally, urine output dropped markedly and gross hematuria was evident. However, some urine production continued even at 2 hr after revascularization. The grafts in both groups 1 and 2 showed almost the same macroscopic findings. There was severe congestion in groups 1 and 2. The macroscopic findings differed between groups 1 and 2, and groups 3 and 4. Although congestion was found in groups 3 and 4, it was less prominent than in groups 1 and 2. One graft of group 4 and the allotransplantation grafts showed no sign of rejection. There was no congestion. These macroscopic findings were well parallel to the urine producing renal function.

2. Urine output

The total urine outputs 2 hr after reperfusion (the total urine outputs) were 97.1 ± 28.4 ml/2hr (allotransplantation), 49.3 ± 28.8 ml/2hr (group 1), 62.8 ± 16.4 ml/2hr (group 2), 92.5 ± 50.9 ml/2hr (group 3), and 183.4 ± 115.6 ml/2hr (group 4). Total urine output was lower in group 1 than in the allotransplanted kidneys and in group 4 ($p<0.05$). One graft from group 4 had a total urine output of 352 ml/2hr.

The urine outputs in the allotransplanted kidneys and in group 4 did not decrease during the 2 hr experimental period (Fig. 1). However, a gradual decrease in urine output over time after reperfusion was observed in groups 1 and 2.

The urine output 90 to 120 min after reperfusion was lower in group 1 than in the allotransplanted kidneys ($p<0.01$) and in group 4 ($p<0.05$). There was also a greater output of urine from 90 to 120 min after reperfusion in group 3

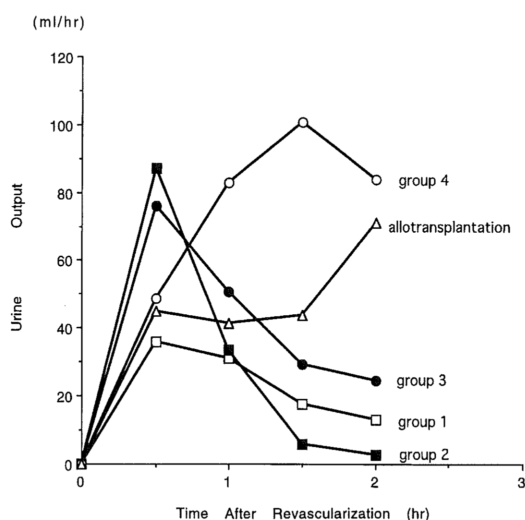

Fig. 1. Changes in urine production after reperfusion.
The urine output was measured every 30 min from the time of reperfusion to 2 hr. Allotransplantation: dog-to-dog kidney transplants were performed. Xenotransplantation: group 1: pig-to-dog renal xenotransplantations were performed. Group 2: dogs were splenectomized prior to pig-to-dog renal xenografting. Group 3: dogs were splenectomized, and ex vivo spleen xenoantibody absorption performed prior to pig-to-dog renal xenografting. Group 4: dogs were splenectomized, and ex vivo spleen and kidney xenoantibody absorption performed prior to pig-to-dog renal xenografting.

than in group 2 ($p<0.05$). During this final time interval there was no difference between groups 1 and 2.

The urine output in one graft of group 4 was 60 ml/hr in the first 30 min, 258 ml/hr at 1hr, and over 200 ml/hr at 2 hr.

3. The hemato biochemical examination of group 4

The WBC counts, platelet counts, and prothrombin times were 5918±3805 /μl, 20.5±11.5 $\times 10^4$ /μl, and 21.1±20.2 sec, respectively, before ex vivo spleen and kidney xenoantibody absorption (Table 1), and 4171±2839 /μl, 13.6±10.3 $\times 10^4$ /μl, and 38.4±76.2 sec, respectively, after ex vivo spleen and kidney xenoantibody absorption. These differences are not significant.

