

The Concurrent Maturation of Mouse and Human Thymocytes in Human Fetal Thymus Implanted in NIH-beige-nude-xid Mice Is Associated with the Reconstitution of the Murine Immune System

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Summary

To determine whether the human thymus provides an environment for the maturation of murine T cells, human fetal thymus and liver (hu-thy/liv) were implanted into congenitally athymic NIH-beige-nude-xid (BNX) mice or C.B-17 *scid/scid* (SCID) mice. 3 mo after implantation, in contrast to the hu-thy/liv implant in SCID mice, which was populated only with human CD4/CD8 single- and double-positive thymocytes, the hu-thy/liv implant in BNX mice contained a chimeric population of human and mouse CD4/CD8 single- and double-positive thymocytes. Immunohistochemical staining of the hu-thy/liv implant in BNX mice indicated that the population of double-positive mouse thymocytes was localized to discrete areas of the human fetal thymus. Quantitative improvements in mouse T cell and immunoglobulin (Ig)G parameters were observed after grafting of the human fetal thymus and liver tissue into BNX mice. In addition, in contrast to the nonimplanted BNX mice, the implanted BNX mice were capable of mounting a keyhole limpet hemocyanin-specific IgG response and their peripheral T cells were responsive to stimulation with mitogens and antibodies directed to the T cell receptor. Furthermore, after *in vivo* priming, T cells present in lymph nodes of the implanted BNX mice were capable of mounting an antigen-induced *in vitro* T cell-dependent proliferative response. Thus, concurrent with the continued maturation of human T cells, murine T cells differentiated within the human fetal thymus implanted in the BNX mice and mediated the phenotypic and functional reconstitution of the murine immune system. Mice with a reconstituted immune system that contain a human thymic implant that is infectible with human immunodeficiency virus (HIV) should prove useful in the investigation of T cell maturation in the thymus and in the evaluation of potential HIV vaccines.

The reduced ability of NIH-beige-nude-xid (BNX)¹ mice and C.B-17 *scid/scid* (SCID) mice to mount an effective cellular and humoral immune response limits their capacity to reject xenografts and therefore permits them to be successfully engrafted with human hematolymphoid cells (1). The etiology of the congenital immunodeficiency of BNX mice differs from that of SCID mice. The BNX strain was derived by crossing congenitally athymic nude mice with NK cell-deficient beige mice and LAK cell-deficient xid mice (2). BNX mice also have a severe Ig deficiency due to the combined effect of a marked decrease in the numbers of helper T cells and in the subset of T cell-independent B cells (1). Since other components of the immune system besides T cells

play a role in xenograft rejection (3), the BNX mice are more tolerant for xenografts than the parental nude mice (1, 4). In contrast to the etiology of the T cell defect in BNX mice, the cause of the T cell defect in SCID mice is not the absence of a functional thymus but rather impaired maturation of T cells secondary to the defective recombination of the antigen receptor genes (5, 6). Therefore, while the immunodeficiency of congenitally athymic mice can be restored by the implantation of either allogeneic mouse thymus (7–10) or xenogeneic rat thymic tissue (11), the intrinsic cellular immune defect of SCID mice is not corrected by thymic transplantation (12).

To investigate whether human thymus could provide an environment wherein mouse and human T cells could concurrently mature, human fetal thymus and liver (hu-thy/liv) were implanted in BNX mice. We postulated that mouse T cells that matured in the human thymic environment would

¹ Abbreviations used in this paper: BNX, NIH-beige-nude-xid; hu-thy/liv, human fetal thymus and liver.

be tolerant of the human donor tissue, as described for nude mice reconstituted with rat thymic tissue (11). In addition, continued maturation of human thymocytes should ensue if fetal liver were coimplanted with the human fetal thymus as described for SCID mice (13). Since the capacity of T cells to distinguish between self and nonself is acquired in the thymus and involves positive and negative selection (14–16), a thymic environment wherein two xenogeneic T cell lineages were maturing should provide a unique model for the study of intrathymic differentiation. We report that after implantation of hu-thy/liv into BNX mice, murine thymocytes matured within the hu-thy/liv graft and mediated the phenotypic and functional reconstitution of the murine immune system concurrent with the continued growth and maturation of human thymocytes.

Materials and Methods

Animals. A colony of SCID mice was established from founder C.B-17 *scid/scid* mice delivered by Caesarean section (Taconic Farms, Germantown, NY) and was maintained in pathogen-free sterile isolators (Standard Safety, Palatine, IL) without prophylactic antibiotic treatment at the Albert Einstein College of Medicine Animal Institute. Homozygous BNX (*bg/bg-nu/nu-x^h/x^h*) mice were obtained from the National Cancer Institute (Frederick, MD) and were maintained in microisolator cages. Control C.B-17 mice were acquired from Taconic Farms. All of the mice were cared for according to institutional guidelines, and all food, water, caging, and bedding were autoclaved before use.

Implantation of hu-thy/liv Tissue Into SCID or BNX Mice. The consent forms and procedures used in this study were reviewed and approved by the Albert Einstein College of Medicine Committee on Clinical Investigation. After the elective termination of pregnancy, hu-thy/liv tissue obtained from 17–21-gestational-week fetuses were implanted into male (6–8-wk-old) mice within 8 h of availability. The fetal gestational age was determined by foot length measurements as described (17). After the SCID or BNX mice were anesthetized with pentobarbital (40–80 mg/kg), a 3-cm incision was made in the left flank, the left kidney was exteriorized, and 1-mm³ pieces of hu-thy/liv were implanted with a 16-gauge trocar under the kidney capsule as described (18). The peritoneal layers were approximated with 7-0 nylon sutures and the wound was closed with an Autoclip (Clay Adams, Parsippany, NJ). All surgical procedures were performed in a laminar flow hood using sterile technique. After surgery, the mice were started on trimethoprim/sulfamethoxazole antibiotic (TMS; Schein Pharmaceutical Inc., Port Washington, NY) prophylaxis as described (19) and were housed in bonnetted isolator cages (Lab Products Inc., Federalburg, MD) in an environment that was monitored for mouse pathogens.

