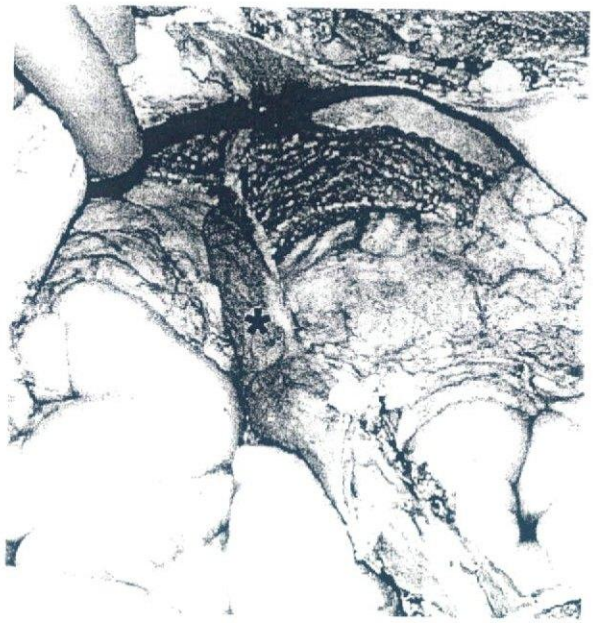

Fig 1. The findings after a laparotomy of patient 1. *A recanalized umbilical vein before procurement.

RESULTS

Patient 1. The first recipient was a 64-year-old man with end-stage liver disease caused by hepatitis C. Abdominal computed tomography (CT) scan showed a large-caliber recanalized umbilical vein as a collateral vein. The donor was his 60-year-old healthy sister whose blood type (O+) was compatible with the patient's (B+). The pretransplant work-up of the donor showed normal liver function tests with an estimated graft volume (a right-lobe graft without MHV) of 483 mL, which accounted for 35% of the recipient's ideal liver volume. The reconstituted image of the hepatic vein by 3-dimensional (3D) CT showed large MHV tributaries draining through segments V and VIII of the liver. The reconstruction of these venous tributaries thus was indicated because of a concomitant small-for-size graft. The operative procedure for the donor has been described in detail elsewhere.[5] The actual graft weighed 510 g, which accounted for 37% of the standard liver volume of the recipient. A segment of the recipient's umbilical vein (Fig 1; 15 cm in length, 20 mm in diameter) was obtained immediately after laparotomy and stored in sterile heparinized saline. The venous tributaries (venous tributaries draining segment V [V5], 8 mm; venous tributaries draining segment VIII [V8], 10 mm) of the graft were anastomosed end-to-side to the umbilical vein graft using 7-0 Prolene (Ethicon Inc, Tokyo, Japan) continuous sutures on the backtable. The implantation of the graft was performed as follows. The donor right hepatic vein was anastomosed to the recipient's right hepatic vein, then the distal end of the interposed umbilical vein was anastomosed to the recipient's MHV orifice with 5-0 Prolene continuous sutures before portal vein anastomosis. The reperfusion of the graft was prompt and smooth. The graft was not swollen on reperfusion and it was very soft on palpation. The interposition graft filled promptly and provided a good outflow for segments V and VIII (Fig 2). The intraoperative color Doppler ultrasonography confirmed excellent flow in the artery, portal vein, right hepatic vein, and in the V5 and V8. The graft functioned quickly with a normalizing prothrombin time by postoperative day 3. Currently, the patient is doing well with a sustained serum creatinine level of around 2.5 mg/dL. Throughout the course, the patency of the interposed graft was confirmed daily.

Patient 2. The second recipient was a 47-year-old man with end-stage liver disease caused by hepatitis B. An emergent LDLT using his 42-year-old wife as a donor was indicated. The estimated graft volume (a right-lobe graft without MHV) was 614 mL, which accounted for 47% of the recipient's standard liver volume. The reconstituted image of the hepatic vein by 3D CT showed large MHV tributaries draining through segment V (V5) of the liver. During the donor hepatectomy, the clamping of the right hepatic artery and segment V5 showed discoloration and congestion of the anterior segment, prompting reconstruction of this venous tributary. A segment of the recipient's umbilical vein (10 cm in length, 12 mm in diameter) was obtained immediately after laparotomy and was stored in sterile heparinized saline. The venous tributaries of the graft were anastomosed end-to-end to the umbilical vein graft using 7-0 Prolene continuous sutures on the backtable. A portion of the left portal vein of the native liver was obtained and anastomosed end-to-end to the distal end of the interposed umbilical vein on the backtable. The implantation of the graft was performed in the same manner. The interposition graft filled promptly and provided good outflow for the anterior segment of the graft. Intraoperative color Doppler ultrasonography confirmed an excellent flow in the right hepatic vein and in V5.

DISCUSSION

The inconstant venous anatomy of the right-lobe graft imposes considerable technical challenges to ensure an adequate outflow of the anterior segment (segments V and VIII) in RL-

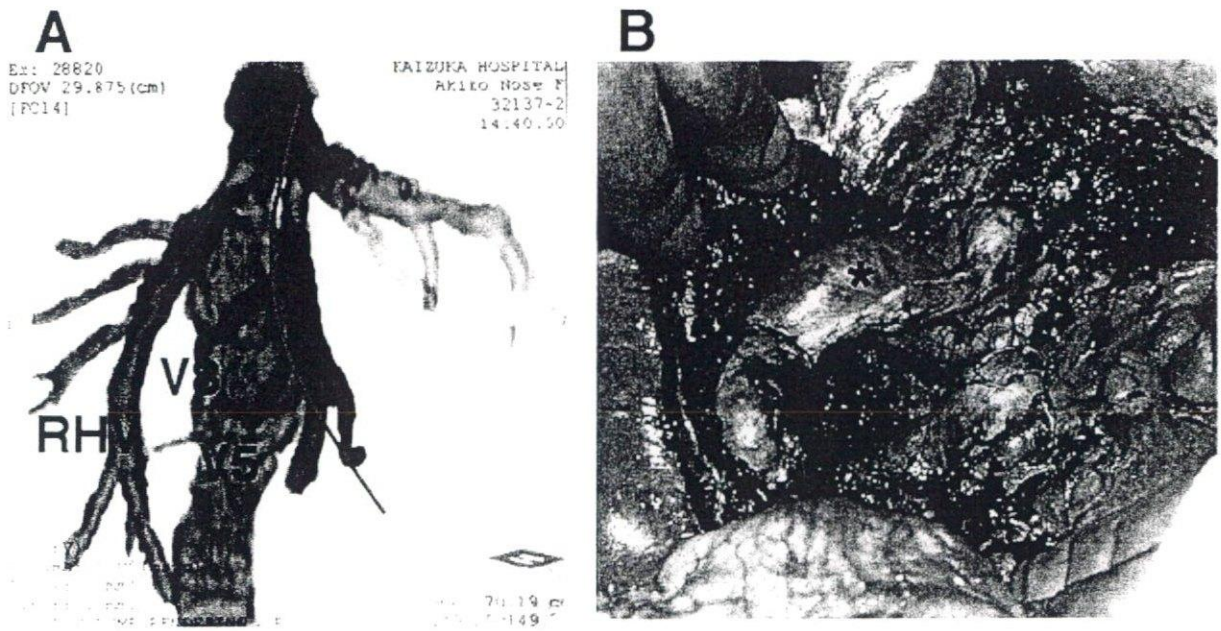

Fig 2. A, A 3D-CT image of the MHV and its tributaries (V5 and V8) and a transection line. **B,** The right lobe graft after reperfusion. *A reconstructed umbilical vein graft.

LDLT. The indications and the methods for reconstructing the MHV or its tributaries remain controversial. However, the importance of obtaining effective drainage of the anterior segment is recognized. In the initial reports regarding RL-LDLT, MHV tributaries were not reconstructed because of fears of increased donor risk and also based on the assumption that intrahepatic collateral circulation between accessory branches and the main right hepatic vein (RHV) provide a retrograde decompression of the anterior segment. However, Lee et al[1] reported that 2 of 5 right liver grafts without MHV drainage reconstruction developed severe congestion of the anterior segment, thus resulting in graft loss. Therefore, they advocated an aggressive approach in reconstructing the MHV tributaries (V5 and V8) using venous interposition grafts such as the greater saphenous vein, the external iliac vein, and a hydrostatically dilated greater saphenous vein graft.[4] Another approach was reported by the Hong Kong group,[3] who routinely used extended right-lobe grafts including MHV drainage. Sano et al[6] proposed criteria for venous reconstruction of the MHV tributaries in RL-LDLT. They assessed the discolored area in the anterior segment by clamping the right hepatic artery concomitant with clamping the MHV or its tributaries at the donor hepatectomy. If the regurgitated portal flow was observed in the right anterior segment by Doppler ultrasonography or the discolored area was large, then the reconstruction of the MHV tributaries was indicated.

Venous drainage by the MHV is complex. The MHV drains all of segments IV and part of segments II and III of the left lobe and segments V and VIII of the right lobe. In 24% of the population, a small RHV is encountered, and right-lobe drainage is achieved predominantly by a large MHV tributary or an inferior RHV. In such patients, the MHV tributaries and the inferior RHV should be reconstructed.[7] Other indications for reconstructing the MHV tributaries may be small-for-size grafts, steatotic or elderly grafts, and grafts with a predominant MHV (large V5 and V8) and a small RHV. Furthermore, the existence of severe portal hypertension also may be an indication for MHV reconstruction. Our current criteria for MHV reconstruction are when the middle hepatic vein is predominant or the MHV tributaries are large ($\geq$5 mm) or the graft size is small (the predicted graft-to-standard liver volume ratio is $\leq$40%). We prefer not to include the MHV with right-lobe grafts, because we maintain donor safety as of paramount importance.

A variety of vein grafts have been used to reconstruct the MHV tributaries. Under circumstances in which a cadaveric venous graft is not available, the use of vein grafts such as the greater saphenous vein, external iliac vein, donor

ovarian vein, or inferior mesenteric vein and explanted left portal vein[8] have been reported. Sugawara et al[9] reported reconstruction of the MHV tributaries using a cryopreserved vein graft. Miller et al[10] reported the use of a prosthetic graft for the reconstruction of the MHV tributaries. Although their long-term patency has not yet been evaluated fully, these techniques are considered to be effective.

We recommend use of the recipient's recanalized umbilical vein, if available, because it usually is long enough for an interposition graft. The umbilical vein graft also offers a large caliber and a thick wall with a well-preserved intima, which all should reduce the risk for thrombosis.