The RBC counts were 644.5±168.4 $\times 10^4$ /μl before, and 428.1±196.7 $\times 10^4$ /μl after ex vivo spleen and kidney xenoantibody absorption ($p<0.01$). The Hgb was 16.0±4.8 g/dl before ex vivo spleen and kidney xenoantibody absorption, dropping to 11.2±5.5 g/dl after xenoantibody absorption ($p<0.01$).

4. Serum titer of antibodies in group 4

As shown in Table 2, the serum titer of the anti-pig lymphocytotoxic and hemagglutination antibodies dropped markedly after ex vivo sequential spleen/kidney (dual organs) perfusion. IgG was also well absorbed by ex vivo dual organ absorption. IgM was most effectively absorbed by

Table 1. Blood examination of group 4

	WBC (/μl)	Platelets ($\times 10^4$/μl)	PT (sec)	RBC ($\times 10^4$/μl)	Hgb (g/dl)	Fibrinogen (mg/dl)
No treatment	6382±3730	25.7±10.3	22.8±23.0	632.3±140.1	14.5±3.9	305.0±191.6
After splenectomy	5918±3805	20.5±11.5	21.1±20.2	644.5±168.4#	16.0±4.8*	259.3±196.1
After ex vivo spleen xenoantibody absorption	6919±7573	15.1± 8.5	23.7±17.4	664.4±298.0	14.5±4.8	179.5± 95.2
After ex vivo kidney xenoantibody absorption	4171±2839	13.6±10.3	38.4±76.2	428.1±196.7#	11.2±5.5*	123.6± 78.7

#p<0.01, *p<0.01
WBC: White blood cell, PT: Prothrombin time, RBC: Red blood cell, Hgb: Hemoglobin.

Table 2. The antibody examination of group 4

	Lymphocytotoxic antibody titer	Hemagglutination antibody titer	IgG (mg/dl)	IgM (mg/dl)
No treatment	X45.3±19.4	X3.4±5.0	398.0±122.6	62.4±22.5
After splenectomy	X36.0±21.5*	X3.3±3.8**	341.1±145.6***	53.7±20.9@
After ex vivo spleen xenoantibody absorption	X22.7± 9.7#	X2.1±2.6	276.5±146.0	46.5±20.2##
After ex vivo kidney xenoantibody absorption	X 7.0± 4.6*#	X0.7±1.1**	172.2±157.0***	26.4±16.7@##