Recovery of Tissue and Cells from Mice. Blood was obtained from the retro-orbital sinus of anesthetized mice at the indicated times, and serum was obtained or peripheral leukocytes were isolated as described (20). Selected mice were killed by lethal ether inhalation and the hu-thy/liv implants, spleens, and lymph nodes were removed, dissected free of connective tissue, and divided. A portion of the tissues was immediately embedded in OCT, snap frozen in liquid nitrogen, and stored at –70°C for immunohistochemical analysis. Single cell suspensions were obtained from another portion of the tissues by gently teasing it apart, and then filtering the suspension through a sterile stainless steel mesh. After lysis of red blood cells by incubation in Tris-buffered ammonium chloride, the

cells were washed three times in ice-cold PBS containing 1% BSA and 0.01% NaN₃ wash buffer, and then resuspended for flow cytometric analysis.

Antibodies. For flow cytometric analysis, cells were stained with PE-conjugated mouse mAb to human CD4 (Leu 3a; Becton Dickinson & Co., Mountain View, CA), FITC-conjugated mouse mAb to human CD8 (Leu 2a; Becton Dickinson & Co.), PE-conjugated rat mAb to mouse CD4 (Pharmingen, San Francisco, CA), or FITC-conjugated rat mAb to mouse CD8 (Pharmingen). For immunohistochemical staining, sections were either incubated with biotin-conjugated mouse mAb to human CD45 (Pharmingen), or incubated first with rat mAb to mouse CD4 (Boehringer Mannheim Biochemicals, Indianapolis, IN) or rat mAb to mouse CD8 (Boehringer Mannheim Biochemicals), and then incubated with biotinylated rabbit antibodies (mouse adsorbed) to rat IgG (Vector Laboratories, Burlingame, CA). For ELISAs, alkaline phosphatase-conjugated goat (human adsorbed) antibody to mouse IgG, goat (mouse adsorbed) antibody to human IgG, and alkaline phosphatase-conjugated goat (mouse adsorbed) antibody to human IgG were obtained from Jackson Immunolabs (West Grove, PA). For stimulation of mouse T cells, hamster antibody directed specifically against the CD3 ϵ component of the mouse TCR-CD3 complex was obtained from Boehringer Mannheim Biochemicals. All the antibodies used were species specific and noncrossreactive as determined by performing the appropriate control experiments.

Flow Cytometric Analysis. The surface expression of mouse CD4 and CD8 or human CD4 and CD8 by lymphocytes was determined by two-color flow cytometric analysis. Briefly, single cell suspensions (5×10^4 cells) were incubated with the indicated antibodies for 30 min at 4°C, washed in wash buffer, fixed in 1% paraformaldehyde in PBS for 12 h, and then washed and resuspended in wash buffer. The indicated cell population was analyzed using a FACScan[®] cell analyzer with Lysis-II software (Becton Dickinson & Co.). After nonviable cells and unlysed red blood cells were gated out based on their forward and side scatter profiles, 20,000 events per sample were acquired. Lymphocyte gates were set on the basis of forward and side scatter profiles to correspond to gates set to include both control human (from healthy adult volunteer) and mouse (from healthy C.B-17 mice) lymphocytes. After compensation for PE vs. FITC emission based on the analysis of single and double staining of positive and negative control samples (human adult and C.B-17 mouse mononuclear cells) and analysis of the appropriate isotype controls, cut off values for the quadrants were set.

Mitogen Stimulation Assays. Mouse spleen mononuclear cells were isolated by centrifugation over a Ficoll-Hypaque gradient, washed, suspended in RPMI 1640 with added 10% heat-inactivated FCS and 2-ME (50 μ M), plated (2.5×10^5 cells/well) in 96-well round-bottomed microplates, and cultured at 37°C for 3 d. Cells in quadruplicate wells were stimulated with either PHA (0.25 μ g/well) or Con A (0.5 μ g/well). Cellular proliferation was assessed by determining the incorporation of [³H]thymidine (1 μ Ci/well) added during the last 6 h of culture as described (21).

T Cell Stimulation Assay. The responsiveness of T cells present in the mice to stimulation with an antibody directed against the CD3 ϵ component of the mouse TCR-CD3 complex was assessed as described (22, 23). Indicated wells of flat-bottomed microplates were coated with hamster anti-mouse CD3 ϵ (2.5 μ g/well) in 50 μ l of PBS for 1 h at 37°C, and unbound antibody was washed away. Splenocytes (2×10^5 /well) were cultured in anti-mouse CD3 ϵ -coated or uncoated wells in RPMI 1640 with added 10% heat-inactivated FCS, 2-ME (50 μ M), and 1 ng/ml PMA (Sigma Chemical Co., St. Louis, MO) for 72 h. Cellular proliferation was

assessed by measuring the incorporation of [³H]thymidine (1 μCi/well) added during the last 24 h of culture.

Immunohistochemistry. Serial frozen sections (5 μm) of the hu-thy/liv implants were mounted on slides coated with poly-L-lysine (Sigma Chemical Co.) and then fixed in cold acetone at 4°C for 10 min. The sections were incubated overnight at 4°C with either biotin-conjugated mouse mAb to human CD45, rat mAb to mouse CD4, or rat mAb to mouse CD8. Sections to which primary rat mAbs had been applied were incubated with biotinylated rabbit antibodies to rat IgG for 30 min. The sections were stained by the avidin-biotin-peroxidase complex (ABC) method using an ABC kit (Vector Laboratories), and bound antibodies were visualized using the substrate diaminobenzidine (Sigma Chemical Co.). The sections were washed twice with Tris-buffered saline (pH 7.4) between each step.

Quantitation of Mouse IgG Subclass Antibody Levels. The concentrations of mouse IgG1, IgG2a, IgG2b, and IgG3 antibodies present in serum were measured by using IgG subclass-specific radial immunodiffusion (RID) plates (Tago Inc., Burlingame, CA). An aliquot of appropriately diluted serum was applied to wells in the RID plates, incubated for 24 h at 37°C, and then the concentrations of the IgG subclass antibodies were determined according to the manufacturer's protocol. Crossreactivity with human antibodies was not observed, and proper assay conditions were ensured by the inclusion of internal standards on every plate.

