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原　　著

The Fate of Liver Allografts in Radiation Bone Marrow Chimeras in Mice

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Summary

We attempted to establish a new methodology of tolerance induction in liver allografts. When liver tissue of BALB/c (H-2^d) or C57BL/6J (H-2^b) mice were minced and grafted under the kidney capsules of C3H/HeN (H-2^k) mice, it was rejected. However, when C3H/HeN mice were irradiated and reconstituted with BALB/c nu/nu bone marrow cells, they accepted both bone marrow donor-type (BALB/c) and host (thymus)-type (C3H/HeN) liver tissue. Assays for both mixed-lymphocyte reaction (MLR) and induction of cytotoxic T-lymphocytes (CTL) revealed that the newly-developed T cells are tolerant to bone marrow donor-type and host (thymus)-type major histocompatibility complex (MHC) determinants. Based on these data, we propose that liver allografts combined with bone marrow transplantation should be considered as a viable therapy for humans.

Introduction

Since 1963, when STARZL described the first successful orthotopic liver transplantation in man, more than 500 patients have been treated in this way[3,13,14]. In all species, liver allografts are rejected less aggressively than allografts of other organs, because I-region antigens are less prominent in the liver than in other organ[4,10,15]. In man, however, aggressive destructive rejection of a liver allograft will usually occur if no immunosuppression is induced. It would be of benefit to patients if rejection could be controlled without giving immunosuppressive agents. We have previously reported that fully allogeneic chimeras in mice accept both thymus-type and bone marrow-type skin[6]. In addition, we have recently found that allogeneic bone marrow transplantation can treat autoimmune diseases in MRL/l and BXSB mice without showing graft-versus-host reaction (GVHR), provided that bone marrow cells of young nu/nu mice or

Key words: Liver transplantation, Bone marrow transplantation, Major histocompatibility complex, Graft-versus-host reaction, Tolerance.
索引語：肝臓移植，骨髄移植，主要組織適合遺伝子複合体，移植片対宿主反応，寛容．
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T cell-depleted bone marrow cells are used[8,9]. Furthermore, we have found that the newly-developed T cells are tolerant to both bone marrow donor-type and host-type major histocompatibility complex (MHC) determinants[9].

These data prompted us to examine the fate of liver allografts in radiation bone marrow chimeras in mice. Small animals have been of limited use as a model for liver transplantation because the surgery has been technically difficult. In rats, MITO et al. discovered that splenic pulp is the most suitable location for long-term survival of isolated hepatocytes[11].

In the present study, we attempted to graft liver tissue minced to rice-grain size under the kidney capsules of mice. We show that C3H/HeN mice reconstituted with BALB/c nu/nu bone marrow cells accept both BALB/c bone marrow donor-type and C3H/HeN host-type livers.

Materials and Methods

Animals: Inbred C3H/HeN (H-2^k), BALB/c (H-2^d), and C57BL/6J (H-2^b) mice were used under standard laboratory conditions. BALB/c nu/nu mice were obtained from the Central Institute for Experimental Animals, Tokyo, and maintained under specific pathogen-free conditions in our facilities.

Transplantation of bone marrow cells and liver: Two mo old C3H/HeN mice were exposed to 850 rad from a ^{60}Co source and subsequently injected with 1 to 2×10^7 bone marrow cells obtained from 2 mo old BALB/c nu/nu mice. Two or three months after bone marrow transplantation, the mice were anesthetized with pentobarbital (0.05 mg/g body weight, Pitman-Moore, N.J.). Livers taken from 1 to 4 wk old C3H/HeN, BALB/c, or C57BL/6J mice were minced to about rice-grain size in RPMI-1640 medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan) with 0.01% collagenase (Type I, Sigma Chemical Co., USA). The liver tissue was grafted under the left kidney capsdles of the C3H/HeN mice. Two months later, the mice were sacrificed, and the engrafted liver tissue was examined macroscopically and microscopically.

Cell separation: The spleens were aseptically removed, minced, and gently passed through a fine mesh stainless-steel sieve into phosphate-buffered saline.

