Successful liver allografts in mice by combination with allogeneic bone marrow transplantation

(liver transplantation/major histocompatibility complex/graff-versus-host reaction/tolerance)

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ABSTRACT Successful liver allografts were established by combination with allogeneic bone marrow transplantation. When liver tissue of BALB/c (H-2b) or C57BL/6J (H-2b) mice was minced and grafted under the kidney capsules of C3H/HeN (H-2b) mice, it was rejected. However, when C3H/HeN mice were irradiated and reconstituted with T-cell-depleted BALB/c or BALB/c nu/nu bone marrow cells, or with fetal liver cells of BALB/c mice, they accepted both donor (stem-cell)-type (BALB/c) and host (thymus)-type (C3H/HeN) liver tissue. Assays for both mixed-lymphocyte reaction and induction of cytotoxic T lymphocytes revealed that the newly developed T cells were tolerant of both donor (stem-cell)-type and host (thymus)-type major histocompatibility complex determinants. We propose that liver allografts combined with bone marrow transplantation should be considered as a viable therapy for patients with liver disease such as liver cirrhosis and hepatothymus.

Orthotopic liver transplantation in humans was first successful in 1963 (1). Since then, more than 500 patients have been treated with this method (2, 3). In all species, liver allografts are rejected less aggressively than allografts of other organs (4–6), probably because Ia antigens are less prominent on liver cells than on cells of other organs. In humans, however, aggressive destructive rejection of liver allografts usually occurs if an immunosuppressant is not employed. It would be useful if rejection could be controlled without giving immunosuppressive agents.

Fully allogeneic chimeras in mice accept the skin of both thymus-type and bone marrow-type (7). In addition, allogeneic bone marrow transplantation permits effective treatment of autoimmune diseases in MRL/l and BXSB mice without graft-versus-host reaction, provided that bone marrow cells from young nu/nu mice or T-cell-depleted marrow cells are used (8, 9). Further, we have found that in these mice the newly developed T cells are tolerant of 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. Mito et al. (10) discovered that, in rats, splenic pulp is a most suitable location for long-term survival of dispersed hepatocytes (10).

In the present study, we attempted to transplant liver tissue (minced to rice-grain size) under the kidney capsules of mice. We show that C3H/HeN mice reconstituted with bone marrow or fetal liver cells from BALB/c mice accept both BALB/c donor-type and C3H/HeN host-type liver grafts, which can develop at this site into well-organized liver tissue.

MATERIALS AND METHODS

Animals. Inbred C3H/HeN (H-2b), BALB/c (H-2b), and C57BL/6J (H-2b) mice were used under standard laboratory conditions. BALB/c nude (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-month-old C3H/HeN mice were exposed to 8.5 Gy from a 60Co source and subsequently injected with 1–2 × 107 bone marrow cells from 2-month-old BALB/c nu/nu mice or with 1–2 × 107 T-cell-depleted bone marrow cells of BALB/c mice; the latter cells had been treated with monoclonal anti-Thy-1.2 antibody (clone F7D5, Olic Ltd, Bicester, UK) plus complement. [In some experiments, 1–2 × 107 fetal liver cells (hematopoietic stem cells) from BALB/c embryos were injected instead of bone marrow cells.] Two or three months after bone marrow transplantation, the mice were anesthetized with pento-barbital (0.05 mg/g of body weight, Pitman-Moore, Washington Crossing, NJ). Livers taken from 1- to 4-week-old C3H/HeN, BALB/c, or C57BL/6J mice were minced to about rice-grain size in RPMI 1640 medium (Nissui Seiyaku, Tokyo) with 0.01% collagenase (type I, Sigma). The liver tissue was grafted under the left kidney capsules of the C3H/HeN mice. Two months later, the mice were killed and the engrafted liver tissue was examined macroscopically and microscopically.

Cell Preparation. Spleens were removed aseptically, minced, and gently passed through a fine-mesh stainless-steel sieve into phosphate-buffered saline, as described previously (8).

Mixed-Lymphocyte Reaction (MLR). Triplet cultures were set up in 96-well round-bottom microwell trays (Corning, Cornning, NY). Each well contained 2 × 105 responder cells and 104 stimulator cells in a total of 0.2 ml of RPMI 1640 medium supplemented with 2 mM L-glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml), 5% heat-inactivated human serum, and 50 μM 2-mercaptopethanol (Wako, Osaka, Japan). Stimulator cells were treated with mitomycin C (50 μg/ml) for 30 min at 37°C. The cultures were incubated for 96 hr in a humidified 5% CO2 atmosphere. [3H]Thymidine (0.5 μCi; New England Nuclear, 1 Ci/mmol; 1 Ci = 37 GBq) was present during the last 4 hr of the culturing period. [3H]Thymidine incorporation into trichloroacetic acid-insoluble material was determined by liquid scintillation counting.