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SHORT REPORT

Successful Adult-to-Adult Living Donor Liver Transplantation in a Patient with Moderate to Severe Portopulmonary Hypertension

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Portopulmonary hypertension (PPHTN) is one of the most devastating consequences of end-stage liver cirrhosis. When a patient has moderate to severe PPHTN, his or her candidature for liver transplantation is denied. Here we report a successful adult-to-adult living donor liver transplantation (LDLT) in a patient with moderate to severe PPHTN. The patient was a 58-yr-old female who was diagnosed with end-stage liver cirrhosis due to chronic hepatitis C. Preoperative evaluation revealed that the patient had moderate to severe PPHTN. Her mean pulmonary artery pressure (mPAP) was 35-47 mmHg without treatment. Continuous epoprostenol therapy was introduced to lower the mPAP. She underwent LDLT using an extended right hepatic lobe graft which was donated by her daughter. Prolonged artificial ventilation was necessary until postoperative day (POD) 25, after which her general condition gradually improved. By POD 72, she was in good condition and was allowed to leave the hospital. Currently, 1 yr after the operation, she visits the outpatient clinic regularly and enjoys a normal life. It should be noted, however, that the PPHTN markedly improved but did not completely resolve, as assessed by right heart catheterization 1 yr after successful LDLT. *Liver Transpl 12:481-484, 2006.* © 2006 AASLD.

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Portopulmonary hypertension (PPHTN) is defined as mean pulmonary artery pressure (mPAP) >25 mmHg in patients with a pulmonary capillary wedge pressure <15 mmHg (although this is not an absolute inclusion criterion) in association with portal hypertension, and without any intrinsic or underlying lung disease.[1-5] PPHTN can be categorized into mild (mPAP of 25-35 mmHg) or moderate to severe (mPAP >35 mmHg).[4,5] Its occurrence is estimated at approximately 2 to 10% in patients with end-stage liver cirrhosis.[6] The exact pathogenesis of this disorder remains to be elucidated. It is generally considered that moderate to severe PPHTN is a contraindication for liver transplantation because of increased risk of right-side cardiac failure and other cardiopulmonary complications.[1-3] Krowka et al.[2] reported that an mPAP of 50 mmHg or greater was associated with 100% cardiopulmonary mortality within 2.5 yr of liver transplantation and that all patients died of right heart/cardiopulmonary failure. Recently, continuous epoprostenol therapies for these patients were introduced to lower mPAP and a few successful cases have been reported.[4,7] Here we report 1 case of successful adult-to-adult living donor liver transplantation (LDLT) in a patient with moderate to severe PPHTN.

CASE PRESENTATION

A 58-yr-old female was diagnosed with end-stage liver cirrhosis due to chronic hepatitis C in 1995. She was further observed to have PPHTN in 1999 by chest X-ray. She visited our hospital on December 2000, hoping to

Abbreviations: PPHTN, portopulmonary hypertension; LDLT, living donor liver transplantation; POD, postoperative day; mPAP, mean pulmonary artery pressure; PVR, pulmonary vascular resistance.

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TABLE 1. Pre-, Intra-, and Posttransplantation Cardiopulmonary Hemodynamic Data

Time	mPAP (mmHg)	PCWP (mmHg)	CO (L/minute)	PVR (dynes · second/cm^5)	Epoprostenol dose (ng/kg/minute)
4 months before Tx	47	16	NA	NA	0
2 months before Tx	35	4	4.77	519	0
1 week before Tx	33	10	8.24	223	10
On laparotomy	33	NA	5.45	NA	20
On anhepatic phase	43	NA	9.23	NA	20
Immediately after reperfusion	31	NA	7.5	NA	20
2 months after Tx	30	6	7.67	250	5
1 yr after Tx	30	6	4.68	410	0

Abbreviations: Tx, liver transplantation; mPAP, mean pulmonary artery pressure; PCWP, pulmonary capillary wedge pressure; CO, cardiac output; PVR, pulmonary vascular resistance; NA, not applicable.

undergo LDLT. The initial examination by right heart catheterization revealed that she had a high mPAP (Table 1). Chest X-ray examination showed remarkable dilatation of the pulmonary arteries. The electrocardiogram revealed right ventricular hypertrophy and right axis deviation. The ultrasonic echocardiograph examination showed increased right ventricle-right atrium gradient, right ventricular hypertrophy and tricuspid valve regurgitation. Other causes of pulmonary hypertension were ruled out and she was diagnosed with moderate to severe PPHTN (although pulmonary capillary wedge pressure was mildly elevated), and denied proceeding to LDLT. First, a calcium channel blocker (nifedipine) was given to decrease her mPAP, which was only partially successful (47 → 35 mmHg) but pulmonary vascular resistance (PVR) remained high (519 dynes · second/cm^5). Nitric oxide inhalation was not effective. Continuous epoprostenol therapy was initiated to decrease her PVR. Epoprostenol was gradually increased to 20 ng/kg/minute, at which dose she experienced mild side effects, such as gastrointestinal symptoms, precluding further increases. Epoprostenol at this dose (20 ng/kg/minute) neither affected the platelet count adversely nor decreased the systemic blood pressure. Approximately 2 months after starting epoprostenol therapy, PVR was decreased and finally she was able to undergo LDLT in April 2004, using an extended right lobe graft donated by her daughter. Because the donor had a relatively large left hepatic lobe, the extended right lobe graft was selected. The graft volume was 600 gm and the graft volume/recipient's standard liver volume was 52.0%.

The operation was completed without any adverse event. Pump-driven venovenous bypass was used during the anhepatic phase to maintain hemodynamic stability. A piggyback technique was used. Because there was a large splenorenal shunt with partial portal vein thrombosis, renoportal anastomosis was performed to reconstruct the portal vein, using the gonadal vein as an interposition graft.[8] The operative time was 15 hours 25 minutes and the estimated blood loss was 6348 mL. Immunosuppression was initiated with basiliximab, mycophenolate mofetil and with early withdrawal of steroid followed by maintenance immunosuppression with cyclosporin and mycophenolate mofetil. In the immediate postoperative period, her blood gas analyses showed that oxygen pressure continued to be low, so that a high inspired oxygen concentration of more than 0.5 and long-term management on artificial ventilation were imperative. On postoperative day (POD) 14, tracheostomy was done, and the next day sepsis occurred. Catheter infection seemed to be the cause of sepsis. By use of appropriate antibiotics and removal of catheters, this critical condition gradually improved. On POD 23, because elevation of aspartate transaminase was noted, a liver biopsy was taken, revealing not rejection but ischemic changes (centrilobular ballooning of the hepatocytes). According to the biopsy result, no specific treatment for the elevation of transaminase was given, and there was a gradual decrease of aspartate transaminase level (Fig. 1). On POD 25, artificial ventilation was withdrawn and a rehabilitation program initiated. The dose of continuous epoprostenol was gradually decreased. The fourth examination by right heart catheterization revealed that PVR remained low although the continuous epoprostenol dose was decreased to 5 ng/kg/minute, so that epoprostenol therapy was completely stopped. Finally, the patient was allowed to leave the hospital on POD 72.

One year after the operation, she has been visiting our outpatient clinic regularly and remains in a good condition. The final examination by right heart catheterization was performed on May 2005, which showed mPAP = 30 mmHg and PVR = 410 dynes · second/cm^5. So far, there has not been any marked improvement in her cardiopulmonary profiles.

DISCUSSION

The precise mechanisms by which portal hypertension causes PPHTN remain unclear. Several factors, such as splanchnic volume overload and bowel congestion which result in release of endotoxins and various cytokines, are considered to lead to PPHTN. In the severe

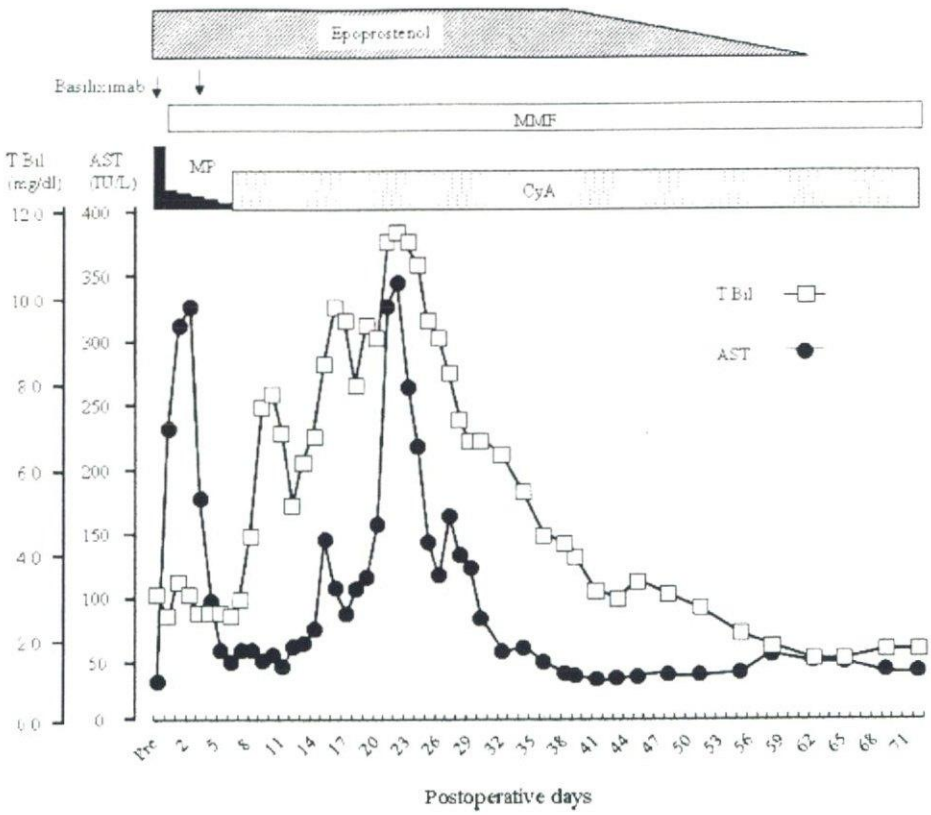

Figure 1. Postoperative course of total bilirubin and aspartate transaminase. Abbreviations: MMF, mycophenolate mofetil; MP, methylprednisolone; CyA, cyclosporine; T.Bil, total bilirubin; AST, aspartate transaminase.

form, as reported by Krowka et al.,[2] the mortality rate for liver transplant recipients is 100%. Some effective interventions are clearly needed to decrease the mortality rate of liver transplant recipients with moderate to severe PPHTN.

Continuous epoprostenol therapy has been shown to have some efficacy for PPHTN.[4,7] However, this therapeutic modality needs long-term venous access and uninterrupted drug infusion, so there are several serious problems in performing this therapy, such as catheter infection, line trouble, pump failure, and so on. Furthermore, there has been no randomized controlled trial on continuous epoprostenol therapy. Mair et al.[9] reported that a liver transplant recipient with PPHTN died of right-sided heart failure 28 days after orthotopic liver transplantation although continuous epoprostenol therapy successfully decreased mPAP. Starkel et al.[10] reported the feasibility of liver transplantation with good long-term outcome in patients with severe PPHTN without the need for pharmacological intervention. Although the patient's PVR decreased with the introduction of continuous epoprostenol therapy, whether it had favorable effects on the patient's outcome remains to be elucidated. However, it may be speculated that the decrease in mPAP and PVR associated with epoprostenol therapy results in a reduction in perioperative mortality in liver transplant recipients with PPHTN. Moreover, there is no effective therapeutic modality for PPHTN other than epoprostenol at the present time. Continuous epoprostenol therapy for liver transplant candidates with PPHTN must be investigated.