Mixed-lymphocyte reaction (MLR): MLR was examined by measuring the incorporation of 0.5 μCi ^{3}H-thymidine (New England Nuclear, Boston, USA) into DNA. Triplicate cultures were set up in 96-well round-bottom microtiter trays (Corning Glass Works 25850). Each well contained 2×10^5 responder cells and 1×10^5 stimulator cells in a total volume of 0.2 ml RPMI-1640 medium that was supplemented with 2 mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 μg/ml), 5% heat-inactivated human serum, and 2-mercaptoethanol (2-ME: 5×10^{-5} M, Wako Pure Chemical Industries, Tokyo). Stimulator cells were treated with 50 μg/ml mitomycin C for 30 min at 37°C. The cultures were incubated for 96 hr in a humidified atmosphere of 5% CO_2 in air. ^{3}H-thymidine was present during the last 4 hr of the culturing period. The number of ^{3}H incorporated into trichloroacetic acid-insoluble material was measured by a liquid scintillation counter.

Cytotoxicity assay: Responder cells (7.5×10^6) and mitomycin C (50 μg/ml)-treated stimulator cells (2.5×10^6) were cocultured in RPMI-1640 medium containing 10% heat-inactivated human

serum, supplemented with 5×10^{-5} M 2ME, penicillin, and streptomycin. Cultures were incubated for 5 days at 37°C in 5% CO_2 incubator. P815 (H-2^d), EL-4 (H-2^b), and X5563 (H-2^k) were used as target cells. They were labelled with 100 μCi Na_2 [^{51}Cr]O_4 (New England Nuclear, Boston, USA) by means of incubation for 1 hr at 37°C. Labelled cells were washed three times. These cells (5×10^4) were mixed with effector cells in 200 μl of RPMI-1640 medium in round-bottomed micro-plates and incubated at 37°C in 5% CO_2 for 4 hr. Using the Titertec Supernatant Collection System (Flow Lab., Irvine, Scotland), supernatant was harvested in order to determine released radioactivity. Specific lysis was calculated according to the following formula:

$$\% \text{ Specific lysis} = \frac{\text{Experimental release} - \text{Spontaneous release}}{\text{Maximal release} - \text{Spontaneous release}} \times 100$$

Histopathology: The left kidney with engrafted liver tissue was obtained at autopsy and prepared for light-microscopical observation.

Results

C3H/HeN (H-2^k) mice reconstituted with BALB/c nu/nu (H-2^d) bone marrow cells survived more than 8 mo without showing graft-versus-host reaction (GVHR). Using anti-H-2^k and anti-H-2^d serum plus complement, we confirmed that more than 95% of spleen cells from the chimeras were donor-derived. The mice possessed normal numbers of Thy-1$^+$ cells in the spleen, and the spleen cells significantly responded to PHA, Con A, and LPS (data not shown). The fate of the engrafted liver tissue is summarized in Table 1. Non-treated C3H/HeN mice rejected allogeneic liver tissue of BALB/c (6/6) or C57BL/6J mice (5/5). In contrast, C3H/HeN mice reconstituted with BALB/c nu/nu bone marrow cells rejected third-party C57BL/6J liver tissue (5/5), whereas they accepted both BALB/c bone marrow donor-type (8/10) and C3H/HeN host-type (3/5) liver tissue. As shown in Fig. 1-A, liver cells and central veins of BALB/c mice were found under the kidney capsules of C3H/HeN mice. However, due to rejection, liver cells of

Table 1. Fate of allografted liver tissue in C3H/HeN mice reconstituted with bone marrow cells of BALB/c nu/nu mice

Mouse	Liver donor	No. examined	No. accepted
C3H/HeN(H-2^k)	C3H/HeN	10	8
C3H/HeN	C57BL/6J(H-2^b)	5	0
C3H/HeN	BALB/c (H-2^d)	6	0
C3H/HeN with BALB/c nu/nu B.M.[a]	C3H/HeN	5	3
C3H/HeN with BALB/c nu/nu B.M.	C57BL/6J	5	0
C3H/HeN with BALB/c nu/nu B.M.	BALB/c	10	8

[a] C3H/HeN (H-2^k) mice were irradiated (850 rad) and reconstituted with bone marrow cells (2×10^7) of BALB/c nu/nu (H-2^d) mice. Two or three months after bone marrow transplantation, livers taken from C3H/HeN, BALB/c, or C57BL/6J mice were minced and grafted under the kidney capsules of the C3H/HeN mice.

