GENERATION OF BILIARY LESIONS AFTER TRANSFER OF HUMAN LYMPHOCYTES INTO SEVERE COMBINED IMMUNODEFICIENT (SCID) MICE

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Severe combined immunodeficient (SCID)1 mice have an autosomal recessive defect that impairs the rearrangement of antigen receptor genes in lymphoid progenitors (1). Consequently, functional lymphocytes do not develop and these animals are severely lymphopenic (2, 3). Recent results have demonstrated that human fetal lymphoid tissue or peripheral blood lymphocytes from adults can survive in SCID mice, and this has raised the possibility that this model system would be valuable for the study of the human immune response and lymphohematopoietic abnormalities (4, 5). A recent report suggests that acute infection of lymphoid cells in these so-called SCID-hu mice with human immunodeficiency virus is possible, but the actual transfer of a lymphoid associated abnormality with human cells has not been reported (6).

To further study this model we injected PBL obtained from both normal volunteers and patients with primary biliary cirrhosis (PBC) into SCID mice. PBC is a chronic autoimmune disease serologically characterized by the presence of autoantibodies to the mitochondrial oxo-dehydrogenase enzymes and histologically by the inflammation, specific obstruction, and eventual obliteration of the intrahepatic bile ducts (7–9).

We report herein the transfer of PBC-like characteristics to the immunodeficient mice with PBLs from patients with PBC. Mitochondrial autoantibodies of human origin were present in the serum of the mice and biliary lesions that were apparently due to human lymphoid cells that had infiltrated around the intrahepatic bile ducts were present. A hepatic inflammatory reaction was also observed in mice that were recipients of PBLs from normal donors, suggesting that human lymphoid cells react against murine tissues and cause a graft-vs.-host (GVH)-like disease in the animals.

Materials and Methods

Mice. Homozygous C.B-17 scid mice, designated SCID mice, were bred and maintained in a barrier facility in the Division of Biomedical Sciences, University of California, River-

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1 Abbreviations used in this paper: AMA, antimitochondrial antibodies; GVH, graft-vs.-host; PBC, primary biliary cirrhosis; PDH, pyruvate dehydrogenase complex; SCID, severe combined immunodeficiency.
Mice were housed in microisolator cages (Lab Products, Maywood, NJ) and kept in a laminar flow portable clean room (Airo Clean, Edgemont, PA). Animals were not treated with antibiotics and were healthy throughout the course of these experiments.

All manipulations were performed aseptically under a laminar flow hood including obtaining blood from the lateral tail vein 4 wk after PBL transfer. 8 wk after PBL transfer, mice were exsanguinated and spleens and femurs were removed and placed in RPMI 1640 supplemented with 5% FCS. Single cell suspensions were prepared by teasing the tissue apart with bent 18-gauge needles. After gentle aspiration with a pipette, cell suspensions were let stand 30 s to allow debris to settle. Supernatants were then decanted. Bone marrow cells were treated in a similar manner after the marrow plug was flushed from the bones (10).

**Preparation of Human PBL.** Blood samples from six patients with a well-established clinical and laboratory diagnosis of PBC and from five normal human volunteers were collected into heparinized tubes. As shown in Table I, the histological stage of disease of the patients with PBC varied. Two patients were stage II, three were stage III, and one was stage IV. PBMC were isolated using Lymphoprep (Nycomed, Oslo, Norway). Plasma from the same patients was frozen and subsequently used for serologic analysis. Based upon the cell dosage used by others (5), between 10 and 42 x 10^6 PBLs in RPMI 1640 were injected intraperitoneally into 5-8-wk-old male or female SCID mice. Up to three SCID mice were able to be reconstituted with the cells of any one patient.

**Detection of Human Ig in SCID Sera.** Human Ig were quantitated by a sandwich ELISA. Polystyrene microtiter plates were coated with the capture reagent, a 1:1,000 dilution of goat anti-human Ig (IgG or IgM; Cappel Laboratories, Malvern, PA) in carbonate coating buffer. After blocking, 10-fold dilutions of SCID sera in 1% BSA and standardized controls were added to the wells. After incubation and washing, peroxidase-conjugated goat anti-human polyvalent Ig (Tago Inc., Burlingame, CA) was used as the detection reagent. Reactivity was visualized with 40.0 mM 2,2'-azinobis 3-ethyl benzothiazoline sulfonic acid (Sigma
Chemical Co., St. Louis, MO) in 0.05 M citric acid buffer, pH 4.2, containing 0.05 M H$_2$O$_2$. The reaction was stopped after 5 min by the addition of 5% SDS.