Measurement of Human IgG Antibody Levels. The serum concentration of human IgG in the BNX-hu and SCID-hu mice was measured by a human IgG-specific ELISA using a variation of a previously described technique (21). Briefly, microtiter plates (Corning, Corning, NY) were coated for 1 h at 37°C with goat anti-human IgG diluted 1:2,000 in 100 mM NaHCO₃ (pH 9.6) and then washed. After incubation of the appropriately diluted serum samples in duplicate for 1 h at 37°C, the wells were washed, and alkaline phosphatase-conjugated goat anti-human IgG was added for 1 h at 37°C. The plates were washed, substrate solution (*p*-nitrophenyl phosphate; Sigma Chemical Co.) was added, and absorbance at 405 nm was measured with an automated spectrophotometer (Titertek Multiscan; Flow Laboratories, Inc., McLean, VA). Human IgG standards were run simultaneously with the serum samples to generate a standard curve, and the IgG concentration of the samples was calculated by a computer program (TiterSoft; Flow Laboratories, Inc.). Crossreactivity of the ELISA with mouse Ig was not observed.

Detection of Anti-KLH-specific IgG. BNX-hu mice and age-matched BNX mice were immunized by intraperitoneal injection with 100 μg of KLH (Pierce Chemical Co., Rockford, IL) emulsified in CFA (Difco Laboratories, Detroit, MI) and boosted 1 mo later with 100 μg of KLH emulsified in IFA as described (24, 25). Anti-KLH mouse IgG was detected using a modification of a previously described technique (26). Briefly, microtiter plates were coated with KLH (10 μg/ml) in 100 mM NaHCO₃ (pH 9.6) overnight at 4°C, and blocked for 1 h at room temperature with PBS containing 1% BSA and 1% goat serum. After overnight incubation of the indicated dilution of the mouse serum sample in the sample wells at 4°C, the plates were extensively washed, and then alkaline phosphatase-conjugated goat anti-mouse IgG or alkaline phosphatase-conjugated goat anti-human IgG was incubated in the plates for 2 h at room temperature. The plates were washed, substrate solution (*p*-nitrophenyl phosphate; Sigma Chemical Co.) was added, and absorbance at 405 nm was measured as described above.

Characterization of In Vitro Responsiveness of Peripheral T Cells after Immunization with KLH. Peripheral T cells were assessed for their ability to generate an antigen-specific response to KLH as described

(27, 28). Mice were primed by injection of KLH (10 μg) emulsified in CFA into their hind footpads. Draining lymph nodes were harvested 9–21 d after priming, a single cell suspension was obtained as described above, and mononuclear cells were isolated by centrifugation over a Ficoll-Hypaque gradient. Highly enriched T cell preparations were obtained by passing the lymph node mononuclear cells through a nylon wool column (27, 28). APC were obtained from each mouse by harvesting their peritoneal exudate cells, ~70% of which were macrophages, by peritoneal lavage with cold PBS (27). Irradiated (4,000 rad) peritoneal exudate cells (2.5×10^5 cells/well) were cultured with the highly purified lymph node T cells (4×10^5 cells/well) and the indicated concentration of KLH in flat-bottomed microtiter plates for 5 d. Cellular proliferation was assessed by determining the incorporation of [³H]thymidine added (1 μCi/well) during the last 24 h of culture. No significant cellular proliferation was detected in control wells to which irradiated peritoneal exudate cells alone were added.

Statistical Analysis of the Data. The student's *t* test for unpaired data was used for all statistical comparisons made.

Results

Cytometric Analysis of the hu-thy/liv Implants. 3 mo after implantation of hu-thy/liv tissue under the kidney capsules of BNX (BNX-hu) and SCID (SCID-hu) mice, selected mice were killed for analysis. At necropsy the implanted tissue in both the BNX-hu and SCID-hu mice had increased from the size implanted (~1 mm³) to ~5 × 10 mm. The histological appearance of the hu-thy/liv implants closely resembled those of normal human thymus with clear demarcation of cortical and medullary regions. The expression of CD4 and CD8 by human thymocytes present in the hu-thy/liv implants was examined by two-color flow cytometry. The human thymus implanted in the SCID-hu (Fig. 1A) and the BNX-

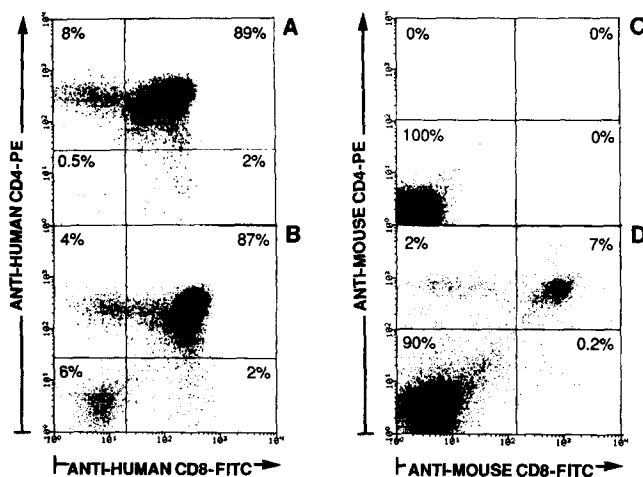


Figure 1. Flow cytometric analysis of the hu-thy/liv implant in SCID-hu mice and BNX-hu mice. Thymocytes from the hu-thy/liv implant from a SCID-hu (A and C) or a BNX-hu mouse (B and D) were analyzed by two-color flow cytometry for the expression of human CD4 and CD8 (A and B) or for mouse CD4 and CD8 (C and D), as described in Materials and Methods. Percentages of cells in each quadrant are indicated. The immunofluorescence profiles shown reflect the profiles obtained from analysis of three SCID-hu and three BNX-hu hu-thy/liv implants.