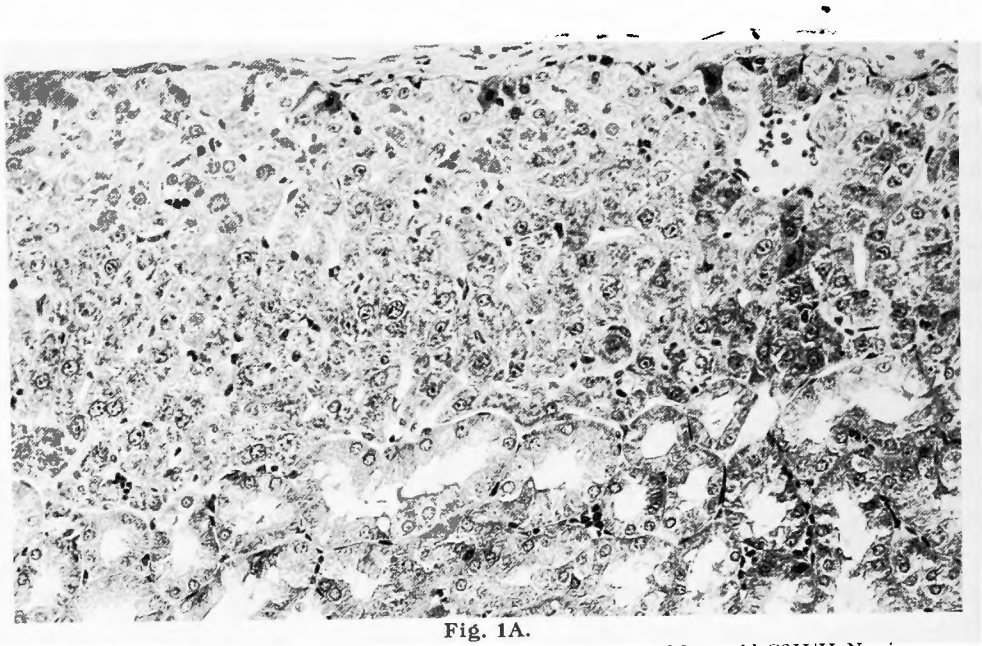

Fig. 1A.

Fig. 1A. Histopathological findings in allografted liver tissue of 6 mo old C3H/HeN mice reconstituted with bone marrow cells of young BALB/c nu/nu (≦2 mo) mice.
A. Non-rejection of the liver tissue from a BALB/c (bone marrow donor-type) mouse grafted under the kidney capsule of a C3H/HeN mouse which had been irradiated and reconstituted with bone marrow cells of BALB/c mice (×200).

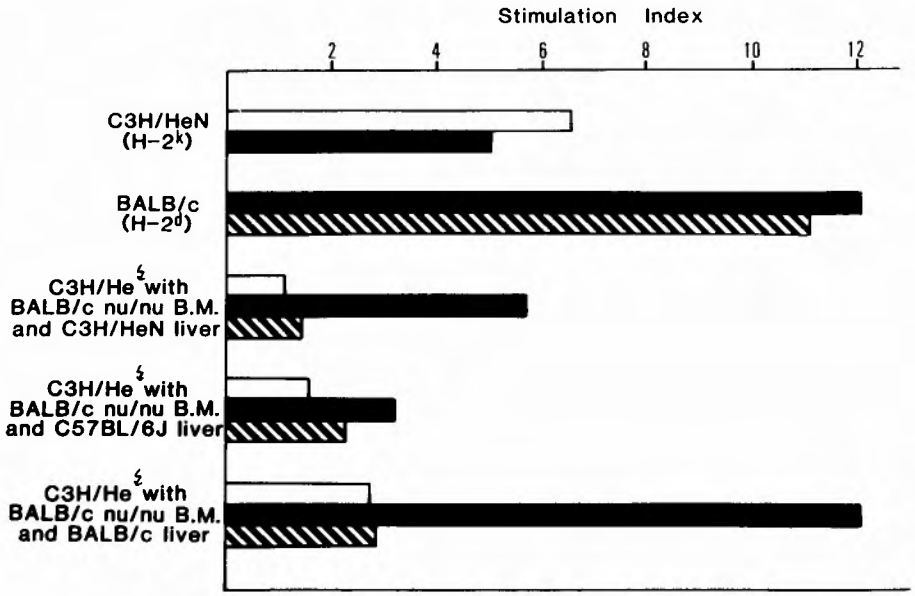

Fig. 2. Allotolerance expressed by T cells after bone marrow transplantation. Mixed-lymphocyte reaction (MLR) reveals that C3H/HeN mice reconstituted with BALB/c nu/nu bone marrow cells are tolerant to both bone marrow donor-type (BALB/c) and host (thymus)-type (C3H/HeN) MHC determinants.
Stimulator: BALB/c (□), C57BL/6J (■), and C3H/HeN (■).

