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Propagation of Cells Expressing Donor Phenotype (MHC Class I, II and Y-Chromosome) From the Bone Marrow of Murine Liver Allograft Recipients in Response to GM-CSF In Vitro

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AN unresolved issue with the recent demonstration of persistent low-level donor leukocyte chimerism following organ transplantation[1,2] has been the means by which the chimeric cells can perpetuate themselves for long periods postoperatively. This question was addressed in mice by transplantation without immunosuppression across MHC class I–II and mHC disparities using a strain combination (B10→C3H) that permits permanent acceptance of the liver, but not other allogeneic organs.[3] A method used previously to propagate dendritic cell progenitors from normal mouse spleen or liver[4] was implemented to ascertain whether, in addition to host-derived cells, cells of donor phenotype could be propagated from the lymphoid tissue (bone marrow and spleen) of liver-transplant recipients. For comparative purposes, similar techniques were applied to cells propagated from the lymphoid tissues of unmodified C3H mice that rejected cardiac allografts from the same donor strain.

MATERIALS AND METHODS

Animals

Ten to twelve-week-old C57BL/10J (B10, H-2^b, I-A^b, I-E^-) and C3H/HeJ (C3H, H-2^k, I-A^k, I-E^k) mice were obtained from The Jackson Laboratory, Bar Harbor, Me. They were maintained in the specific pathogen-free facility of the University of Pittsburgh Medical Center.

Liver and Heart Transplantation

Orthotopic liver transplantation (OLTx) was performed using techniques described previously,[5] with minor modifications. Heterotopic heart transplantation (HHTx) was adapted from the rat procedure of Ono and Lindsey.[6] The heart was transplanted into the abdomen with end-to-side anastomosis of aorta to aorta and pulmonary artery to vena cava. Rejection was defined by the cessation of cardiac contraction after daily palpation through the abdominal wall, and confirmed by histological examination. No immunosuppressive therapy was used and animals (three per group) were sacrificed 14 days after OLTx and 8 days after HHTx.

Culture of Tissue-Derived Cells

Before harvesting of organs, whole body perfusion was performed. Bone marrow, spleen, and thymus cell suspensions were prepared in RPMI-1640 (Gibco, Grand Island, NY), using conventional methods; liver nonparenchymal cells (NPC) (hepatocyte contamination ≤5%) were isolated as described previously.[4] Two-5 × 10^6 cells were cultured in 24-well plates in RPMI-1640, supplemented with 10% fetal bovine serum and 0.4 ng/mL mouse recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) (R&D Systems, Minneapolis, Minn). The procedure used was modified after that described by Inaba et al[7] for the propagation of large numbers of dendritic cell progenitors from mouse bone marrow. The medium containing GM-CSF was refreshed every 2 days; after gentle swirling of the plates, half of the old medium was aspirated and an equivalent volume of RPMI-1640 with GM-CSF was added. An objective of this manipulation was to deplete nonadherent granulocytes, without dislodging clusters of developing dendritic cells that attached loosely to a monolayer of firmly adherent macrophages. Morphological and phenotypic analyses of typical, single, nonadherent mononuclear cells released spontaneously from clusters were performed between 6 and 10 days.

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Flow Cytometric Analysis

Cultured cells (5×10^5/tube) in Hank's balanced salt solution (HBSS) containing 1% w/v bovine serum albumin (Sigma, St. Louis, Mo) and 0.1% sodium azide (Sigma) were stained by direct immunofluorescence. Donor and recipient-specific MHC class I positive cells were identified using FITC-conjugated mouse anti-mouse H-2K^b and H-2K^k monoclonal antibodies (MAbs) (IgG2a; PharMingen, San Diego, Calif), respectively. FITC-conjugated mouse IgG2a was used as a negative isotype control. After staining, the cells were fixed in 1% paraformaldehyde in saline. Flow cytometric analysis was performed using a FACSTAR® flow cytometer (Becton Dickinson, San Jose, Calif) and 5000 events were analyzed for each sample.

Immunocytochemistry

Cytocentrifuge preparations of the cultured cells were processed and stained using the avidin-biotin-peroxidase complex (ABC) procedure, as described elsewhere.[4] Biotinylated mouse IgG2a anti-I-E^k or anti-I-A^b MAbs (PharMingen) were used to demonstrate expression of recipient and donor MHC class II antigens, respectively. Controls included the omission of primary antibody and the use of isotype-matched, irrelevant MAb.

Detection of Donor Male (Y) Chromosome by Polymerase Chain Reaction (PCR)

DNA was prepared from freshly isolated and 10-day GM-CSF-stimulated cells from both normal male B10 and female C3H mice and from female C3H mice 14 or 8 days, respectively, after liver or heart transplantation from male B10 donors. The presence of male donor cells was determined by PCR amplification of the SRY region of the mouse Y chromosome from DNA extracted from the various cell preparations. Primers (CAGCCCTACAGCCACAT and CCACTCCTCTGTGACACTTT) were chosen from the p4.2.2 sequence.[8] The amplified DNA was Southern blotted and hybridized with a homologous radioactive probe, as previously described for human analysis.[9]

RESULTS

Incidence of Donor MHC Class I Positive Cells in Lymphoid Tissues of Liver-Allografted Mice

The incidence of chimeric cells in freshly isolated recipients' bone marrow or spleen 14 days after OLTx was determined by flow cytometric analysis. The results were compared to those obtained using freshly isolated cells from naive unmodified mice of either donor (B10) or recipient (C3H) strain. The proportion of donor (B10) MHC class I positive cells (H-2^{b+}) was low (from 0% to 5%) in the bone marrow, spleen, and thymic cell populations of the transplanted animals.