In some experiments radial immunodiffusion plates to detect human IgG (Diffu-gen; Tago Inc.) and low level human IgM (AccraAssay, ICN ImmunoBiologicals, Lisle, IL) were used. Sera from reconstituted and control SCID mice were incubated in parallel with standards for 18 h to detect IgG and 44 h for IgM. Ig concentrations in SCID sera were determined from a standard curve.

Identification of Human PBLs in SCID Mice by Immunofluorescence and Immunolabeling. Cells that expressed various T cell surface antigens were identified by incubating 10^6 SCID spleen cells in 0.1 ml of 4°C RPMI 1640 with mAbs to the following cell surface determinants: CD3 (Leu 4), CD4 (Leu 3a & 3b), and CD8 (Leu 2a) (Becton Dickinson & Co., Mountain View, CA). After a 30-min incubation at 4°C, cells were washed in RPMI 1640 supplemented with 0.1% NaN$_3$ and incubated an additional 30 min at 4°C with a fluorescein-conjugated anti-mouse Ig (Tago Inc.). Cells were then washed twice in RPMI-Na$_3$, and fixed in 2% paraformaldehyde. A FITC-labeled anti-human Ig antibody (Tago Inc.) was used to identify human B cells by direct immunofluorescence. Cells were enumerated on a Leitz laborlux microscope equipped for epifluorescence. At least 300 cells were counted per sample. In addition, the above protocol was performed on 10^6 cultured splenocytes from 6 PBC-reconstituted and one normal reconstituted mouse in RPMI 1640 supplemented with 10% pooled human AB serum and 100 U/ml human rIL-2 (Cetus Corp., Emeryville, CA). After 2 wk in culture cells were removed, centrifuged onto microscope slides in a Cytospin 2 (Shandon Southern Instruments Inc., Sewickley, PA), and acetone-fixed before indirect immunofluorescence.

Livers specimens were embedded in OCT compound (Lab-Tek, Elkhart, IN), sectioned at 6 μm on a Tissue Tek cryostat (Miles Laboratories, Elkhart, IN) at −20°C, and placed on gelatin-subbed slides. Immediately before use the tissue was fixed for 5 min at room temperature in acetone and then washed in PBS, pH 7.4. Typing of T cell surface markers was performed by indirect immunofluorescence. 100 μl of a 1:200 dilution of anti-CD3, or one drop of anti-CD4 and anti-CD8 were added to individual tissue sections for 30 min at room temperature. Antibody specificity was assessed by using a control serum on replicate sections. After two washes in PBS, goat F(ab')² anti-mouse Ig-FITC was added for 30 min at room temperature.

To detect Ig-bearing cells, liver specimens were embedded in paraffin, sectioned on a rotary microtome, and mounted on gelatin-subbed slides. Slides were dried at 55°C for 2 h before deparaffinization and rehydration. 20 μl of a 1:100 dilution of polyvalent goat anti-human FITC conjugate (Tago Inc.) was overlayed into slides placed in a room temperature humidified incubator for 30 min. After washing in PBS, the sections were cover-slipped with PBS-buffered glycerol. Sections were read on a Zeiss microscope equipped for epifluorescence.

Autoantibodies to Mitochondria. The presence of autoantibodies to the mitochondrial PDH-E2 antigen in the sera of SCID mice was determined by ELISA and immunoblotting. For the ELISA, pyruvate dehydrogenase (PDH; Sigma Chemical Co.) was used to coat polystyrene microtiter plates (Immulon, Dynatech Laboratories, Alexandria, VA) as previously described (11–13). Plates were coated with 2 μg/ml of protein in carbonate coating buffer and incubated at 4°C overnight after which time the plates were washed with 0.5% PBS-Tween and blocked with 1% BSA-PBS. Two fold dilutions of serum from PBL reconstituted SCID mice, plasma from the patients who donated cells, or normal control serum were dispensed into individual wells in a volume of 0.1 ml in 1% BSA-PBS for 1 h at room temperature. After washing, the plates were incubated for 30 min with 100 μl of a goat anti-human peroxidase conjugate diluted 1:4000 (Tago Inc.).

An ELISA for quantitative determination of antimitochondrial antibody (AMA) isotypes recognizing PDH was similar to a previously described assay with the following modifications (11). After incubation with sera from reconstituted SCID mice and sera from PBC patients, plates were incubated with mAbs specific against human heavy chain isotypes: SG-11 for IgG1, GOM-1 for IgG2, SJ-33 for IgG3, SK-44 for IgG4, and MB-11 for IgM (Miles Scientific, Naperville, IL, and ICN, Lisle, IL). The binding of these mouse mAbs was detected with peroxidase-conjugated goat anti-mouse IgG and IgM (Tago Inc.).