*p<0.05, #p<0.01, **p<0.05, ***p<0.01, @p<0.01, ##p<0.01,

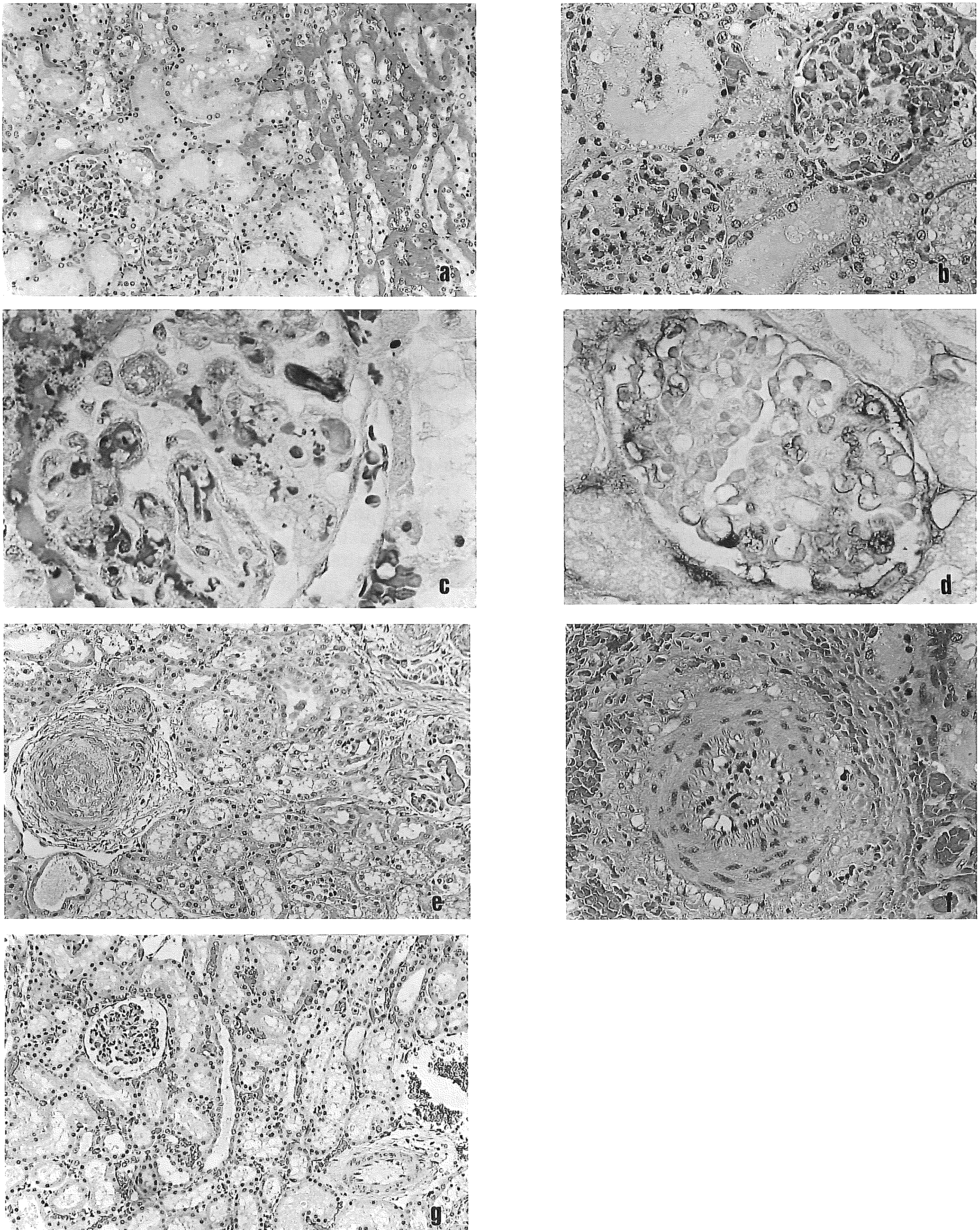

Fig. 2. a: Massive congestion and interstitial hemorrhage of a transplanted pig kidney from group 1. Two glomeruli contain fibrin thrombi. HE staining, ×150. b: Fibrin thrombi of glomeruli (group 1). Note pyknotic nuclei of dilated tubules. HE staining, ×300. c: Fibrin thrombi of glomerulus (group 1). PTAH staining, ×600. d: Linear deposits of IgM (group 2). Immunostaining, ×600. e: Fresh thrombus in artery. Glomerulus contains fibrin thrombi (group 1). HE staining, ×150. f: Detachment of endothelia from the lamina elastica interna. Perivascular recent hemorrhage is also observed (group 2). HE staining, ×300. g: Relatively well preserved transplanted pig kidney from group 4. Mild congestion and dilated tubules with a few pyknotic nuclei are noted. HE staining, ×150.

both single (spleen) organ and dual organ absorption.