Propagation of Donor and Recipient MHC Class I or MHC Class II Positive Cells From Bone Marrow and Spleen of Liver Allograft Recipients in Response to GM-CSF

GM-CSF-stimulated cells derived from freshly isolated bone marrow, spleen, or thymus of liver-allografted mice grew more actively than cells from naive animals. Moreover, in the former cultures, more clusters developed from which larger numbers of cells, with similar dendritic morphology, were released. Nonadherent cells were harvested 10 days after initiation of the cultures. The expression of donor or recipient MHC class I antigens was determined using appropriate MAbs and flow cytometric analysis. In addition to the propagation of recipient cells in response to GM-CSF, donor-derived cells expressing H-2K^b were detected both in the bone marrow and in the spleen cell cultures. The presence of cells expressing donor MHC allo-antigens was confirmed by immunocytochemical analysis of cultures stained for donor MHC class II (H-2^b). Detection of donor Y chromosome by PCR analysis (Fig 1) confirmed the growth of donor-derived cells in the 10-day bone marrow cell cultures and reinforced the immunocytochemical observations. Although Y chromosome was also detected in 10-day spleen cell cultures, the signal was reduced substantially compared to that with bone marrow (Fig 1), indicating that comparatively few donor-derived, GM-CSF-responsive precursors reached the spleen. The apparent diminished signal for donor cells in the GM-CSF-stimulated liver NPC population (Fig 1) may actually reflect death of contaminating hepatocytes; in addition, in situ replacement of donor with recipient GM-CSF-responsive cells may contribute to a reduced donor signal from liver NPC on day 10. Y chromosome was also detected in freshly isolated bone marrow and spleen cells from unmodified heart allograft recipients isolated 8 days posttransplant. In contrast to cells from liver-transplanted mice, however, Y chromosome was not detected in 10-day cultures propagated from either bone marrow or spleen after heart transplantation (data not shown).

DETECTION OF THE Y CHROMOSOME IN CELLS CULTURED FROM FEMALE RECIPIENTS OF MALE LIVERS

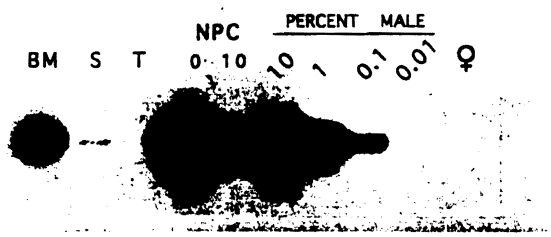

Fig 1. Detection of the Y chromosome in cells cultured from female recipients of male livers. One-μg aliquots of DNA obtained from 10-day bone marrow (BM), spleen (S), thymus (T), or from day 0 and day 10 liver NPC cultures were amplified with 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds in buffer consisting of 50 mmol/L KCl, 10 mmol/L Tris-HCl pH 8.3, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L deoxynucleotides, 0.1% gelatin, 0.2 μmol/L primers, and 2.5 units of Taq polymerase (Perkin Elmer, Norwalk, Conn). The products were separated in a 1% agarose gel, transferred to nylon membrane, and hybridized with an identical, purified, and radiolabelled PCR product from male mouse DNA. Single copy sensitivity and male specificity were verified with control samples consisting of serial dilutions of male mouse DNA into female mouse DNA.

DISCUSSION

We have reported previously that, after local or systemic injection of GM-CSF-stimulated, liver dendritic cell progenitors (MHC class $II^{-/dim}$) into unmodified, MHC-disparate recipients, donor cells (MHC class II^{bright}) can be identified within recipient central lymphoid tissues.[4] These observations have now been extended to the context of whole organ transplantation. Cells harvested from the bone marrow and spleen 14 days after liver transplantation and cultured for 10 days in GM-CSF-supplemented medium, included a minor population of cells with DC characteristics expressing donor phenotype (MHC class I/II and Y chromosome). These findings offer an explanation for the persistence of low-level chimerism in B10→C3H liver transplant recipients,[3] which do not require host immunosuppression. In contrast, in unmodified hosts rejecting their cardiac allografts (day 8), propagation of donor cells ex vivo could not be irrefutably demonstrated. This finding is consistent with the liver containing comparatively large numbers of potential migratory hematopoietic cells, including DC progenitors that have the capacity to propagate ex vivo (as demonstrated herein), or in vivo in response to the appropriate growth factors. The functional role of the precursors of chimeric DC is currently being investigated. The observation that donor as well as recipient cells undergo reciprocal migration after liver transplantation is congruent with the paradigm of bidirectional immune reactivity, which may play a major role in whole organ graft acceptance and acquired tolerance.

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