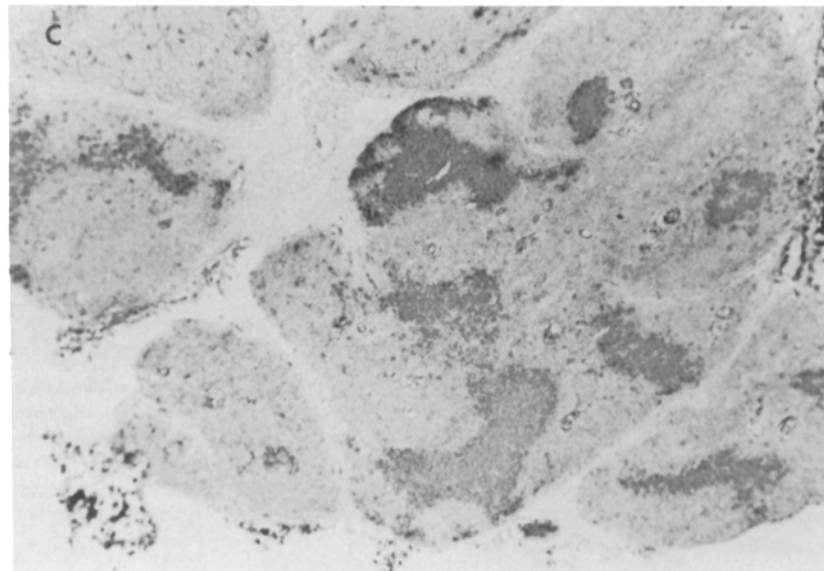
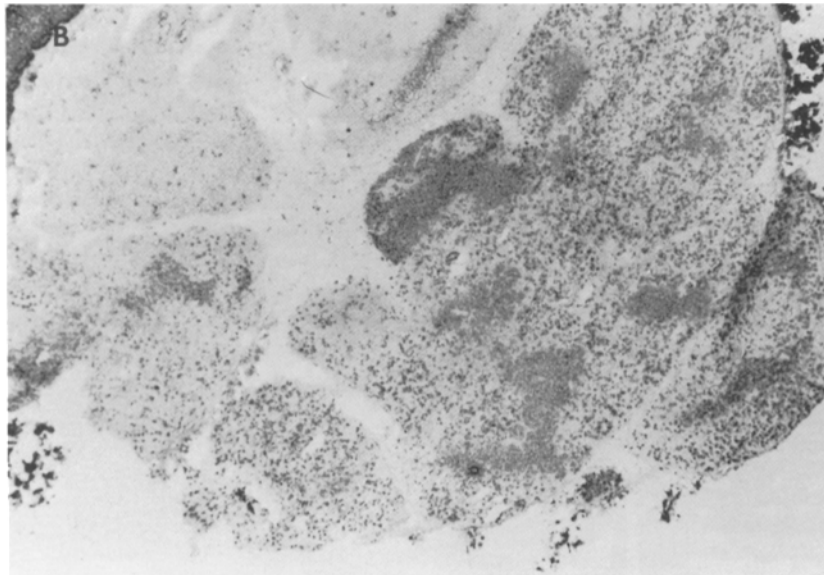
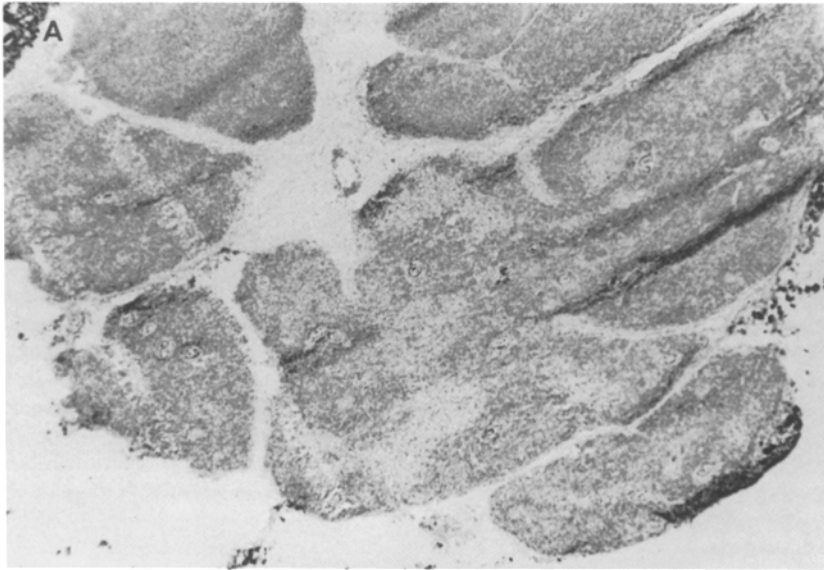
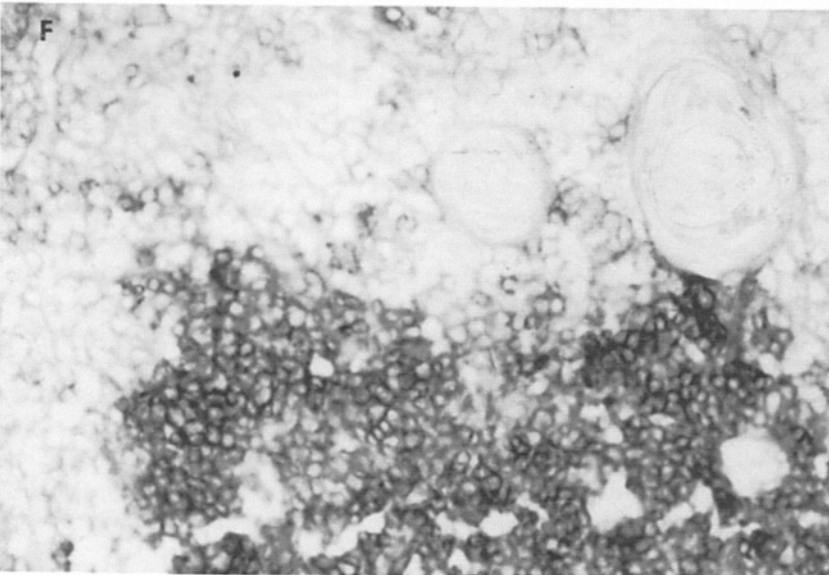
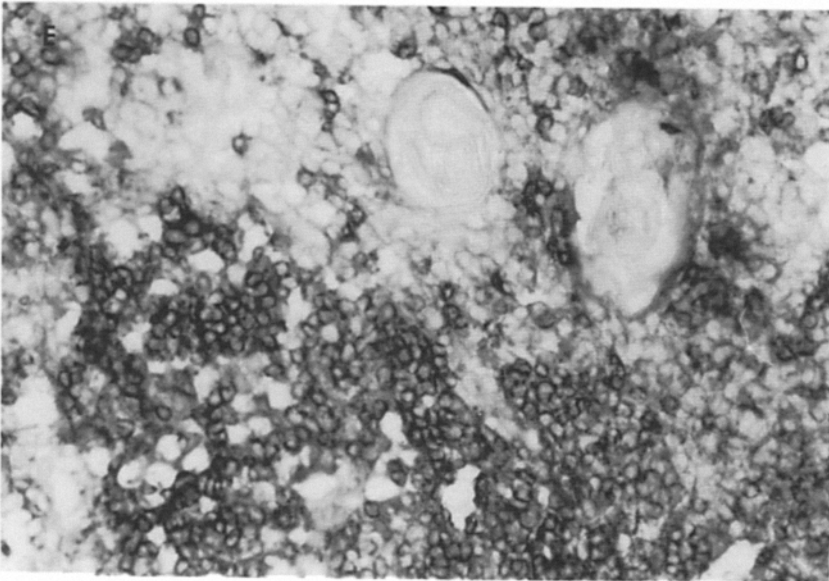
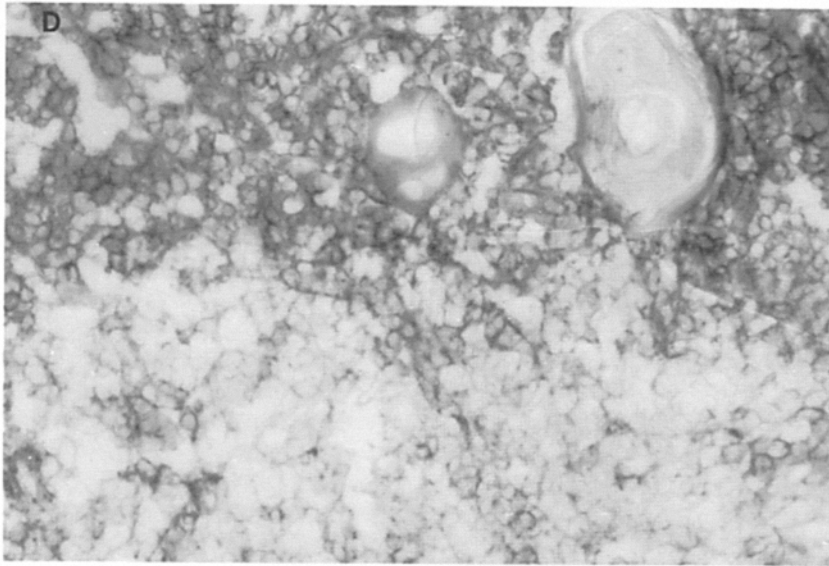