For the detection of AMAs by immunoblotting, samples were boiled for 3 min in SDS
sample buffer with 2-ME and the proteins were separated by 10% SDS-PAGE as previously described (12). Each lane contained 30 μg of protein in the case of beef heart mitochondria (a gift from Dr. Brian Akrell, University of California, San Francisco, San Francisco, CA) and 1 μg of protein for PDH (a gift from Dr. Tom Roch, Kansas State University, Manhattan, KS). Separated proteins were then transferred to nitrocellulose paper (Schleicher & Schuell, Inc., Keene, NH). 4-mm strips of the blotted nitrocellulose were cut, blocked with "Blotto" (3% nonfat milk powder in PBS) for 30 min, and incubated for 1 h with 1:100 dilution of sera from mice reconstituted with PBL from PBC patients. After three washes, the strips were incubated with 0.3 μCi 125I-labeled goat anti-human Ig for 1 h (Amersham Corp., Arlington Heights, IL). After three washes, the strips were dried and exposed to x-ray film overnight. Plasma from the same patients with PBC and the same normal controls were used as positive and negative controls.

Results

Human Ig are Present in PBL Reconstituted SCID Mice. Human Ig were detected in the serum of 10 of 10 and 5 of 6 SCID recipients of PBLs from patients with PBC or normal donors, respectively (Table I). The SCID recipient of PBLs from PBC patient 5 was not analyzed serologically. IgG levels ranged from 0.1 to 3.6 mg/ml in SCID recipients of PBL from patients with PBC and from 0.0 to 6.3 mg/ml in SCID mice that received PBLs from normal donors. In three PBL reconstituted SCID mice, human IgM levels were higher than concentrations of IgG. For example, SCID mice that received PBL from PBC patient 6 have serum IgM levels of 3.2 mg/ml and 2.5 mg/ml, while the IgG levels were only 2.4 mg/ml and 1.0 mg/ml, respectively. In all of the SCID recipients of PBL from normal donors analyzed, IgG levels were considerably higher than IgM (Table II).

| Table II
| Levels of Human Ig in the Sera of SCID Sera |

| Sera       | Donor | IgG | IgM |
|------------|-------|-----|-----|
|            |       | mg/ml | mg/ml |
| PBC-SCID   | 1A*   | 0.6  | 0.2  |
|            | 1B    | 0.1  | 0.0  |
|            | 6A    | 2.4  | 3.2  |
|            | 6B    | 1.0  | 2.5  |
|            | 6C    | 0.2  | 0.0  |
|            | 3A    | 0.5  | 0.1  |
|            | 3B    | 0.6  | 0.3  |
|            | 3C    | 0.1  | 0.0  |
|            | 2     | 0.1  | 0.1  |
|            | 4     | 3.6  | 4.8  |
|            | 5     | ND   | ND   |
| Nor-SCID   | 1     | 1.0  | 0.1  |
|            | 2     | 6.3  | 0.9  |
|            | 3     | 1.8  | 0.6  |
|            | 4     | 0.4  | ND   |
|            | 5     | 0.6  | ND   |
| Unreconstituted SCID | | 0.0 | 0.0 |

* All sera were from animals 8 wk after being reconstituted.
* A, B, and C were used to designate different mice that received PBLs from the same donor.
**Presence of Human PBLs in SCID Mice.** As shown in Table I, human lymphoid cells were detected in the spleens of three of six of the SCID recipients of PBLs from normal donors. Similar results were obtained with mice that received PBLs from patients with PBC; CD3+ and CD8+ cells were detected in the spleens of 4 of 10 of these PBC-SCID mice, while CD4+ cells were present in the spleens of 2 of the 4 CD3+ mice. Further evidence that human T cells were present in the spleen was obtained by indirect immunofluorescence on cytospin preparation prepared from cultured splenocytes (data not shown). Thus, the extent of reconstitution was variable in these SCID mice even when healthy animals of the same litter and the number of cells from the same donor were used. None of the cells in the spleen suspensions stained with anti-human Ig, although Ig+ cells were present in the liver (Fig. 1).

**AMAs of Human Origin are Present in PBC Reconstituted Mice.** 10 of 10 reconstituted mice that were serologically analyzed and received an injection of cells from the patients with PBC had serum antibodies that reacted with the PDH complex (Fig. 2). Furthermore, immunoblots with beef heart mitochondria and purified pyruvate dehydrogenase confirmed specific reactivity to the mitochondrial antigens (Fig. 3).