It has been speculated that nitric oxide may decrease PVR in patients with PPHTN. However, the efficacy of nitric oxide in PPHTN is controversial. Ramsay et al.[11] demonstrated that nitric oxide did not reverse PPHTN associated with end-stage liver disease. In fact, we also tried to decrease mPAP by nitric oxide inhalation, but this was ineffective in our patient.

Other drugs such as iloprost[12] and bosentan[13] are used for the treatment of pulmonary arterial hypertension, showing some beneficial effects. Randomized controlled trials for these drugs in patients with PPHTN are warranted.

There have been several reports on the reversibility of PPHTN after liver transplantation.[14-16] In contrast, Prager et al.[17] reported that pulmonary hypertension associated with liver disease was not reversible after liver transplantation. In our experience, PPHTN was partially, but not completely reversed, 1 yr after successful LDLT. It can be considered that the more severe the PPHTN, the longer it takes to be reversed. How should recipients with PPHTN be monitored over the long term after liver transplantation? We will have this patient visit our outpatient clinic once every 3 months, undergo ultrasonic echocardiograph examination and blood gas analyses every 6 months, and undergo right heart catheterization every year to determine whether her cardiopulmonary profiles improve. If the latter fail to improve or even worsen in most recipients with

PPHTN over the long term, even after successful liver transplantation, this approach by itself cannot represent an accepted indication for such patients (unlike hepatopulmonary syndrome). Further follow-ups are necessary for these patients with PPHTN.

A report of the Multicenter Liver Transplant Database demonstrated that transplant mortality was 36% (13/36) in patients with PPHTN.[5] All deaths in such patients occurred within 18 days of liver transplantation; 5 of the 13 deaths occurred intraoperatively.[5] Safer strategies for liver transplantation in patients with PPHTN are mandatory to reduce this high mortality rate. Our current strategies for LDLT in patients with moderate to severe PPHTN are as follows: 1) Try to decrease mPAP to less than 35 mmHg using epoprostenol, calcium channel blockers and other agents. If mPAP cannot be lowered by pharmacological interventions, liver transplantation is denied. 2) Use veno-venous bypass to maintain hemodynamic stability. 3) Prepare a heart assist device in case of cardiac arrest due to right-sided heart failure. 4) Never use a small-for-size graft to avoid small-for-size graft syndrome.[18]

To the best of our knowledge, this is the first report of a successful adult-to-adult LDLT in a patient with moderate to severe PPHTN. Because the number of recipients with PPHTN is relatively low, it is very difficult to decide optimal management of liver transplant candidates with PPHTN. Collaboration between major liver transplant centers regarding the management of PPHTN is therefore highly desirable.

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Critical Roles of Memory T Cells and Antidonor Immunoglobulin in Rejection of Allogeneic Bone Marrow Cells in Sensitized Recipient Mice

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Background. Allosensitization is a major risk factor for graft failure in clinical bone marrow transplantation, even with an human leukocyte antigen (HLA)-matched combination under radiation-based conditioning regimens. The critical components of immunological memory in donor bone marrow graft rejection in allosensitized hosts remain unclear at present.
Methods. C57BL/6-recipient mice, which had been intraperitoneally injected with splenocytes from donor C3H mice on day −35 (sensitized recipients), had been lethally irradiated with 10-Gy whole-body irradiation and were intravenously injected with T-cell-depleted bone marrow cells (TCD-BMC) from C3H mice or third-party SJL mice.
Results. Lethally irradiated recipient mice, which had been sensitized by donor splenocytes 5 weeks before the transplantation of TCD-BMC, completely rejected the donor-BMC in a donor-specific manner, whereas none of the nonsensitized recipient mice, all of which showed full allogeneic chimerism, rejected the donor TCD-BMC. Antibody-mediated T cell and/or Natural Killer (NK) cell depletion did not improve the ability of the sensitized recipients to overcome the rejection even when a megadose of TCD-BMC was administered to the sensitized recipients. Furthermore, BMC rejection occurred in sensitized B cell-deficient mice. In adoptive transfer experiments, naive mice, which received a transfer of purified T cells from sensitized mice, rejected the donor BMC, but not those from nonsensitized mice. Moreover, naive mice, which received a transfer of serum containing antidonor immunoglobulin, rejected the donor BMC.
Conclusions. These findings suggest that alloreactive memory T cells and antidonor immunoglobulin independently function to reject donor BMC in sensitized recipients.

Keywords: Bone marrow transplantation, Sensitized recipient, Memory T cell, Alloantibody, Rejection.

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Allogeneic hematopoietic cell transplantation is a curative therapy for hematologic malignancies and hematologic disorders (*1–6*). In rodent experiments, donor-derived hematological chimerism has been shown to induce in recipients to a state of stable tolerance to donor antigens (*7–10*). Therefore, the allogeneic hematopoietic cell transplantation may induce tolerance in patients undergoing organ transplantation, thus enabling the avoidance of harmful chronic nonspecific immunosuppression. Compared clinical outcomes with experimental results using murine bone marrow transplantation (BMT) models, there is a clear distinction in difficulty to overcome immunological obstacles between human and specific pathogen-free mice. In murine BMT models, lethal irradiation alone suffices for the recipient to overcome recipient alloreactivity in BMT involving major histocompatibility complex (MHC) and multiple minor disparate combination, so that the subsequent transplant of donor-derived hematopoietic cells leads to complete reconstitution, provided that a sufficient dose of bone marrow cells (BMC) is used to overcome Natural Killer (NK) cell alloreactivity (*11–13*). On the other hand, in the case of clinical allogeneic BMT, graft failure is a significant problem among patients who receive a human leukocyte antigen (HLA)-mismatched stem cell transplantation using standard successful conditioning regimens, that are used in BMT involving a HLA-identical donor-recipient combinations (*14, 15*). Adams and colleagues proposed that the critical distinction in the ability to induce tolerance in human or large animals and specific pathogen-free mice

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may be the immunological history of former groups, which involves the generation of crossreactive memory T cells previously primed by viral antigens and other pathogens (*16*). Therefore, in cases of human BMT with a MHC incompatible combination, such crossreactive memory T cells may be responsible for the observed difficulty in overcoming allogeneic barriers. In addition, host sensitization to alloantigens due to blood transfusions is also a critical problem in BMC rejection even in BMT using HLA-identical stem cell transplant to treat certain hematologic disorders, such as severe anaplastic anemia (*2, 17, 18*). The clinical data imply that the BMC rejection may be mediated by conventional T cells, antibody-dependent cell cytotoxicity, or complement-dependent cell cytotoxicity in highly sensitized recipients (*17, 19, 20*). However, these propositions are based on the results including experimental data in vitro in a few cases, yet the critical components that contribute to the BMC rejection in vivo remain obscure. In the clinical setting, numerous factors related to BMC rejection, such as BMC dose, myeloablative regimen, aging, and various degrees of graft-versus-host disease (GVHD), prevent the identification of an unequivocal contribution of immunological components to allogeneic BMC rejection. It is important to clarify the precise mechanism of BMC rejection in allosensitized hosts in order to accomplish the following goals, namely, to broaden the application of BMT for the treatment of various diseases, to improve the success rate, and to decrease the mortality of patients waiting for treatment.

In studies of experimental BMT models using naive mice, the components responsible for the rejection of allogeneic BMC are the NK cells and the T cells. NK cell is a well-known major component in hybrid resistance, and high doses of transfer of BMC overcome this resistance (*12, 13, 21, 22*). T cell is the most important component in the rejection of allografts. In nonsensitized murine BMT, conditioning regimens to manipulate T cells, namely, lethal irradiation or additional treatments such as the administration of T-cell-depleting antibody, thymic irradiation, or cyclophosphamide under nonmyeloablative conditions, are needed to overcome the T cell resistance (*7, 8, 23–25*). In contrast to the detailed analysis in BMC rejection in BMT using naive mice, the mechanism of BMC rejection in sensitized recipients remain unclear. A few studies have suggested that T cell components, most likely radioresistant memory T cells, are responsible for the rejection of donor BMC (*26, 27*). In the studies, sensitized T cell transfer models were used, yet it remains only partly understood how multiple alloreactive immunological components affect rejection within the sensitized host. In particular, the role played by antiallogeneic antigen antibodies (alloAbs), which is not present in naive mice, is of great interest in this context.

In the present study, we investigated the impact of allosensitization on BMC engraftment and we attempted to identify the key effectors in donor BMC rejection using a conventional fully allogeneic BMT model. To avoid an indefinite nonspecific drug effect and the GVHD reaction, lethal irradiation and T cell-depleted BMC (TCD-BMC) were used as a basic protocol.

MATERIALS AND METHODS

Sensitization of Recipient Mice and BMT

C57BL/6 (B6; H-2^b, Ly-5.2)-recipient mice were intraperitoneally injected with 1×10^7 splenocytes from C3H/HeJ (H-2^k, Ly-5.2) mice on day −35 (sensitized recipients). On day 0, B6-recipient mice had been lethally irradiated with 10 Gy whole-body irradiation (WBI, ^{137}Cs; Gammacell 40, Atomic Energy of Canada Limited, Ottawa, Canada) and the animals were intravenously injected with 2×10^7 or 10×10^7 donor C3H or third-party SJL/J (H-2^s) TCD-BMC, or with mixed BMC which consisted of 10×10^7 TCD-BMC from C3H mice and 5×10^6 TCD-BMC from Ly-5.1-congenic B6 mice (online supporting information for materials).

T-cell Isolation and Adoptive Transfer to Naive Mice

Spleens and lymph nodes were collected from nonsensitized or sensitized B6 mice five weeks after alloantigen exposure, and the T cells were separated from the collected cells using the MACS separation system according to the manufacturer's instructions (online supporting information for materials). Naive B6 mice were intravenously injected with 4×10^7 purified T cells 24 hr before allogeneic BMT using 2×10^7 TCD-BMC from C3H mice.

Serum Transfer Experiments

Sera were aseptically collected from B6 mice which had previously been injected intraperitoneally with one of the following: 1) no splenocytes (naive-serum); 2) 1×10^7 splenocytes from C3H mice five days (5D-serum) before the serum sample collection; or 3) 1×10^7 splenocytes from C3H mice five weeks (5W-serum) before the serum sample collection. Sera containing no Ig were obtained from B cell-deficient C57BL/6 mice (μMT; H-2^b) mice, which had been injected intraperitoneally with 1×10^7 splenocytes from C3H mice five days before the serum sample collection (nonIg-serum). The titers of alloAbs in the serum were determined as mentioned in supporting information for the Web site, and only the following two types of sera were used for the serum-transfer experiments: 1) 5D-serum containing abundant donor reactive-IgM (allo-IgM) and little donor-reactive IgG (allo-IgG), and 2) 5W-serum containing no allo-IgM and abundant allo-IgG. The serum samples were stored at −20°C until use. Naive B6 mice were intravenously injected with 600 μl of sera 24 hr and 1 hr before allogeneic BMT using 2×10^7 TCD-BMC from C3H mice.