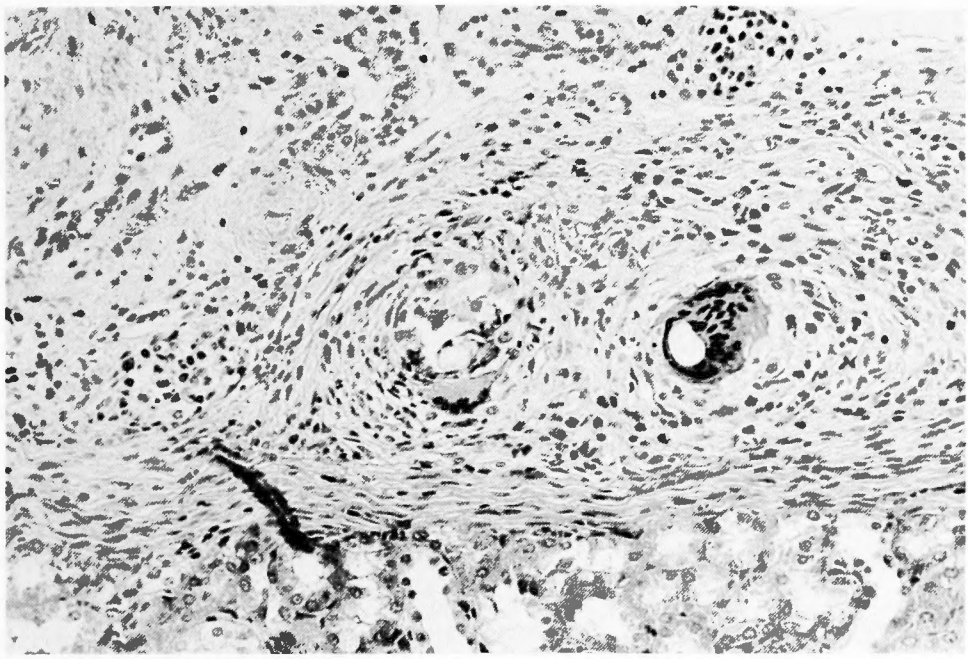

Fig. 1B.

Fig. 1B. Rejection of the liver tissue from a C57BL/6J (third party) mouse grafted under the kidney capsule of a C3H/HeN mouse which had been irradiated and reconstituted with bone marrow cells of BALB/c nu/nu mice. The liver tissue was replaced by fibrous tissue containing multinucleated giant cells (×200).

Table 2. Generation of cytotoxic T-lymphocytes from C3H/HeN mice reconstituted with bone marrow cells of BALB/c nu/nu mice[a]

Spleen cell	Liver donor	E/T[b] ratio	% specific release from target cells (mean±s.d.)		
			X5563 (H-2^k)	EL-4 (H-2^b)	P815 (H-2^d)
C3H/HeN(H-2^k)	–	4/1	0	25.7±0.4	40.9± 6.3
C57BL/6J(H-2^b)	–	4/1	54.7±10.1	0	94.4±23.9
BALB/c(H-2^d)	–	4/1	71.9±15.4	66.9±9.1	0
C3H/HeN with BALB/c nu/nu B.M.[b]	C3H/HeN	2/1	0	22.0±7.5	0
C3H/HeN with BALB/c nu/nu B.M.	C57BL/6J	2/1	0	7.6±0.3	5.7± 8.4
C3H/HeN with BALB/c nu/nu B.M.	BALB/c	2/1	0	21.0±1.6	0.3± 3.4

[a] Responder cells (7.5×10^6) and mitomycin C (50 μg/ml)-treated stimulator cells (2.5×10^5) were cocultured in RPMI-1640 medium containing 10% heat-inactivated human serum, supplemented with 100 μg/ml streptomycin, 100 I.U./ml penicillin, and 5×10^{-5} M 2-mercaptoethanol. After 5 days of co-culture in a humidified CO_2 incubator, the cells were collected, and their cytotoxic activity was determined by ^{51}Cr release assay as described previously[9].