Figure 2. Photomicrographs of serial sections of the BNX-hu hu-thy/liv graft immunoperoxidase stained for the detection of human CD45, mouse CD4, and mouse CD8 expression. (A) Antibodies to human CD45 stained the majority of the cells in the graft. Several clusters of nonstaining cells were distributed throughout the implant. The nonstaining human CD45 clusters corresponded to cells that stained with (B) antibodies to mouse CD4 and (C) antibodies to mouse CD8. High-power views of the cell clusters using two Hassal's corpuscles as landmarks in serial sections showed (D) human CD45 staining of cells in the upper half, and (E) mouse CD4 and (F) mouse CD8 staining of cells in the lower half. The photographs shown are representative of three BNX-hu hu-thy/liv implants studied (A-C) 30 × ; (D-F) 300 × .



hu mice (Fig. 1 B) contained a mixed population of human thymocytes. Over 85% of the human lymphocytes in the hu-thy/liv implants from the SCID-hu and BNX-hu mice were double positive for CD4 and CD8, and the remainder were single positive for either CD4 or CD8. Thus, 3 mo after implantation into BNX-hu and SCID-hu mice, phenotypic maturation of human T cells was continuing to occur in the hu-thy/liv implant comparable to differentiation of thymocytes in normal human thymus (29). However, whereas almost all of the lymphocytes in the SCID-hu hu-thy/liv implant were positive for either human CD4 or CD8, a significant percentage of lymphocytes in the BNX-hu hu-thy/liv implant were negative for these markers. To determine whether mouse T cells were present in the BNX-hu hu-thy/liv implant and accounted for the population of cells that did not express human CD4 or CD8, we assessed the expression of mouse CD4 and CD8 by thymocytes in the hu-thy/liv implant. Whereas no mouse T cells were observed in the SCID-hu hu-thy/liv implant (Fig. 1 C), 9.2% of the cells detected in the BNX-hu implant (Fig. 2 D) were mouse T cells that were either single positive for CD4 or CD8 (2.2%), or double positive for CD4 and CD8 (7.0%). The relative percentages of the major murine thymocyte subpopulations detected in the BNX-hu hu-thy/liv implant were comparable to that which occurs in the normal mouse thymus (16).

Immunohistochemical Assessment of the Thymic Implants. The anatomical distribution of the mouse thymocytes in the hu-thy/liv implant was determined by immunohistochemical staining of serial sections of the hu-thy/liv implants for expression of mouse CD4 and CD8. In addition, to delineate the location of mouse thymocytes relative to human thymocytes, serial sections of the hu-thy/liv implants were evaluated for the expression of the human leukocyte common antigen, CD45, which is present on human thymocytes at all stages of differentiation (30). Almost all of the cells present in the hu-thy/liv implant in SCID-hu mice expressed human CD45, and no thymocytes positive for mouse CD4 and CD8 were observed (data not shown). In contrast, although the majority of cells present in the hu-thy/liv implant in BNX-hu mice were positive for human CD45, clusters of cells were observed in some thymic lobes that did not express human CD45 (Fig. 2 A), but rather expressed both mouse CD4 (Fig. 2 B) and mouse CD8 (Fig. 2 C). Examination of serial sections of the BNX-hu hu-thy/liv implant under higher power demonstrates the reciprocal distribution of human cells expressing CD45 (Fig. 2 D) and murine cells expressing CD4 (Fig. 2 E) or CD8 (Fig. 2 F). Taken together these results indicate that mouse thymocytes that were double positive for CD4 and CD8 were localized in discrete regions of the human thymus. However, although most murine CD8 cells in the implant were confined to the human CD45-negative and murine CD4-positive regions, low numbers of mouse CD4-positive cells were also found dispersed throughout the implant. Examination of expression of mouse Ia in the hu-thy/liv implants indicated that dendritic-appearing cells positive for mouse Ia were scattered in the SCID-hu (Fig. 3 A) and BNX-hu (Fig. 3 B) implants.