Statistics

The statistical significance of the survival curves was determined by the log-rank test and that of the ELISPOT assay was determined by one-way ANOVA and Tukey's multiple comparison test (a posthoc test) using GraphPad PRISM 4 software (version 4, GraphPad Software, Inc. San Diego, CA). A value of $P<0.05$ was considered to be statistically significant.

Additional Information

In detail, additional supplemental information for Materials and Methods is available at the *Transplantation* web site: http://www.transplantjournal.com/.

RESULTS

Protocol for Allosensitization and Kinetics of Alloantibody Production

Allosensitization was induced by intraperitoneal administration of C3H/Hej (C3H) donor splenocytes into C57BL/6 (B6) recipients. To confirm the successful adaptive immune response to the alloantigen, recipient sera were obtained following the administration of donor splenocytes, and we measured the presence of serum alloAbs against the donor splenocytes. The levels of allo-IgG peaked at four to seven weeks after allosensitization and then slowly decreased, but the allo-IgG was present for at least nine weeks thereafter (Fig. 1A). The titers of allo-IgM increased immediately after allosensitization, and arrived at the maximum level within the first week; thereafter, the allo-IgM rapidly decreased to undetectable levels within three weeks. The detected antibodies were alloantigen-specific, as there was no detectable antibody to either syngeneic B6 splenocytes or to third-party SJL/J splenocytes (data not shown).

Donor-specific BMC Rejection in Allosensitized Recipients

In this study, in order to investigate the potential immunological components in allosensitized mice that are responsible for the rejection of allogeneic BMC, we used a myeloablative lethally irradiated BMT model in a fully allogeneic murine combination, in which BMC rejection exerted a lethal effect due to a failure of bone marrow function. Lethal irradiation is a method for the induction of a donor chimera in naive recipient mice. BMT from C3H mouse donors was performed to nonsensitized B6-recipients and sensitized B6-recipients at five weeks after immunization by C3H splenocytes. All recipients were lethally irradiated (10 Gy) and reconstituted with 2×10^7 donor TCD-BMC of C3H or SJL mice. Because this dose of BMC suffices for the recipient to overcome the rejection mediated by NK cells, all nonsensitized recipients reconstituted with BMC from C3H mice survived (MST [mean survival time] > 100 days; Figure 1B). On the other hand, none of sensitized recipients survived (MST ± SEM [standard error of the means] = 10.2 ± 1.7 days; Figure 1B). The rejection of BMC was found to be donor-specific because all sensitized recipients that received third-party SJL-BMC survived (MST > 100 days; Figure 1B). This rejection of BMC was not overcome by the administration of a mega-dose of 1×10^8 BMC (MST ± SEM = 8.3 ± 2.1 days; Figure 1B). Moreover, the increased dose of irradiation, i.e., the maximum possible radiation dose (*28*), did not suffice for the recipient to overcome the rejection of BMC (data not shown). We also confirmed by autopsy that this lethal effect was not induced by a GVHD reaction (data not shown).

Cellular Components Are Not Solely Responsible for BMC Rejection in Sensitized Recipients

Initially, we investigated which critical cellular components might be responsible for BMC rejection in allosensitized recipients. Because the potential cellular components, which are known to have allorecognition systems and cytotoxic effector function, consist of NK cells and T cells, we conducted antibody-mediated depletion studies in BMT models using allosensitized recipient mice. The allosensitized recipient mice treated with anti-ASGM1 antiserum (NK-cell depletion) died on approximately day 10 after allogeneic BMT (MST ± SEM = 8.9 ± 2.1 days; Figure 2A). This rejection could not be overcome by the use of 1×10^8 C3H-BMC (MST

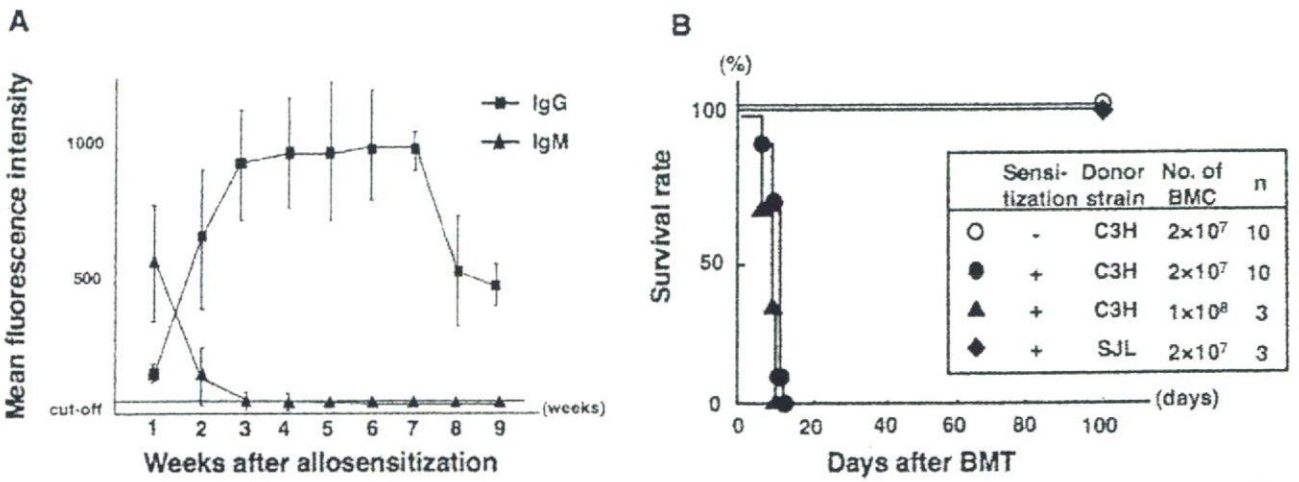

FIGURE 1. Donor-specific BMC rejection in allosensitized recipient mice. (A) The kinetics of allo-IgG and allo-IgM in sensitized recipients. Sera from B6-recipients injected with splenocytes from donor C3H mice, were collected at the indicated time points after allosensitization, and levels of allo-IgG (■) and allo-IgM (▲) were determined using flow cytometry, as described in online supporting information for materials. The data are presented as the average of the mean fluorescence intensity ± SEM of a total of six mice from three independent experiments. (B) B6-recipient mice were sensitized with splenocytes from donor C3H mice at five weeks before allogeneic BMT. On day zero, the allosensitized mice were irradiated with 10 Gy WBI, and injected with 2×10^7 (●: n=10) or 1×10^8 (▲: n=3) donor C3H or 2×10^7 third-party SJL BMC (◆: n=3). As a control group, non-sensitized mice were irradiated with 10 Gy WBI, and injected with 2×10^7 (○: n=10) donor C3H on day zero. The data indicate the survival rates of recipient mice after BMT and were collected from two independent experiments.

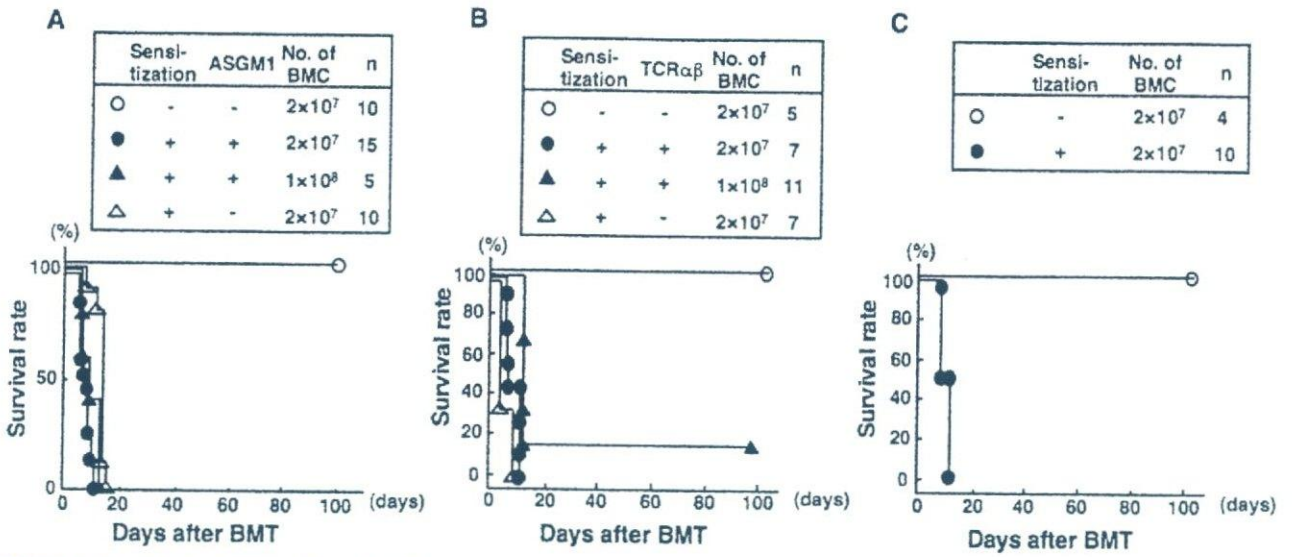

FIGURE 2. The depletion effect of cellular components in BMC-rejection of allosensitized recipients. (A) The depletion of NK cells in BMC rejection in allosensitized hosts. B6-recipients were sensitized with splenocytes from C3H mice at five weeks before allogeneic BMT. To deplete the NK cells, allosensitized mice were intraperitoneally injected with 100 μl of anti-ASGM1 mAb on days −2, 0, 3, and 6. On day zero, allosensitized mice, treated with anti-ASGM1, were irradiated with 10 Gy WBI and intravenously injected with 2×10^7 donor C3H BMC (●: n=15, MST ± SEM=8.9 ± 2.1 days) or 1×10^8 donor C3H BMC (▲: n=5, MST ± SEM=9.6±3.3 days). As control groups, non-sensitized (○: n=10, MST > 100 days) and allosensitized (△: n=10, MST ± SEM=10.4±1.7 days) mice, which had not been treated with anti-ASGM1, were irradiated with 10 Gy WBI and intravenously injected with 2×10^7 donor C3H BMC on day zero. The data indicate the survival rates of recipient mice after BMT and were collected from three independent experiments. (B) The depletion of $\alpha\beta$ T cells in BMC rejection in allosensitized hosts. To deplete the T cells, allosensitized mice were injected with 400 μg of anti-TCR-$\alpha\beta$ mAb on days −3 and −2. On day zero, allosensitized mice, treated with anti-TCR-$\alpha\beta$ mAb, were irradiated with 10 Gy WBI and were intravenously injected with 2×10^7 donor C3H BMC (●: n=7, MST ± SEM=8.9±0.6 days) or 1×10^8 donor C3H BMC (▲: n=11, MST ± SEM=25.7±11.0 days). As control groups, non-sensitized (○: n=5, MST >100 days) and allosensitized (△: n=7, MST ± SEM=9.3±2.4 days) mice, which had not been treated with anti-TCR-$\alpha\beta$ mAb, were irradiated with 10 Gy WBI and intravenously injected with 2×10^7 donor C3H BMC on day zero. The data indicate the survival rates of recipient mice after BMT and were collected from three independent experiments. (C) BMC-rejection in sensitized B cell-deficient mice. μMT mice were sensitized with splenocytes from donor C3H mice at five weeks before allogeneic BMT. On day zero, non-sensitized (○: n=4, MST >100 days) and allosensitized B cell-deficient mice (●: n=10, MST ± SEM=9.0±0.5 days) were irradiated with 10 Gy WBI, and injected with 2×10^7 donor C3H BMC. The data indicate the survival rates of recipient mice after BMT.