[b] Effector/target cell ratio.

[c] C3H/HeN(H-2^k) mice were irradiated and reconstituted with bone marrow cells of BALB/c nu/nu mice.

C57BL/6J mice were replaced by fibrous tissue containing multinucleated giant cells (Fig. 1-B).

It is well known that precursor T cells of donor bone marrow migrate into the host thymus and then differentiate into mature T cells. In order to verify whether or not newly-developed T cells are tolerant to both host-type and donor-type MHC determinants, MLR and CTL assays were performed. Fig. 2 shows that spleen cells of chimeras significantly respond to the thrid party cells, whereas they do not respond to either bone marrow donor-type or host-type MHC determinants. As shown in Table 2, the assay for induction of CTL also revealed that the T cells are tolerant to donor-type as well as host-type MHC determinants.

Discussion

It has been reported that liver allografts in all species are rejected less aggressively than allografts of other organs[4]. In the present study we demonstrated that C3H/HeN (H-2^k) mice reject allogeneic liver tissue of BALB/c (H-2^d) and C57BL/6J (H-2^b) mice. By contrast, when C3H/HeN mice were irradiated and reconstituted with BALB/c nu/nu (H-2^d) bone marrow cells, the C3H/HeN mice accepted both BALB/c bone marrow donor-type and C3H/HeN host (thymus)-type liver tissue, but rejected third party C57BL/6J liver tissue (Table 1). Using assays for both MLR (Fig. 2) and induction of CTL (Table 2), we clearly demonstrated that the newly-developed T cells are tolerant to both donor-type and host-type MHC determinants. Slavin et al. have also reported that radiation bone marrow chimeras accept donor-type as well as host-type skin[12]. Thus, it is likely that donor stem cells (or precursor T cells) migrate into the host thymus and acquire self-tolerance during the differentiation in the thymus[1].

Immunosuppressive agents such as azathioprine, steroid hormones, and anti-lymphocyte globulin[2] have been used in order to prevent allograft rejection. However, these agents have cytotoxic effects on lymphocytes, especially T cells. Therefore, most patients die of infection. A new immunosuppressive drug, Cyclosporin A (Cy A), has been shown to be eflective in prolonging kidney, heart, pancreas, and liver allograft survival in man[3] as well as animals[5]. However, it has been reported that CyA has toxic eflects on kidney and liver. In the present study, we showed that liver allografts combined with bone marrow transplantation have no side eflects. We have previously reported that MHC-across bone marrow transplantation can treat autoimmune diseases in mice without GVHR, provided that T cells contained in bone marrow are entirely depleted[9]. Good et al. reported that more than 20 diseases otherwise fatal for man can be treated by bone marrow transplantation from HLA-matched or mismatched donors[7]. Based on our experiments, we think that organ allografts combined with bone marrow transplantation will become a viable therapy for humans.

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和文抄録

マウスにおける同種肝移植法の確立
—骨髄移植による免疫学的寛容を利用して—

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同種肝移植の際の拒絶反応を抑制するため，免疫学的寛容を誘導する試みとして骨髄移植を併用した．免疫学的機構の解析には，マウスが一番適しているが，マウスで血管縫合により肝を移植することが困難であるため腎被膜下に細切した肝を移植した．

C3H/HeN(H-2^k) マウスに C57BL/6J(H-2^b) や BALB C(H-2^d) マウスの肝を移植しても拒絶された．しかしながら，放射線照射し BALB/C nu/nu(H-2^d) マウスの骨髄を移植した C3H/HeN マウスでは骨髄 doner-type の BALB/C マウスの肝も，host-type の C3H HeN マウスの肝も拒絶されなかった．リンパ球混合培養法 (MLR) や killer T 細胞の誘導で解析すると，新しく分化して来たT細胞は骨髄 doner-type のみならず，host-type の組織適合抗原に対して免疫学的寛容が誘導されていることが判明した．

人では HLA を一致させた肝を入手することが困難なことや，免疫抑制剤を長期間投与すると肝や腎に毒性を有すること，また免疫抑制の結果感染による死亡が増加していることから肝移植と同種骨髄移植の併用療法は今後の臓器移植に新しい道を拓くものと考えられる．