Abbreviations: MLR, mixed-lymphocyte reaction; CTL, cytotoxic T lymphocytes; MHC, major histocompatibility complex.

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Induction of Cytotoxic T Lymphocytes (CTL). Responder cells \((7.5 \times 10^6)\) and mitomycin C (50 \(\mu g/ml\))-treated stimulator cells \((2.5 \times 10^6)\) were cocultured in RPMI 1640 medium containing 10% heat-inactivated human serum, supplemented with 2-mercaptoethanol (50 \(\mu M\)), penicillin, and streptomycin. Cultures were incubated for 5 days at 37°C in a 5% \(CO_2\) atmosphere. P815 (H-2k), EL-4 (H-2d), and X5S63 (H-2d) were used as target cells. They were labeled by incubation for 1 hr at 37°C with 100 \(\mu M\) of NaC\(^{51}\)CrO\(_4\) (New England Nuclear). Labeled cells were washed three times. These cells \((5 \times 10^4)\) were mixed with effector cells in 100 \(\mu l\) of RPMI 1640 medium in round-bottom microwells and incubated at 37°C in 5% \(CO_2\) for 4 hr. A Titertek supernatant-collection system (Flow Laboratories, Irvine, CA) was used to harvest supernatant for determination of released radioactivity. Percent specific lysis was calculated as \([\text{experimental release} - \text{spontaneous release}] / [\text{maximal release} - \text{spontaneous release}]\) \(\times 100\).

**Histopathology.** The left kidney with engrafted liver tissue was obtained at autopsy and prepared for observation by light microscopy.

**RESULTS**

Reconstitution of Immunological Functions in Radiation Bone Marrow Chimeras. C3H/HeN (H-2k) mice reconstituted with BALB/c nu/nu (H-2k) bone marrow cells survived more than 8 months without showing graft-versus-host reaction. Using anti-H-2k and anti-H-2d 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 responded significantly to phytohemagglutinin, Con A, and bacterial lipopolysaccharide (data not shown).

**Fate of the Engrafted Liver Tissue.** As shown in Table 1, untreated C3H/HeN mice rejected allogeneic liver tissue of BALB/c (6 of 6) or C57BL/6J mice (5 of 5). In contrast, C3H/HeN mice reconstituted with BALB/c nu/nu bone marrow cells rejected the third-party C57BL/6J liver tissue (6 of 6), whereas they accepted both BALB/c bone marrow donor-type (8 of 10) and C3H/HeN host-type (3 of 5) liver tissue. C3H/HeN mice reconstituted with T-cell-depleted BALB/c bone marrow cells or with fetal liver cells of BALB/c mice also accepted liver tissue of BALB/c bone marrow donor-type (5 of 6) or BALB/c fetal liver donor-type. BALB/c liver tissue, including organization with lobules and central veins, was observed under the kidney capsules of C3H/HeN mice (Fig. 1 Upper). However, due to rejection, grafting liver tissue of C57BL/6J mice was regularly replaced by fibrous tissue (Fig. 1 Lower).

**Evidence for Induction of Tolerance.** It is known that precursor T cells of donor bone marrow migrate into the host thymus and there differentiate into mature T cells. To ascertain whether or not newly developed T cells are tolerant of both host-type and donor-type MHC determinants, MLR and CTL assays were performed. The MLR assay showed that spleen cells of the chimeras respond significantly to the third-party cells, whereas they do not respond to MHC determinants of either bone marrow-donor type or host type (Fig. 2). In accord with these results, the assay for induction of CTL showed that the T cells from C3H/HeN mice reconstituted with BALB/c nu/nu or BALB/c bone marrow cells, or with BALB/c fetal liver cells, are tolerant in each model, of donor-type as well as host-type MHC determinants (Table 2).