± SEM=9.6±3.3 days; Figure 2A). Furthermore, all of the allosensitized recipient mice treated with anti-TCR-$\alpha\beta$ mAb also rejected the donor BMC when 2×10^7 of C3H-BMC were used (MST ± SEM=8.9±0.6 days; Figure 2B). Two of eleven recipients that had been treated with anti-TCR-$\alpha\beta$ mAb and had received a transplant of 1×10^8 C3H BMC showed long-term survival (Fig. 2B) and exhibited durable multilineage chimerism including T cells, B cells, macrophages, and granulocytes (data not shown); however, the survival period did not significantly differ from that of the recipients when 2×10^7 C3H BMC were used (P=0.1596). In addition, allosensitized recipient mice treated with both anti-TCR-$\alpha\beta$ mAb and anti-ASGM1 also rejected the donor BMC (MST ± SEM=8.0±0.6 days; n=3, data not shown).

Because the early death of recipient mice prevented the direct assessment of donor BMC rejection in the above experiments, we performed a mixed BMT experiment using 5×10^6 syngeneic Ly 5.1 B6-TCD-BMC and 1×10^8 of TCD-C3H-BMC. Using this system, we were able to detect residual cells, including NK, NKT, and T cells from B6-recipients (Ly-5.2) after lethal irradiation (Fig. 3, A and B). In agreement with the results obtained from the full-donor BMT experiment, sensitized recipient mice rejected the C3H BMC, and anti-ASGM1 or anti-TCR-$\alpha\beta$ mAb treatment did not lead to an avoidance of BMC rejection in the sensitized recipient mice (Fig. 3C). On the other hand, non-sensitized recipient mice exhibited multilineage mixed chimerism 6 weeks after BMT (Fig. 3C and data not shown). Whereas recipient-derived cellular components (Ly-5.2$^+$H-2Kb$^+$), including NK, NKT, and T cells, were clearly detected in nonsensitized as well as in sensitized recipient mice, no recipient-derived NK cells were detected in recipients treated with anti-ASGM1 and no recipient-derived T and NKT cells were detected in recipients treated with anti-TCR-$\alpha\beta$ mAb (Fig. 3, A and B). This result indicated that treatment with either antibody sufficed to delete radioresistant cellular components, and the failure to prevent the BMC rejection was not due to the inadequate effects of the depleting antibodies.

Taken together, the results of these antibody-mediated depletion studies have indicated that cellular components, including NK, NKT, and T cells, are not solely responsible for the rejection of donor BMC in sensitized recipient mice.

B Cells and AlloAbs Are Not Solely Responsible for BMC Rejection in Sensitized Recipients

Because sensitized recipient mice had high titers of the alloAbs and they rejected transplanted BMC in a donor-specific manner (Fig. 1), donor-specific alloAbs, which are absent in naive mice, were possibly responsible for the donor BMC rejection. To assess the contribution of alloAbs produced by B cells and its finally differentiated plasma cells in BMC rejection in sensitized recipients, BMT using C3H TCD-BMC was performed on nonsensitized and sensitized μMT mice at five weeks after immunization with C3H splenocytes. Although all nonsensitized μMT recipients exhibited both reconstitution of the C3H BMC and long-term survival (MST > 100 days), none of the sensitized μMT recipients survived (MST ± SEM=9.0±0.5 days; Figure 2C). This finding suggests that B cells and alloAbs were not solely responsible for the donor BMC rejection in sensitized recipient mice.

Radioresistant Memory T Cells from Sensitized Recipient Mice Can Lead to the Rejection of Donor BMC after Lethal Irradiation

In the current study, sensitized recipient mice rejected the BMC in a manner dependent on the sensitizing donor strain (Fig. 1B), which indicated that immunological memory compartments capable of distinguishing donor BMC from third-party BMC, play a role in the rejection of donor BMC. To date, no such components other than memory T cells and immunoglobulin have been identified in the process of sensitization. These considerations, taken together with the results of these antibody-mediated depletion studies and experiments using μMT mice, suggest the possibility that the memory T cells and antidonor immunoglobulin act independently to reject the donor BMC in sensitized recipient mice. It is likely that memory T cells may be more radioresistant than naive T cells, and sensitized mice may have a larger number of T-cell, which recognize donor antigens, than naive mice following lethal irradiation. In fact, abundant recipient-derived radioresistant T cells were detected in sensitized recipients, even after the recipients were exposed to lethal irradiation (Fig. 3B). We therefore conducted an analysis of the expression of CD44 and CD62L in surviving T cells after lethal irradiation (*29–31*). In both CD4 T cells and CD8 T cells, the respective percentages of the naive phenotype, $CD44^{low}CD62L^{high}$, were remarkably reduced, and the percentages of the effector/memory phenotype, $CD44^{high}CD62L^{low}$, were clearly increased in the allosensitized recipients after lethal irradiation (Fig. 3D). The percentage of the central memory phenotype, $CD44^{high}CD62L^{high}$, in CD8 T cells was slightly increased, but this phenotype in CD4 T cells was hardly detected. The radiation-mediated reduction rates in the number of effector/memory phenotypic T cells were lower than those of the naive T cell population (Table 1). As regards the nonsensitized recipients, although the number of splenocytes obtained was too small to precisely quantify the reduction in T cells, the radiosensitivity of each T cell phenotype was compatible with that of each T cell phenotype in sensitized mice (data not shown). Furthermore, to assess whether or not the radio-resistant T cells in sensitized mice included donor-antigen-responsive memory T cells at a high frequency, we performed an ELISPOT assay for T cells producing IFN-γ, an important functional cytokine in transplantation immunology, using a short-term culture system, in which memory T cells can be distinguished from naive T cells by their ability to rapidly produce cytokines (*32, 33*). The ELISPOT assay for the detection of IFN-γ-production of memory murine and human T cells against allogeneic antigens is thought to provide high levels of sensitivity and specificity, as previously described (*32, 33*). As expected, in the nonirradiated groups, the frequency of IFN-γ-producing T cells that responded to donor antigens (NK- and T-cell- depleted splenocytes) was significantly increased in sensitized B6 mice (mean spot number ± SEM per 100,000 purified T cells=121.7±16.0) as compared to that of responsive cells in untreated B6 mice (31±4.6, $P<0.05$, Figure 3E). This result was suggestive of successful sensitization to the donor antigens. Notably, lethal irradiation in vivo resulted in a remarkable increase in the frequency of IFN-γ-producing T cells to donor antigens in sensitized mice (409.0±32.1, $P<0.001$), as compared to that of responsive cells in nonirradiated sensitized mice; however, this increase was not observed in nonsensitized mice (irradiated mice vs. untreated mice=20.3± 7.3 vs. 31.0±4.6, $P>0.05$, Figure 3E). In all groups, IFN-γ-producing T cells in response to syngeneic splenocytes were hardly detected (Fig. 3E). These findings suggest that donor antigen-reactive memory T cells were relatively radioresistant and maintained the ability to produce IFN-γ after lethal irradiation in vivo.

To assess whether or not radioresistant memory T cells were a critical component in the rejection of donor BMC, we also performed an adoptive transfer experiments using purified T cells, which were isolated from nonsensitized or sensitized B6 mice. Recipients with T cells transferred from naive mice showed long-term survival (MST > 100 days; Figure 4) and durable multilineage chimerism (data not shown). However, the recipients with T cells transferred from allosensitized mice rejected the C3H BMC (MST=7.0±1.7 days; Figure 4). These findings suggest that donor antigen-reactive memory T cells are radio-resistant, and these cells act as effectors in cases of donor BMC rejection in sensitized recipients.

Allo-IgM and Allo-IgG Antibodies Contribute to Donor BMC Rejection in Sensitized Mice

To investigate whether or not alloAbs are involved in the rejection of donor BMC, naive mice that received a transfer of serum from either sensitized or nonsensitized mice were lethally irradiated and transplanted with donor BMC. In the experiments described above, we had used sensitized recipients that had been immunized five weeks before BMT. The sensitized recipients had only allo-IgG, but not allo-IgM (Fig. 1A). In the preliminary experiments, the recipient mice, which had been sensitized by C3H splenocytes one week before BMT, also rejected the C3H BMC, in spite of treatment with anti-ASGM1 and/or anti-TCR-$\alpha\beta$ mAb (data not shown). The sensitized recipients had high titers of allo-IgM and low titers of allo-IgG (Fig. 1A). Therefore, we separately assessed the roles played by the allo-IgM and allo-IgG in cases of BMC rejection in sensitized recipients. We used serum obtained from B6 mice, which had been allosensitized five days before (5D-serum) and five weeks before (5W-serum) the sample collection; these samples contained predominantly allo-IgM and allo-IgG antibodies, respectively. Here, we used only the 5D-serum samples containing abundant