Reconstitution of the Murine Immune System in BNX-hu Mice. The effect of implantation of hu-thy/liv tissue on repopulation of the murine peripheral cellular immune system of the mice was examined by evaluating the mouse CD4- or CD8-positive lymphocyte population present in the peripheral blood, spleen, and lymph nodes of nine BNX-hu mice. The BNX-hu mice displayed a significant increase in CD4- or CD8-expressing murine lymphocytes in the peripheral blood, spleen, and lymph nodes ($p < 0.01$) when compared with age-matched control BNX mice (Table 1). In contrast, the number of mouse CD4- or CD8-positive lymphocytes in SCID-hu mice was not affected by the hu-thy/liv implant (data not shown).

The function of the reconstituted peripheral T cells in BNX-hu mice was assessed by investigating their responsiveness to stimulation with ligands that interact with the TCR-CD3 complex, such as PHA, Con A, or antibodies directed to the CD3 ϵ component of the mouse TCR-CD3 complex (31). As shown in Fig. 4, the response of splenic T cells from BNX-hu mice to PHA stimulation was significantly greater than that of splenic T cells from BNX mice ($p < 0.001$), as was the response of splenic T cells from three BNX-hu mice (nos. 1, 2, and 4) to Con A ($p < 0.01$). To further assess the function of T cells in the BNX-hu mice, splenocytes were stimulated with immobilized anti-mouse CD3 ϵ and a surrogate second signal, PMA (31). In contrast to splenocytes from BNX mice, which exhibited minimal response to stimulation with anti-mouse CD3 ϵ and PMA, splenocytes from BNX-hu mice proliferated vigorously in response to stimulation with anti-mouse CD3 ϵ and PMA (Table 2). Little cellular proliferation was observed in the absence of cellular stimulation or if the cells were stimulated with PMA alone. The degree of functional reconstitution of T cell immunity in BNX-hu mice was further investigated by assessing their ability to mount an antigen-induced *in vitro* T cell-dependent proliferative response after *in vivo* priming (24, 25). After immunization of BNX-hu and BNX mice with KLH, a highly enriched T cell population was obtained from their draining lymph nodes and stimulated in culture with KLH. As shown in Fig. 5, in contrast to T cells from BNX mice that did not respond to KLH, T cells from BNX-hu mice displayed a significant proliferative response to KLH in a dose-responsive manner.

The effect that reconstitution of the T cell compartment of the BNX-hu mice had on mouse Ig production was assessed by determining the concentration of mouse IgG subclasses in serum from BNX-hu mice. In contrast to the markedly reduced levels of all IgG subclasses observed in sera from age-matched control BNX and preimplant BNX-hu mice, all mouse IgG subclasses were detected at either normal or elevated levels in the postimplant BNX-hu mice (Table 3). The concentration of mouse IgG in the serum of the SCID-hu mice was not affected by implantation of hu-thy/liv tissue. The B cell function of the BNX-hu mice was further evaluated by examining their capacity to mount an antigen-specific humoral immune response to KLH. After BNX and BNX-hu mice received two immunizations with KLH, the presence of mouse IgG directed to KLH was assessed. Whereas