The isotype distribution of AMAs in the PBC reconstituted animals was further analyzed by ELISA. As shown in Fig. 4, AMAs of IgM and all the IgG subclasses were detected. Patients with PBC typically have AMAs that are predominantly of the IgG3 and IgM isotypes (11). In three of four cases, PBC-reconstituted mice with AMAs that were predominantly IgM also had high levels of total human IgM immunoglobulin.

**Histological Appearance of Tissue.** In 8 of 11 SCID recipients of PBLs from patients...
FIGURE 2. Detection of AMAs in reconstituted SCID sera by ELISA. The mean optical density ± SD values are for triplicate wells at a 1:100 dilution of SCID sera. Sera from mice reconstituted with PBL from normal healthy donors and unreconstituted SCID sera did not give optical density values above those of background controls. (A, B, and C) Different mice that received PBL from the same donor.

FIGURE 3. Reactivity of SCID sera against (1) Purified pyruvate dehydrogenase complex, and (2) beef heart mitochondria. The proteins were separated by SDS-PAGE, blotted onto nitrocellulose paper, and probed with 1:100 dilution of PBC plasma (lane A), 1:100 dilution of reconstituted SCID sera from the same patient (lane B), 1:100 dilution of normal reconstituted SCID sera (lane C), 1:100 dilution of PBC-reconstituted SCID sera from a different patient with PBC (lane D), or a 1:100 dilution of plasma from a normal donor. Note that the serum from the reconstituted SCID mouse reacts with the same proteins as does the plasma from the patient with PBC; the 74-kD dihydrolipoamide acetyltransferase, the 56-kD antigen is the protein X of the PDC that possesses crossreactive AMA epitope(s) with the 74-kD antigen (9). The 48-kD antigen may be the Ela of the PDC. The remaining bands are presumed to be degradation products as previously reported (11, 12).
with PBC, an infiltration of inflammatory cells consisting primarily of lymphocytes including plasma cells was observed around the portal areas of the liver. The mononuclear infiltrate was consistently localized around the bile duct and ranged from mild to severe (Fig. 5, A, B). Degenerative changes or destruction of the bile ducts were observed in 7 of 11 PBC-reconstituted mice with cellular infiltrates. Fibrosis and necrosis were also noted in these sections (Table III).

The liver sections from SCID mice reconstituted with normal PBLs also exhibited mild to moderately severe mononuclear cell infiltrates (Fig. 5 C), but bile duct damage and hepatocyte necrosis were only seen in one of six mice with the most intense infiltration. No evidence of inflammation or a mononuclear infiltrate was present in the liver of unreconstituted SCID mice (Fig. 5 D). In addition, tissue sections of skin, lung, and gut from five PBC-reconstituted mice and five normal reconstituted mice revealed no abnormalities.

**Immunohistochemical Evaluation of Liver Sections.** The lymphoid infiltrate surrounding the bile ducts in the liver of normal and PBC reconstituted SCID mice was examined using specific mAbs. Liver sections from three of three PBC-reconstituted SCID mice and three of three normal reconstituted SCID mice contained CD3⁺, CD4⁺,
and CD8' T cells (data not shown). Ig' cells were seen in four of four PBC-reconstituted SCID mice and three of four normal-reconstituted SCID mice analyzed.

Discussion

The data herein confirm a previous report that human peripheral blood lymphocytes can reconstitute T and B cells and serum Ig of human origin in mice with severe combined immunodeficiency disease. The purpose of the present study was to extend these observations and investigate whether immune system abnormalities could be duplicated in SCID mice by transfer of PBL. The data indicate that PBL from patients with the autoimmune condition PBC transferred many of the characteristics of the disease to the immunodeficient mice. A second important finding from these studies was that the transfer of PBL, even from normal donors, produced a GVH-like condition in the liver of the animals.

Attempts to develop an animal model of PBC have been made, but these have duplicated only selected aspects of the disease (14–16). For example, we have previously demonstrated that immunization of various species of animals with recombinant dihydrolipoamide acetyltransferase resulted in the generation of AMAs, but hepatic pathology was not exhibited (12). The PBC-SCID mice described herein contained the characteristic AMAs reactive with the major mitochondrial autoantigen, dihydrolipoamide acetyltransferase (E2, EC 2.3.1.12) of the PDH complex. In addition, the animals presented with marked hepatic infiltrates of human mononuclear cells that were localized around the intrahepatic bile ducts of the PBC reconstituted mice. The lesions associated with these infiltrates consisted of lymphoid cell invasion of the bile duct epithelium accompanied by subsequent cellular destruction. These infiltrating lymphocytes consisted of CD3', CD4', and CD8' cells, as well as plasma cells. This is consistent with the fact that mononuclear infiltrates in the early stages of PBC contain T lymphocytes, most of which are CD4' with a smaller number of CD8' cells immediately surrounding the bile duct (7). A recent report demonstrates that CD8' cells are the predominant T cell subset that migrates out of stage II or III liver in culture (17). Thus, the above findings are the first to report an animal model in which both the cellular and humoral aspects of the disease are present.