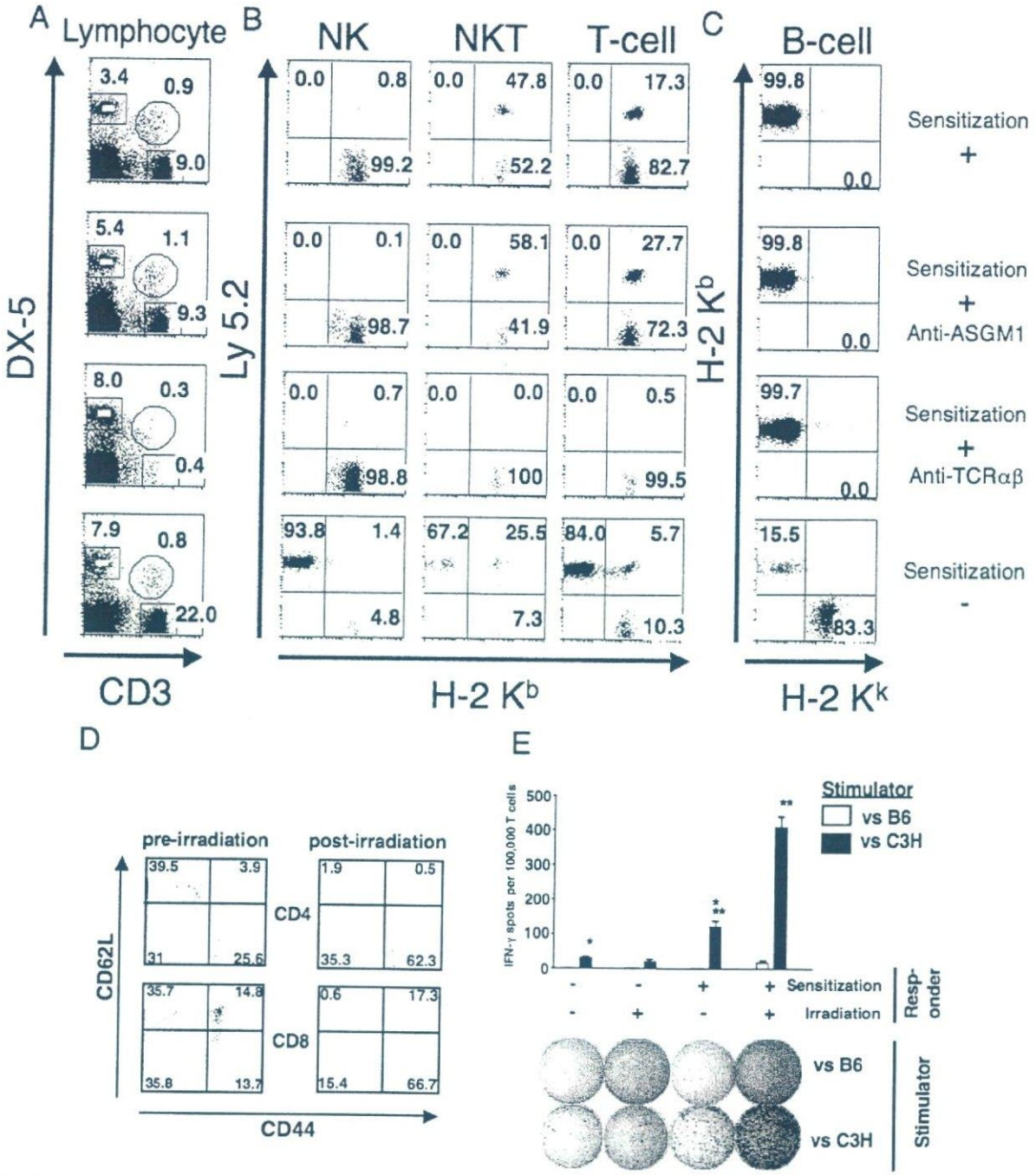

FIGURE 3. Assessment of mixed chimerism and recipient-derived radio-resistant cellular components. (A-C) B6 (H-2^b, Ly-5.2)-recipient mice were sensitized with splenocytes from donor C3H mice at five weeks before allogeneic BMT. On day zero, non-sensitized mice (bottom panels) and allosensitized mice without depletion (top panels), with NK cell depletion (second to top panels), or with T cell depletion (third to top panels) were irradiated with 10 Gy WBI, and transplanted with 10×10^7 TCD-BMC from C3H mice (H-2^k, Ly-5.2) and 5×10^6 TCD-BMC from Ly-5.1-congenic B6 mice (H-2^b, Ly-5.1). On week six after BMT, the peripheral blood lymphocytes were stained with PE-conjugated anti-CD49b/Pan-NK (DX-5) mAb, APC-conjugated anti-CD3 mAb, FITC-conjugated anti H-$2K^b$ mAb, and biotin-conjugated anti-Ly-5.2 mAb, followed by PerCP-streptavidin staining (A, B), or the cells were stained with PE-conjugated anti-B220 mAb, FITC-conjugated anti-H-$2K^b$ mAb, and biotin-conjugated H-$2K^k$ mAb, followed by APC-streptavidin staining (C). The dot plots show the expression of CD3 and DX-5 (CD49b/Pan-NK) among the lymphocyte-gated population (A), the expression of Ly-5.2 and H-$2K^b$ on NK cells (DX-5[CD49b/Pan-NK] $^+$CD3$^-$ cell-gated population in [A]), NKT cells (DX-5[CD49b/Pan-NK] $^+$CD3$^+$ cell-gated population in [A]), or T cells (DX-5[CD49b/Pan-NK] $^-$CD3$^+$ cell-gated population in [A]) (B), and the expression of H-$2K^k$ and H-$2K^b$ on B220^{high} cells in a lymphocyte-gated population (C). H-$2K^{b+}$Ly-5.2$^+$, H-$2K^{b+}$Ly-5.2$^-$, and H-2Kb$^-$Ly-5.2$^+$ cells were derived from B6-recipients, syngeneic B6 (Ly5.1)-BMC, and donor C3H-BMC, respectively (B). The indicated numbers represent the percentages of each population. The data are representative of three independent experiments.

TABLE 1. Naive/memory phenotypic analysis of radioresistant T cells

T cell population	CD4			CD8		
	Pre-Ra ($\times 10^5$)	Post-Ra ($\times 10^3$)	Reduction rate	Pre-Ra ($\times 10^5$)	Post-Ra ($\times 10^2$)	Reduction rate
$CD44^{low}CD62L^{high}$	36.0±4.6	9.1±0.4	1/396	22.5±2.8	4.3±0.2	1/5232
$CD44^{high}CD62L^{high}$	3.4±0.3	2.1±0.3	1/1619	8.0±1.4	108.8±20.0	1/74
$CD44^{high}CD62L^{low}$	29.9±3.7	347.0±15.8	1/9	9.2±0.5	456.7±57.7	1/20
$CD44^{low}CD62L^{low}$	32.7±4.4	203.2±10.9	1/16	27.9±3.0	95.5±3.0	1/292

The data are indicated as the mean cell number of indicated population of splenocytes ± SEM. The data are representative of two independent experiments. B6 mice, which had been immunized with C3H spleen cells five weeks before irradiation, were or were not irradiated with 10 Gy WBI. The spleens were collected from the irradiated (n=3) or nonirradiated (n=3) B6 mice, on day three after WBI. The splenocytes were stained with FITC-conjugated anti-CD44 mAb, PE-conjugated anti-CD62L mAb, APC-conjugated anti-CD3 mAb, and biotinylated CD4 or CD8 mAb, followed by PerCP-streptavidin staining. CD4 T cells and CD8 T cells were defined as $CD3^+CD4^-$ cells and $CD3^+CD8^+$ cells, respectively.

Pre-Ra, nonirradiated B6 mice; Post-Ra, irradiated B6 mice; Reduction rate, (mean cell number of indicated population of splenocytes in irradiated B6 mice)/(mean cell number of indicated population of splenocytes in non-irradiated B6 mice).

allo-IgM and little allo-IgG. To rule out the possible effects of other soluble factors in the serum, we also used the serum of naive B6 mice (naive-serum) or μMT mice, which had been sensitized five days before the sample collection (nonIg-serum). It was found that all recipients that had received a transfer of 5D-serum, and three of six recipients that had received a transfer of 5W-serum rejected the donor BMC, but this was not the case with the recipients that had received a transfer of naive-serum or nonIg-serum (Fig. 5). These findings thus suggest that both the allo-IgM and allo-IgG contributed to donor BMC rejection in the sensitized mice.

DISCUSSION

Positive and negative selection of T cells involves the cell's response to its own MHC, such that the peripheral T cells cannot directly react to one's own MHC, yet numerous T cells can still directly react to allogeneic MHC (direct recognition) (*34*, *35*). It is reasonable that memory T cells primed by exogenous antigens will show crossreactivity to allogeneic MHC. Therefore, in the context of clinical transplantation, it is possible that recipients have many more immunological memory compartments reactive to donor antigens than expected, especially in cases involving MHC-disparate combinations. Indeed, in clinical organ transplantation and BMT, aging is a known risk factor (*15*, *36*, *37*). Thus, a recipient's immunological history might contribute to the difficulty of controlling a rejection. In our BMT models using sensitized recipient mice, lethal irradiation alone did not suffice to delete memory T cells, and the residual T cells then facilitated the rejection of donor BMC (Figs. 3 and 4). This rejection was not overcome by the administration of a megadose of BMC, and it also occurred in B cell-deficient recipient mice, thus indicating that such radioresistant T cells exhibited substantial capacity for rejection of donor BMC, independent of the effect of alloAbs. Therefore, a critical obstacle to BMT in sensitized recipients could be the memory T cells. This interpretation of the findings is compatible with clinical BMT outcome, as well as canine BMT models, in which T cell immunotoxin, such as ATG, have been used to reduce the graft rejection rate in MHC-disparate combinations and in highly sensitized recipients (*2*, *38–40*).

It is possible that not only memory T cells, but also circulating alloAbs, could be obstacles to successful BMT in sensitized recipients. In the present study using sensitized recipients, we clearly demonstrated that allo-IgM had a stronger impact on BMC rejection than did allo-IgG, independent of the effect of memory T cells. This finding suggests that the precise assessment of allo-IgG and allo-IgM may contribute to the successful prediction of donor BMC rejection; moreover, selective adsorption may contribute to reducing the risk of infection. Furthermore, it was found that the administration of anti-TCR-$\alpha\beta$ mAb and BMT using megadose BMC resulted in the survival of 2 of 11 sensitized recipients for more than 100 days, and these recipients showed multilineage chimerism (Fig. 2B). In addition, the transfer of serum containing allo-IgG resulted in the rejection of donor BMC among half of the B6-recipients; the lower rate of rejection of BMC among the B6-recipients may have been due to an insufficient amount of transferred IgG, as compared with that of the sensitized recipients (Fig. 5). These findings suggest

FIGURE 3. Continued. (D) Naïve/memory phenotypic analysis of radio-resistant T cells. B6 mice had been immunized with C3H spleen cells five weeks before irradiation, and the splenocytes were collected from the non-irradiated (left panel) or irradiated sensitized B6 mice (right panel), on day three after WBI. The splenocytes were stained with FITC-conjugated anti-CD44 mAb, PE-conjugated anti-CD62L mAb, APC-conjugated anti-CD3 mAb, and biotinylated CD4 or CD8 mAb followed by PerCP-streptavidin staining. The dot plots show the expression of CD62L and CD44 on $CD3^+CD4^+$ (upper panels) or $CD3^+CD8^-$ (lower panels) lymphocytes. The indicated numbers represent the percentages of each population. These data are representative of five independent experiments. (E) ELISPOT assay for IFN-γ-producing T cells. 1×10^6 cells from NK and T cell-depleted irradiated (3000 rad) splenocytes (stimulator) from B6 mice (white bars) or C3H mice (black bars) were cocultured for eight hr with 1×10^5 purified splenic T cells (responder cells) from B6 mice in the indicated groups (shown on the X-axis), as described in Materials and Methods. The data are indicated as mean spot number ± SEM per 100,000 purified T cells from triplicate wells. The asterisks indicate statistical significance by Tukey's multiple comparison test (*$P<0.05$, **$P<0.01$). Images of representative ELISPOT wells for each of the responders against a B6 stimulator (upper panels) or C3H stimulator (lower panels) are shown below the groups indicated on the X-axis of the bar graph.