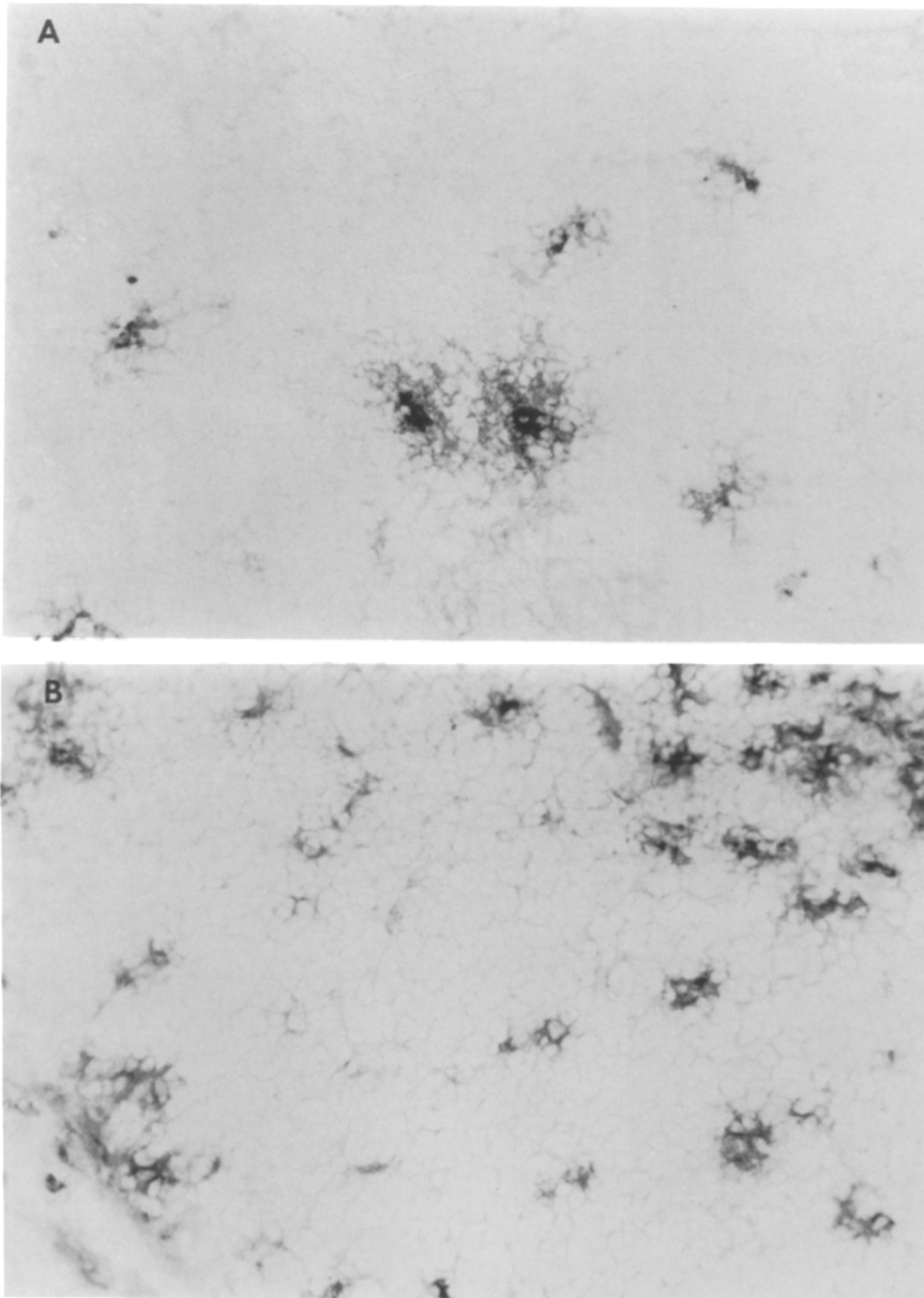


Figure 3. Photomicrographs of immunoperoxidase-stained sections for the detection of mouse Ia expression. Cells expressing mouse Ia were detected in the hu-thy/liv implant from the (A) SCID-hu mouse and the (B) BNX-hu mouse. The elongated cell processes of the stained cells were suggestive of dendritic type cells. $\times 300$.

no IgG response directed to KLH was detected in the serum of BNX mice immunized with KLH, a significant murine IgG response to KLH was observed in KLH-immunized BNX-hu mice (Fig. 6). No human IgG antibodies directed to KLH were detected.

Detection of Human T Cells and IgG in SCID-hu and BNX-hu Mice. Since human T cells and IgG can be detected in the blood of SCID-hu mice (19), the degree of reconstitution of BNX-hu mice with the human immune system was com-

pared with that observed in SCID-hu mice. As shown in Table 4, although cells positive for human CD4 and CD8 were detected in the peripheral blood of both SCID-hu and BNX-hu mice, a significantly higher percentage of human CD4-positive cells was detected in SCID-hu than in BNX-hu mice ($p < 0.05$). No human CD4- or CD8-positive cells were detected in the spleen or lymph nodes of BNX-hu mice. 6 wk after implantation, the concentration of human IgG present in the serum of BNX-hu and SCID-hu mice was determined

Table 1. Flow Cytometric Analysis of Blood, Lymph Node, and Spleen of BNX, BNX-hu, and C.B-17 Mice

Mouse	Peripheral blood		Spleen		Lymph node	
	CD4	CD8	CD4	CD8	CD4	CD8
BNX	1.13 ± 0.19	0.53 ± 0.15	3.12 ± 1.18	1.22 ± 0.67	4.55 ± 0.73	1.95 ± 0.62
BNX-hu	17.33 ± 2.08	7.81 ± 1.91	14.99 ± 1.57	5.54 ± 1.34	34.01 ± 3.15	8.98 ± 1.21
C.B-17	28.5 ± 1.03	5.25 ± 1.43	24.50 ± 0.75	9.00 ± 0.35	40.50 ± 3.05	25.50 ± 3.34

Mononuclear cells obtained from the blood, lymph nodes, and spleen of either BNX mice ($n = 6$), BNX-hu mice 3 mo after implantation ($n = 9$), or C.B-17 mice ($n = 4$) were analyzed by flow cytometry. The mean percentage of cells within the lymphocyte gates expressing mouse CD4 or CD8 of the analyzed mice ± SEM is shown.

Table 2. Proliferative Responses of Splenocytes after Activation with Antibodies Directed against the CD3 ϵ Component of the Mouse TCR-CD3 Complex

Mouse	Unstimulated	PMA	Anti-mouse CD3 ϵ + PMA
BNX no. 1	131 ± 22	307 ± 44	905 ± 204
BNX no. 2	151 ± 33	392 ± 51	519 ± 50
BNX-hu no. 1	139 ± 5	1079 ± 133	106,894 ± 3,055
BNX-hu no. 2	2,085 ± 55	3,289 ± 385	119,440 ± 7,230
BNX-hu no. 3	381 ± 59	1,471 ± 154	76,887 ± 1,255

Splenocytes (2×10^5 /well) from the indicated mouse were either not stimulated, stimulated with PMA (1 ng/ml), or stimulated with PMA (1 ng/ml) and immobilized antibody to mouse CD3 ϵ (2.5 μ g/well) for 3 d as described in Materials and Methods. Results are reported as cpm ± SEM of triplicate wells.