5. Histological findings

The histopathological findings clearly differed between groups 1 and 2, and groups 3 and 4. The xenografted pig kidneys in both groups 1 and 2 showed almost the same findings: massive and diffuse congestion with interstitial hemorrhage, evident even at low magnification (Fig. 2-a). Most glomeruli contained fibrin thrombi without polymorphonuclear leukocytes (Fig. 2-b). PTAH staining confirmed the presence of microvascular glomerular thrombi occluding the capillary lumens (Fig. 2-c). Immunohistochemically, a few glomeruli contained linear deposits of IgM along capillary basement membranes (Fig. 2-d). Fresh thrombi were also detected in a few arteries (Fig. 2-e), while in other arteries, detachment of endothelia from the lamina elastica interna was noted (Fig. 2-f). Dilated tubules contained pyknotic nuclei (Fig. 2-a, b, c, e), and occasionally evidence of recent cell death. No infiltration by lymphocytes was observed in any of the specimens examined. Although glomerular thrombi, interstitial hemorrhage and tubular degeneration were also found in groups 3 and 4 (Fig. 2-g), these features were less prominent than in groups 1 and 2. The histopathological and immunohistochemical features of groups 1 and 2 were the same as those seen in hyperacute rejection in experimental xenotransplantation and in clinical allotransplantation[19]. On the other hand, the xenotransplanted kidneys of group 4 had less marked pathological changes, although congestion and thrombi were noted in a few cases. These findings are reminiscent of accelerated rejection, which occurs 2 or 3 days after renal allotransplantation[19].

DISCUSSION

The severe hyperacute rejection in pig-to-dog renal xenotransplantation is mainly caused by xenoreactive NAb[1,3–5,8–10,13–18,20]. This antibody-mediated response activates an inflammatory reaction and coagulation cascades that ultimately result in ischemic necrosis of the xenograft[5]. The combination of the recipient's NAb plus the activated complement leads to activation of the endothelial cells lining the vessels of the graft[2]. Progressive endothelial cell activation leads to complications associated with hyperacute rejection[2]. Most of the components of a typical acute inflammatory response are invoked, and the classical pathway of complement is activated[2,11]. This effector cascade invokes most of the classical mediator systems of the acute inflammatory process (i. e., coagulation, fibrinolytic, and kallikrein-kinin systems)[11]. During this process, a large number of biologically active molecules are responsible for the increased vascular permeability, arterial vasoconstriction, damage to the cytoskeleton, fibrin deposition, generation of oxygen-free radicals, and the recruitment, activation, and release of various inflammatory cells[11]. The accumulation of platelet-polymorphonuclear leukocyte-erythrocyte plugs in small arteries and capillaries is followed by intravascular coagulation and ischemic necrosis[11].

The initiating event is NAb-mediated injury directed against antigens on the vascular endothelium of the xenograft[11]. Therefore, it is important to define appropriate therapeutic interventions aimed at removing NAb. To date, the two most common methods used to deplete xenoreactive NAb from a prospective recipient are plasmapheresis and organ absorption[2]. In addition to NAb, other factors such as complement and coagulation factors are also depleted by these treatments[2]. Plasmapheresis is an excellent intervention for removing high concentrations of natural antibody with repeated plasma exchanges[2]. However, as it involves nonspecific antibody removal, it is not particularly good at lowering the level of NAb already present in low concentrations to exceedingly low levels, or removing the last measurable traces of NAb. In contrast, organ absorption is possibly very effective at removing small residual amounts of NAb, because NAb is specifically absorbed in the xenoantigen of a donor species organ. However, little is known of the exact efficiency of organ absorption. In the present study, the effect of organ absorption was examined using a pig-to-dog renal xenotransplantation model. This model offers one of the harshest examples of hyperacute rejection[5]. We examined not only the hemagglutination antibodies but also IgG and IgM.

Ex vivo organ xenoantibody absorption experiments using single organs, such as kidney[9], liver[4,5,15,17,21], spleen[4,5] and lung[18] have been reported to effectively prolong the function of pig-to-dog renal xenografts. Linn et al[9] performed ex vivo kidney xenoantibody absorption to decrease the anti-pig antibodies to –75%. Theirs was considered the first successful study on ex vivo kidney absorption in pig-to-dog renal xenotransplantation. Moberg et al[15] utilized ex vivo liver xenoantibody absorption to reduce the pretransplant titer of antibody to 65%. However, their paper contained no precise data on urine producing renal function or on the value of antibodies, fibrinogen and WBC. As NAb is absorbed in the xenoantigen of the endothelial cells lining the vessels, the total amount of NAb removed is limited by the number of endothelial cells available in that organ. Since the spleen is rich in vessels, it was used for organ absorption. Sequential spleen/kidney absorption was more effective than single organ (spleen) absorption. Few studies have been done on dual organ absorption. Slapak et al[21] reported on kidney (9.4 ± 6 min) and liver