**DISCUSSION**

Liver allografts in every species studied have been reported to be rejected less aggressively than allografts of other organs (4). In the present study, we demonstrated that C3H/HeN (H-2k) mice reject allogeneic liver tissue of BALB/c (H-2k) or C57BL/6J (H-2d) mice. By contrast, when C3H/HeN mice
were irradiated and reconstituted with BALB/c nu/nu (H-2b) bone marrow cells, the C3H/HeN mice accepted liver tissue of either BALB/c bone marrow-donor type or C3H/HeN host (thymus) type, but rejected vigorously the third-party (C57BL/6J) liver tissue (Table 1). Using assays for both MLR (Fig. 2) and CTL (Table 2), we found that the newly developed T cells are tolerant of both donor-type and host-type MHC determinants. Slavin et al. (11) have also reported that irradiation bone marrow chimeras accept donor-type as well as host-type skin. Thus, it is likely that donor stem cells (or precursor T cells) migrate into the host thymus and there acquire self-tolerance of the recipient strain during differentiation in the thymus (12).

Immunosuppressive agents, such as azathioprine, steroid hormones, antilymphocyte globulin, and, more recently, cyclosporine, have been used to prevent allograft rejection (13). However, these agents exert cytotoxic effects on lymphocytes, especially T cells. Therefore, patients treated with these agents over long periods often suffer from life-threatening infections, and many die of these infections. Cyclosporine has been impressively effective in prolonging kidney, heart, pancreas, and liver allograft survival in humans (2) as well as animals (14). However, cyclosporine may produce serious damage to kidney and liver. In the present study, we showed that liver allografts coupled with simultaneous bone marrow or fetal liver transplantation produce no harmful side effects. We have previously reported that bone marrow transplantation can be used to treat and apparently cure autoimmune diseases in mice without inducing any graft-vs.-host reaction, provided that T cells and committed T-cell precursors have been entirely eliminated from the bone marrow (9). Good et al. (15), in summarizing extensive research with bone marrow transplants, have reported that more than 20 otherwise fatal human diseases can be treated by bone marrow transplantation from HLA-matched or -mismatched donors. This number of diseases now can be extended to at least 50 otherwise lethal diseases.

Based on these experiments, we think that liver allografts combined with bone marrow transplantation could become a viable therapy for many patients suffering from irreversible liver disease such as liver cirrhosis or liver cancer. Further, hematopoietic cells from fetal liver allografts conjoined with transplantation of fetal liver cells obtained from an aborted fetus might represent a valuable strategy that could be used to treat children with congenital liver diseases such as biliary atresia.

**Table 2. Generation of CTL from C3H/HeN mice reconstituted with bone marrow cells of BALB/c nu/nu or BALB/c mice and with fetal liver cells of BALB/c mice**

<table>
<thead>
<tr>
<th>Spleen cell source</th>
<th>Liver donor</th>
<th>E/T*</th>
<th>% specific release from target cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>X563 (H-2b)</td>
</tr>
<tr>
<td>C3H/HeN (H-2b)</td>
<td>-</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>C57BL/6J (H-2b)</td>
<td>-</td>
<td>4</td>
<td>54.7 ± 10.1</td>
</tr>
<tr>
<td>BALB/c (H-2b)</td>
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<td>4</td>
<td>71.9 ± 15.4</td>
</tr>
<tr>
<td>C3H/HeN with BALB/c nu/nu marrow cells†</td>
<td>BALB/c</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>C3H/HeN with BALB/c marrow cells†</td>
<td>BALB/c</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>C3H/HeN with BALB/c fetal liver cells†</td>
<td>BALB/c</td>
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Responder spleen cells (7.5 × 10⁶) and mitomycin C (50 μg/ml)-treated stimulator cells (2.5 × 10⁶) were cocultured in RPMI 1640 medium containing 10% heat-inactivated human serum, supplemented with streptomycin (100 μg/ml), penicillin (100 units/ml), and 50 μM 2-mercaptoethanol. After 4 days of coculture in a humidified 5% CO₂ incubator, the cells were collected, and their cytotoxic activity was determined by ⁵¹Cr-release assay as described (9).

*Effector/target cell ratio.
†C3H/HeN (H-2b) mice were irradiated and reconstituted with bone marrow cells of BALB/c nu/nu or BALB/c mice or with BALB/c fetal liver cells.
We also propose that consideration be given to the possibility that organ allografts of heart, kidney, or pancreas may be accomplished without a long-term immunosuppression if the organ transplantation is done simultaneously with allogeneic bone marrow transplantation from the same donor. Since recipients of such combined transplants would need to be treated only once, such a strategy could prove to be an advantage in managing patients for whom transplantation of organs or tissues must be undertaken.

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