SCID mice that received a graft of PBL from normal donors also developed a hepatic inflammatory reaction. The strongest reaction occurred in a SCID mouse that received cells from a healthy female who has routinely handled mice for the
FIGURE 5. Histologic analysis of liver obtained from SCID mice. (A) Portal triad of a SCID mouse reconstituted with PBLs from PBC patient 6 from Table 1 (×150); (B) view of A showing the mononuclear cell infiltrate into the epithelium of the bile duct (×350); (C) portal triad of SCID mouse reconstituted with PBLs from normal donor 1 from Table 1 (×150); (D) portal triad of unreconstituted SCID mouse (×350).
past 10 yr, and GVH response mediated by sensitized T cells must be considered in this case. The lesions resembled those in patients with GVH disease during hepatic allograft rejection (18). It has been hypothesized that the bile duct lesions that characterize the GVH reaction seen in patients after liver transplantation are due to sensitized graft T lymphocytes selectively attacking MHC class II bearing bile duct epithelial cells (19, 20). Aberrant expression of MHC class II antigens on bile duct epithelium has been observed in patients with GVH disease after bone marrow transplantation as well (20–22). Minor histocompatibility antigens expressed in host biliary epithelial cells are thought to account for the preferential homing of mononuclear cells to the hepatic portal spaces and the resulting GVH in a recently described murine chronic GVH disease (23). These data thus indicate that reconstitution of SCID mice with human cells may prove to be an important model of value in GVH studies.

Bile duct lesions resembling chronic nonsuppurative destructive cholangitis seen in PBC was recently reported in a murine model in which B6 splenocytes were injected into B6 x bm12 F1 recipients (24). These mice developed an inflammatory infiltrate, consisting of lymphocytes, plasma cells, and eosinophils, around the small bile ducts and were hypothesized to serve as a model of PBC even though no AMAs were observed. It is possible that the bile duct lesions in the PBC SCID mice are due to a similar GVH-like reaction. This could, in fact, prove to be an important event in the development of AMAs in the mice. Upon destruction of the bile duct cells, mitochondrial antigens could become accessible to the autoreactive B cells present in the patient's PBLs that were transferred into the mice. Since the mitochondrial antigens to which the AMAs react are highly conserved, the presence of mouse antigen would be expected to stimulate the human B cells. In this case, then, the GVH-like liver damage and the development of AMAs would be linked and the mice actually develop a PBC-like condition.

If the above events actually do occur, then the transfer of PBLs result in the development of disease. This remains highly speculative, however. It remains to be formally proven whether actual disease is duplicated in the mice or they simply serve as an in vivo reservoir in which abnormal lymphoid populations are maintained. In the latter case the presence of AMAs would not be due to reactivity against a conserved murine antigen but rather to spontaneous secretion of autoantibodies by the human cells. This and other important issues, such as the variability of reconstitution even when animals of the same litter are used as recipients of comparable numbers of cells from the same donor, remain to be resolved in the use of these mice for the study of human lymphoid abnormalities.

Summary

Human PBL have been reported to reconstitute B and T cells as well as human serum Ig in mice with severe combined immunodeficiency disease (SCID). To confirm these observations and attempt the transfer of an autoimmune disease to the immunodeficient animals, groups of SCID mice received an injection of PBL from patients with primary biliary cirrhosis (PBC) or from normal volunteers. By 8 wk after the injection of 10–42 x 10^6 PBL into the mice, human lymphoid cells were detected in the spleen of approximately half of the animals and all had detectable serum levels of human IgG. Moreover, the sera of SCID mice that received cells
from patients with PBC contained human antimitochondrial antibodies (AMA) to dihydrolipoamide acetyltransferase, the major mitochondrial autoantigen of PBC. Histologically, a human mononuclear cell infiltrate was present around the portal areas of the liver and inflammation, bile duct atypica, and necrosis of bile duct cells were observed. While the biliary lesions in the SCID recipients of PBC cells were more severe, a mononuclear infiltrate was clearly evident in mice that received cells from normal donors, suggesting the presence of a graft-vs-host-like disease. While these data are the first to describe an animal model with both the humoral and cellular characteristics of PBC, they also raise an interesting question regarding the preferential localization of lymphoid cells to the biliary system.

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