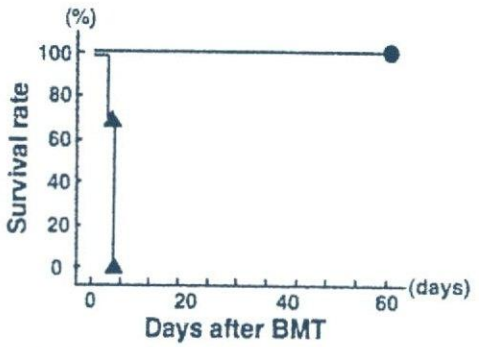

FIGURE 4. Naive mice with transferred T cells purified from allosensitized mice reject allogeneic BMC. Naive B6 mice that had received a transfer of 4×10^7 purified T cells derived from nonsensitized (●: n=3, MST > 100 days) or sensitized B6 mice (five weeks after allosensitization, ▲: n=4, MST ± SEM=7.0±1.7 days) on day −1, were irradiated with 10 Gy WBI, and transplanted with 2×10^7 donor C3H-BMC on day zero. The data indicate the survival rates of recipient mice after BMT and are representative of two independent experiments.

that the capacity of alloAbs, especially IgG, to contribute to rejection, may be weaker than that of T cells, and the balance between the alloAbs titer and the amount of injected BMC may be predictably associated with a rejection of donor BMC.

In general, antibodies exhibit effector functions via complement-dependent mechanisms and complement-independent mechanisms (*41*, *42*). There is known to be a clear functional distinction between IgG and IgM. The former exerts effector function via the two mechanisms, and the latter exerts effector function mainly via complement-dependent mechanisms (*41*). Because IgM is more capable of fixing complement than is IgG (*43*), and serum transfer experiments showed a higher rate of donor BMC rejection associated with allo-IgM than with allo-IgG, it is likely that complement dependent mechanisms play an important role in alloAbs-mediated BMC rejection. On the other hand, we clearly showed that memory T cells were radio-resistant and the frequency of T cells that produced IFN-γ in response to donor antigens were increased after lethal irradiation. In this study, we only assessed IFN-γ-production in radioresistant memory T cells. It is possible that other cytokines or cytotoxic molecules were involved in the BMC rejection in our system. Komatsu and colleagues reported that CD8 T cell-mediated BMC rejection was not entirely dependent on already known cytotoxic molecules such as TNF-α, the perforin-granzyme system, Fas ligand, and TRAIL (*26*). Their findings suggest that unknown molecules may be involved in the BMC rejection by memory T cells. To elucidate the precise mechanisms of alloAbs-mediated and radio-resistant memory T cell-mediated BMC rejection, important issues in the development of alternative BMT strategies for treating highly sensitized recipients, further investigations will still be needed.

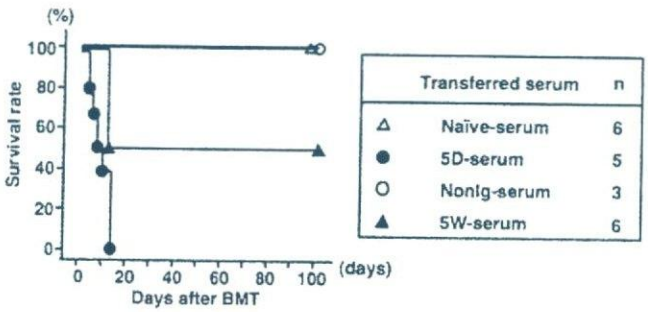

FIGURE 5. Role of AlloAbs in the BMC rejection. On days −1 and 0, naive B6 mice were injected with 600 μl of serum from the following groups: naive B6 mice (naive-serum, △: n=6, MST >100 days), B6 mice sensitized with C3H spleen cells 5 days before serum-sampling (5D-serum, ●: n=5, MST ± SEM=8.6±4.2 days), μMT mice sensitized with C3H spleen cells five days before serum-sampling (nonIg-serum, ○: n=3, MST > 100 days), or B6 mice sensitized with C3H spleen cells five weeks before serum-sampling (5W-serum, ▲: n=6, MST=82.8 days). All mice were irradiated with 10 Gy WBI and injected with 2×10^7 donor C3H BMC on day zero. The data indicate the survival of recipient mice after BMT and were collected from two independent experiments.

Effector memory T cells are present in the blood, spleen, and nonlymphoid tissue, and they rapidly respond to antigens by producing effector molecules, whereas central memory T cells are present in the secondary lymphoid organs, and they produce cytokines and become killer cells in recall response, albeit at a slower tempo than effector memory T cells (*44*). Although it is known that radioresistant T cells are detected after lethal irradiation in murine BMT models, no phenotypic analyses have been reported to date (*45*, *46*). In this study, T cells showing the naive phenotype were almost eradicated by lethal irradiation, but CD4 T cells showing the effector/memory phenotype and CD8 T cells exhibiting the effector/memory phenotype or the central memory phenotype were detected in the spleen of sensitized B6 mice on day three after lethal irradiation (Fig. 3D, Table 1). In general, cells with great proliferative capacity are radio-sensitive. However, this is not the case with T cells, because memory T cells proliferate at a slow rate maintaining a stable population for a long period of time; in contrast, naive T cells do not divide and have a limited lifespan (*47*). The maintenance of memory T cells are responsible for IL-7, IL-15, and/or OX-40-OX-40L interaction (*47*). It remains of interest why quiescent naive T cells are more radiosensitive than are memory T cells; moreover, the reasons for the differences in the radiosensitivity of each phenotype of memory CD4 and CD8 T cells remain unclear. The factors determinative of the radiosensitivity of T cells may include cytokines and/or costimulatory molecules that maintain memory T cells, regardless of their proliferative capacity. In addition, the $CD44^{low}CD62L^{low}$ T cell population in both CD4 and CD8 T cells were detected in wild-type B6 mice, as previously noted (*48*, *49*); the $CD62L^{low}CD44^{low}$ CD8 cells were more radiosensitive than their CD4 counterparts in our system. The $CD44^{low}CD62L^{low}$ T cell population contained $CD3^+TCR\alpha\beta^+DX5^-TCR\gamma\delta^-AnnexinV^-PI^-$, viable αβ T cells (data not shown). Although the functional differences among naive T cells, central memory T cells, and effector/memory T cells have been extensively investigated in a number of studies (*44*, *47*, *49*), little is currently known about the significance of the $CD44^{low}CD62L^{low}$ T cell. It also remains unclear whether or not donor reactive memory T cells were present among the $CD44^{low}CD62L^{low}$ T cells that contributed to the re-

jection donor BMC in this study. Further extensive investigations will be needed in the future to clarify these issues.

In the present series, we used lethal irradiation alone as a immunosuppressant, in order to assess the critical components of BMC rejection, avoiding complex effects associated with other drugs (e.g., cyclophosphamide) or antibodies (e.g., anti-CD40 ligand mAb). However, it is an important issue to clarify how memory T cells are affected by other conditioning regimens. In addition, because not all memory T cells are radioresistant, it still must be clarified which T cells are of the conditioning-resistant type(s) that are classified in detail according to the expression of protein in memory T cells and what mechanisms are involved in radioresistance of memory T cells. To elucidate these issues, comprehensive molecular assays focusing on the molecules related to DNA repair and/or survival factors will be needed. Such information might enable us to delete radioresistant T cells in a donor specific manner in BMT recipient; for example, the recipients could be injected with tolerogeneic donor-derived dendritic cells (*50*) that are genetically modified in order to convert the radioresistance of the alloreactive memory T cells to radiosensitivity prior to BMT. In any case, this application of such information would be very useful in clinical BMT, since the selective inhibition of such T cells would avoid nonselective T cell depletion (e.g., ATG) among recipients, in whom recipient-derived memory T cells would play a role in control of infection, and donor-derived T cells are required to elicit graft vs. leukemia effect and to enhance accommodation of donor-derived stem cells. Furthermore, BMT experiments using mixed BMC have revealed that the population of recipient-derived $B220^+$ cells was extremely small, as compared to that of recipient-derived T cells six weeks after BMT (data not shown), thus indicating that B cells are more radiosensitive than are T cells. It would also be of interest in this context to determine whether or not plasma cells that produce high levels of secretory antibodies are radiosensitive.

In conclusion, we reported here for the first time that donor-reactive radioresistant memory T cells and alloAbs were two independent critical components in an outcome of graft rejection in BMT using sensitized recipient mice. We expect that this BMT model will continue to be useful for the elucidation of the complex mechanisms of BMC rejection in cases of human BMT with HLA-incompatible combination and in highly sensitized patients. In addition, it is expected that elucidation of precise mechanism of BMC rejection, mediated by the two independent components discussed here, can contribute to the broadened application of BMT for the treatment of various diseases.

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Hyperbaric oxygen induces vascular endothelial growth factor and reduces liver injury in regenerating rat liver after partial hepatectomy

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***Background/Aims*: The aim of this study was to investigate the effect and the mechanism of hyperbaric oxygen treatment on regenerating rat liver after partial hepatectomy (PH).**

***Methods*: Wistar rats underwent a 70% PH, followed by treatment with hyperbaric oxygen starting 8 h after PH. The regenerated liver weight and serum parameters were compared. Proliferation of both hepatocytes and sinusoidal endothelial cell (SEC) was also monitored by evaluating the proliferating cell nuclear antigen (PCNA) labeling index. Furthermore, the hepatic adenosine triphosphate levels and vascular endothelial growth factor (VEGF) protein expression were analyzed at different times.**

***Results*: Hyperbaric oxygen treatment significantly reduced the serum alanine aminotransferase levels at 24 h, total bilirubin and total bile acid levels at 48 and 72 h, respectively. No significant differences in the hepatic adenosine triphosphate levels, the restitution of liver weight, or PCNA positive hepatocytes were observed between the two groups. The PCNA positive SEC, in contrast, was significantly increased in the hyperbaric oxygen group at 48 h, furthermore, the hyperbaric oxygen treatment significantly increased the expression of VEGF protein in the regenerating liver at 24 and 48 h.**

***Conclusions*: Hyperbaric oxygen treatment can be considered as a therapeutic modality after massive PH.**

***Keywords*: Liver regeneration; Hyperbaric oxygen; Vascular endothelial growth factor**

1. Introduction

Hepatic resection has become safer and a massive hepatectomy has been aggressively performed for hepatocellular carcinoma, however, liver failure remains one of the major complications and has a high mortality rate [1,2]. The ability of the liver to restore major tissue loss is an important component of the recovery process after a partial hepatectomy (PH), and it is regulated by various growth factors [3]. After a 70% PH in rats, the mass restoration is complete in approximately 7 days [4]. Although the rate of DNA synthesis in hepatocytes begins to increase after 12 h and reaches a peak around 24 h after PH, sinusoidal endothelial cell (SEC) proliferation is delayed in comparison to the hepatocytes and peaks around 96 h [4,5]. This cellular order of proliferation results in the formation of avascular clusters of 10 to 12 hepatocytes [6,7]. The subsequent reconstruction of normal vascular architecture is accompanied by proliferation and infiltration of surrounding endothelial cells into avascular clusters of hepatocytes, and this angiogenic event appears to play an important role in the lobular remodeling after PH [8].