(1 hr) xenoantibody absorption. The survival time was 37.4±8 min, and the average urine output was only 12 ml. The best kidney retained a good color for 20 min. It seems that the time taken for their ex vivo kidney xenoantibody absorption was insufficient. They assumed that organ absorption was effective and that the cause was probably antibody absorption. Theirs is considered the first successful report of ex vivo dual organ absorption in pig-to-dog renal xenotransplantation. However, only globulin was used and no precise data on urine output, peripheral blood, or antibody change was obtained. Moreover, the length of time for ex vivo kidney absorption was not sufficient to allow comparison of mean rejection times between the first and second kidneys. To our knowledge, since this report, there has been no further study of ex vivo dual organ absorption in pig-to-dog renal xenotransplantation. Little is known of the exact efficiency of dual organ absorption using sequential spleen/kidney. We studied ex vivo dual organ absorption, in terms of urine producing renal function and antibody absorption. This study is the first to clarify the effect of dual organ absorption (sequential spleen/kidney) using a pig-to-dog renal xenotransplantation model.

The serum level of IgM was lowered by using ex vivo kidney and dual organ absorption. In pathological specimens, the deposits of IgM in the xenograft were reduced. There seems to be general agreement that IgM can function as a xenoreactive NAb[2]. Some studies of the immunopathology of the rejection of pig heart in rhesus monkey showed primarily IgM deposition on the endothelium of the donor organ[2]. Human xenoreactive NAb derived from individuals with relatively high titers of these antibodies represented only approximately 0.1% of the total IgM, but approximately 100,000 IgM molecules can deposit on a single pig endothelial cell in vitro[2]. Human serum xenoreactive NAb variation in titer did not correlate with either the total amount of IgM in serum or the titers of the ABO isohemoagglutinins[2]. In our study, IgM absorption appears to be more specific than anti-pig hemagglutination antibodies or IgG absorption. In pathological specimens, there were linear deposits of IgM in the glomeruli. IgM is considered to play an important role as NAb.

We splenectomized the recipient dogs in addition to organ absorption. Most experimental animals used as recipients of xenotransplant are splenectomized[2]. Bach et al have shown that splenectomy is associated with a reduction of B cells, since the spleen is a major source of these cells[2]. NAb is considered to be produced by genetically programmed B cells[2].

Dual organ absorption markedly decreased the severity of hyperacute rejection. However, in the pathological findings of group 4 xenografts, evidence of accelerated rejection was observed. This seems to have been caused by residual xenoreactive humoral factors. In order to remove these factors, a combination of plasmapheresis and organ absorption may be a potential therapy. Henry et al performed both plasmapheresis and ex vivo organ absorption[5]. They reported three cases of ex vivo spleen (2 hr) and liver (2 hr) absorption combined with plasmapheresis and a survival time of 460±93 min. The antibody depletion was 95±1.5% by ex vivo spleen and liver absorption combined with plasmapheresis[5]. They did not examine kidney absorption or sequential spleen/kidney absorption. The resultant reduction of NAb markedly prolongs xenogeneic kidney graft survival in pig-to-dog xenocombination, and a delayed form of hyperacute rejection is observed[5].

Bach et al have referred to the survival of a graft under such circumstances as "accomodation"[2]. A combination of plasmapheresis, inhibitors of complement activation, immunosuppressants and ex vivo organ absorption might be effective in achieving so-called "accommodation"[2]. Further studies will be needed to make accommodation a potential therapeutic goal for xenotransplantation.

In conclusion, xenoreactive NAb concentrations dropped markedly after ex vivo sequential spleen/kidney (dual organs) perfusion, and the severity of hyperacute rejection was markedly decreased in comparison with the control. The effect of xenoreactive NAb absorption using dual organs was clearly more effective than that of conventional single organ absorption.

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