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The role of angiogenesis is to provide nutrition and oxygen to growing tissues, and it has been shown to mediate the physiologic condition of wound healing [9,10]. This phenomenon is regulated by several important angiogenic factors after PH [11,12]. Vascular endothelial growth factor (VEGF) is the most specific known angiogenic factor that has a powerful growth-stimulatory effect on endothelial cells [13], and is also a potent mediator of vascular permeability [14]. Moreover, recent studies have shown that VEGF has protective activities in the liver [15,16].

Hyperbaric oxygen (HBO), exposure to oxygen at a pressure greater than one atmosphere absolute (ATA), has been widely used as a primary therapy in patients with carbon monoxide poisoning, decompression sickness, and arterial gas embolism, and as an adjunctive therapy for the treatment of various diseases accompanied by impaired oxygen delivery [17]. Mazariegos et al. have reported that HBO treatment was also effective for hepatic artery thrombosis after liver transplantation [18]. HBO treatment increases the partial pressure of oxygen in body tissues and has been shown to cause upregulation of growth factors [19], down regulation of inflammatory cytokines [20], and increased angiogenesis [21], and is also known as a therapeutic modality for wound healing [22].

Although it has been demonstrated that HBO treatment has several effects on regenerating liver [23,24], there is no in vivo evidence that intrahepatic angiogenesis occurs after PH. The purpose of the present study was to investigate whether HBO treatment correlates with angiogenesis and has protective effects on regenerating rat liver after PH.

2. Material and methods

2.1. Animals and Surgery

The Kyushu University Institutional Animal Care and Use Committee approved all animal protocols, according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Institutes of Health. Male Wistar rats weighing between 260 to 320 g (Kyudo, Fukuoka, Japan) were used in all experiments (n = 7). All animals were housed in temperature- and light-controlled environmental conditions with a 12-hour light and dark cycle and fed standard rat chow and water as desired. A two-thirds PH was performed under light ether anesthesia as described previously [25]. After a midline laparotomy, the liver was exposed and the median and the left lateral liver lobes were ligated and resected. All animals were allowed free access to standard chow and water after the operation. Rats were sacrificed at various time points (24, 48, 72, and 96 h after PH), by puncture of the aorta and exsanguination under ether anesthesia. Liver samples were obtained, some for histological analysis and some were frozen and stored at −80 °C.

2.2. HBO treatment

Animals were divided into two groups, the control group and the HBO group. In the HBO group, rats were placed into a hyperbaric chamber designed for small animals (Model P-5100S; Barotec Hanyuda Co. Ltd., Tokyo, Japan), pressurized to 2.5 ATA with 100% oxygen for 70 min. Compression and decompression were carried out at a rate of 0.15 ATA/min. The HBO treatment was started at 8 h after PH, and it was performed once a day until postoperative day 4.

2.3. Regenerated Liver Weight

The restitution of the liver weight was determined as the percentage of regenerated liver mass by the following equation: $100\times(C-(A-B))/A$, A being the estimated total liver weight at the time of resection, B being the excised liver weight, and C being the weight of the regenerated liver at the time of sacrifice [26].

2.4. Serum Parameters

The degree of hepatic injury and biliary function was assessed by serum levels of alanine aminotransferase (ALT), total bilirubin, and total bile acid. They were determined by routine clinical chemistry.

2.5. Measurement of the Hepatic adenosine triphosphate (ATP)

For the assay of hepatic ATP levels, the liver tissue was obtained from the right lateral lobe and immediately stored at −80 °C until analysis. The ATP levels were quantified with a commercially available kit (ATP bioluminescent assay kit; SIGMA diagnostics, St. Louis, USA) ($n = 6$).

2.6. Immunohistochemistry

Formalin-fixed liver specimens were embedded in paraffin and 3 μm sections were cut. Proliferating cell nuclear antigen (PCNA) expression was detected by immunostaining using monoclonal anti-PCNA antibody (NCL-PCNA; Novocastra Laboratories, New-castle upon Tyne, UK). The number of PCNA positive cells was counted in 30 random high-power fields. Data were expressed as the percentage of PCNA positive cells per total number of cells. In this study, the spindle-shaped sinusoid-lining cells in the open sinusoids were regarded as SEC, while the more rounded cells were regarded as Kupffer cells [5]. Immunohistochemical staining for VEGF was performed with anti-VEGF antibody (1: 200 dilution; Santa Cruz Biotechnology, Inc., Delaware, CA).

2.7. Western Blot Analysis

Frozen liver tissues were lysed in cold-lysis buffer (100 mmol/L NaCl, 50 mmol/L HEPES, 1% Nonidet P-40, 1 mmol/L EDTA, 0.2 mmol/L phenylmethylsulfonyl fluoride, 1.5 mmol/L pepstatin A, 50 mg/mL TPCK, 0.2mmol/L sodium orthovanadate, 1 mg/mL bestatin, and 1 mg/mL leupeptin). Normalized lysates were boiled in sample buffer, run on a 10% SDS-PAGE gel, and transferred to a polyvinylidine difluoride membrane (Invitrogen, Co., CA, USA). Membranes were blocked for 50 min, in Tris-buffered saline containing 5% skim milk with 0.1% Tween 20. The membrane was probed with anti-VEGF antibody, anti-Angiopoietin-2 antibody (R&D Systems, Inc., Minneapolis, USA), and anti-basic fibroblast growth factor (bFGF) antibody (CHEMICON International, Inc., California) (dilution: 1: 500, respectively) overnight at 4 °C.

2.8. Statistical analysis

All values were expressed as mean ± SEM. Statistical significance was determined by the Mann-Whitney U test. $P < 0.05$ was considered significant.

3. Results

Liver Injury and Biliary Function After PH. The effect of HBO treatment on hepatocyte injury was assessed by

measuring peak serum ALT levels 24 h after PH (Fig. 1A). The serum total bilirubin and total bile acid levels rose after PH, reaching a maximum level after 48 h, and the levels declined thereafter (Fig. 1B, C). In the HBO group, they were significantly lower than in the control group at 48 and 72 h, respectively. The biliary function was significantly improved by HBO treatment after PH.

Hepatic ATP concentration After PH. Because it was reported that the regenerating liver required increased levels of energy charge [28], and HBO treatment improved energy metabolism after PH [24], we then analyzed the hepatic ATP levels (Fig. 2). The hepatic ATP levels of the remnant liver decreased after PH, and

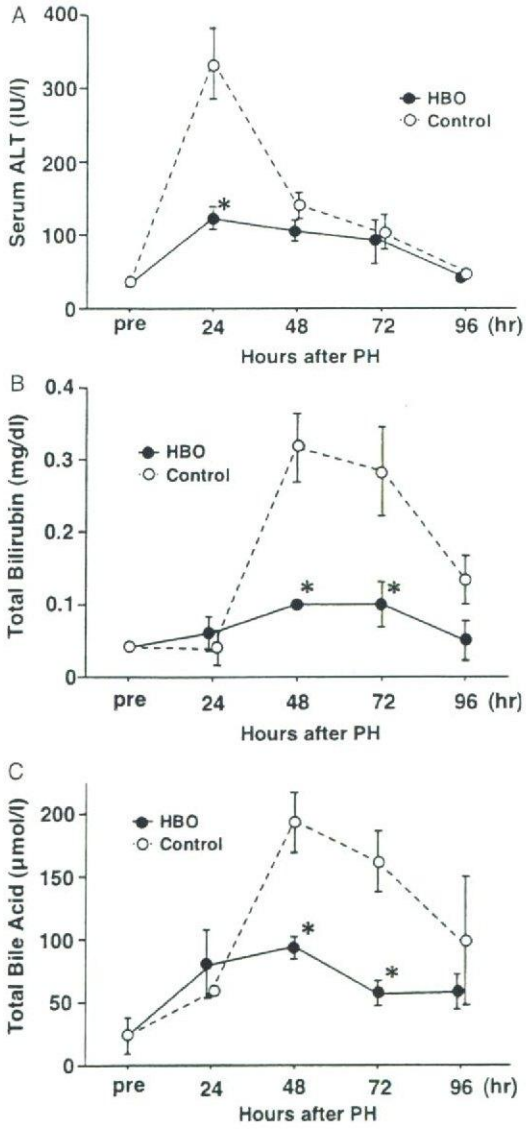

Fig. 1. Serial changes in serum ALT (A), total bilirubin (B) and total bile acid (C) levels after PH. Values are expressed as mean ± SEM. $^*P < 0.05$ versus control group at the same time point.

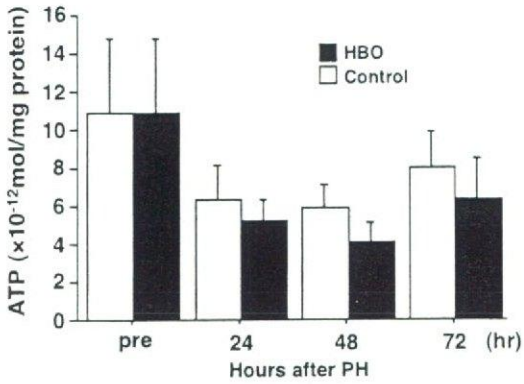

Fig. 2. Changes in the ATP levels of the remnant liver after PH. There were no significant differences in the hepatic ATP levels of both groups. Values are expressed as mean ± SEM.

reached the lowest level at 48 h, then increased at 72 h in the both groups. HBO treatment, however, did not significantly affect the hepatic ATP levels after PH at any time point studied.

Hepatic Regeneration After PH. No significant differences in the restitution of liver weights rates (Fig. 3A) and PCNA labeling index of hepatocytes (Figs. 3B, 4) were observed between the two groups at any time points studied. The number of labeled hepatocytes reached a peak at 48 h after PH in the both groups. Meanwhile, our findings suggest that HBO treatment accelerates the SEC proliferation after PH (Figs. 3C, 4). In control group, increased expression of PCNA labeling index of SEC was found until 72 h after PH, however, in HBO group, it was accelerated (peak at 48 h after PH) compared with that in control group. As presented in Fig. 3C, HBO treatment significantly increased PCNA positive SEC at 48 h, in contrast, it significantly decreased at 72 h after PH.

Effect of HBO on VEGF Expression After PH. Previous studies have demonstrated that HBO induces several angiogenic factors [19,29,30] and is associated with angiogenesis [21], and we found that HBO treatment accelerated the SEC proliferation after PH by PCNA labeling index. To analyze whether HBO treatment also induces angiogenic factors during liver regeneration, we examined their expression in the remnant liver by western blot analysis. VEGF expression in the remnant liver reached a peak at 48 h after PH in the both groups, and HBO treatment significantly increased VEGF expression at 24 and 48 h after PH (Fig. 5A, B). Although HBO treatment caused a small decrease at 72 h, no significant difference was observed in the both groups. On the other hand, HBO treatment did not significantly affect the expression of other angiogenic factors, bFGF and Angiopoitin-2, in the remnant liver at any time point after PH (data not shown).

Immunohistochemical staining for VEGF After PH. We then analyzed the VEGF expression in the remnant