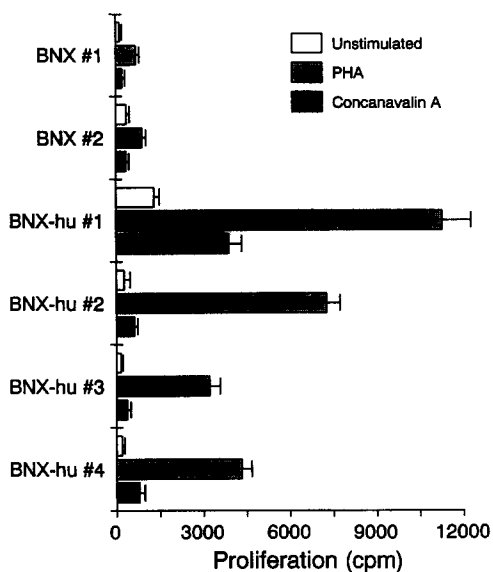
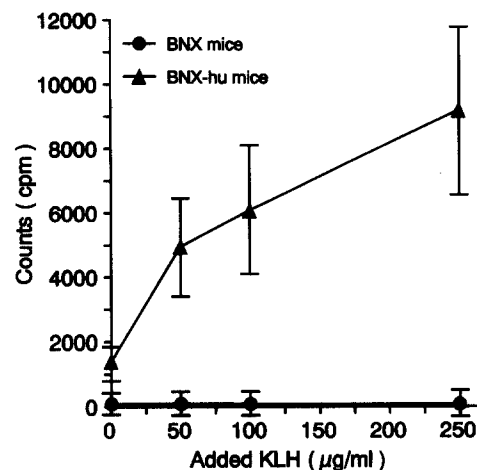
**Figure 4.** Proliferative responses to mitogens. The response of spleen cells from the indicated mice to stimulation with either PHA (0.25 μ g/well) or Con A (0.5 μ g/well) determined as described in Materials and Methods. Results are reported as cpm ± SEM of quadruplicate wells.**Figure 5.** Response of T cells from KLH-immunized mice to stimulation with KLH. Highly enriched T cells (4×10^5 cells/well) isolated from the lymph nodes of three immunized BNX mice and five immunized BNX-hu mice were cultured with irradiated (4,000 rad) APC (2.5×10^5 peritoneal exudate cells/well) and the indicated concentration of KLH for 5 d as described in Materials and Methods. Results from three immunized BNX mice and five immunized BNX-hu mice are shown as mean cpm ± SEM triplicate cultures of the studied mice.

Table 3. Quantitation of Mouse IgG Subclass Antibodies in BNX and Pre- and Postimplant BNX-hu and SCID-hu Mice

Mouse	IgG1	IgG2a	IgG2b	IgG3
	mg/ml			
BNX	0.20 ± 0	0	0	0
BNX-hu (preimplant)	0.23 ± 0.06	0	0	0
BNX-hu (postimplant)	22.32 ± 4.16	12.83 ± 3.27	2.02 ± 0.33	1.50 ± 0.06
SCID-hu (preimplant)	0	0	0	0
SCID-hu (postimplant)	0.04 ± 0.04	0	0	0

The concentrations of mouse IgG1, IgG2a, IgG2b, and IgG3 in the serum of SCID-hu mice before and 6 wk after hu-thy/liv implantation ($n = 6$), BNX-hu mice before and 6 wk after hu-thy/liv implantation ($n = 6$), and BNX mice ($n = 2$) housed together with the BNX-hu mice were measured and are shown as mean ± SEM.

using an ELISA specific for human IgG. As shown in Fig. 7, low quantities of human IgG were detected in the sera from both the SCID-hu and the BNX-hu mice. However, significantly more human IgG was present in SCID-hu than in BNX-hu mice ($p < 0.05$). Taken together these results indicate that although some degree of reconstitution of the human immune system was occurring in BNX-hu mice, significantly more reconstitution occurred in SCID-hu mice.

Discussion

In the present study, we investigated whether mouse thymocytes could differentiate in a human thymic environment. Although only a minority of the thymocytes present in the hu-thy/liv implant were murine, the relative percentage of murine thymocytes double positive for CD4 and CD8 (76%) was comparable to that present in the normal adult mouse thymus (80%) (16). Thus, human thymus may substitute for the congenitally absent mouse thymus in providing an environment for mouse thymocyte differentiation. After thymic implantation in BNX mice, quantitative increases in the

numbers of mouse T cells present in the peripheral blood, spleen, and lymph nodes were observed. These peripheral T cells in the BNX-hu mice had the functional capacity to respond to stimulation with mitogens and antibodies directed against the CD3 ϵ component of the mouse TCR-CD3 complex. In addition, T cells isolated from lymph nodes of BNX-hu mice immunized with KLH displayed an antigen-specific proliferative response to KLH. It is unlikely that the response observed reflected the proliferation of human T cells, as no human T cells were detectable by flow cytometric anal-

Table 4. Percentage of Human CD4- and CD8-expressing Leukocytes in the Peripheral Blood of SCID-hu and BNX-hu mice

Mouse	Human CD4	Human CD8
SCID	0 ± 0	0 ± 0
SCID-hu	3.98 ± 0.24	0.10 ± 0.04
BNX	0 ± 0	0 ± 0
BNX-hu	0.17 ± .05	0.08 ± 0.03

PBL from SCID mice ($n = 2$), BNX mice ($n = 2$), SCID-hu ($n = 6$), and BNX-hu ($n = 6$) were stained for the expression of human CD4 or CD8 and analyzed by flow cytometry as described in Materials and Methods. The mean ± SEM percent positive cells is shown.

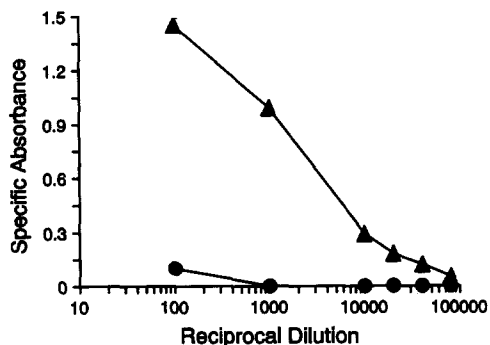


Figure 6. Detection of KLH-specific mouse IgG by ELISA. Murine IgG antibodies directed to KLH in serum obtained from KLH-immunized BNX-hu (\blacktriangle) and BNX mice (\bullet) ($n = 2$) were assessed by ELISA as described in Materials and Methods. Values shown are the mean of specific absorbance at 405 nm of quadruplicate determinations for each reciprocal dilution of mouse serum assayed and were normalized for mouse IgG concentration.

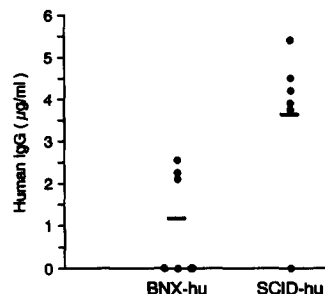


Figure 7. Measurement of human IgG in BNX-hu and SCID-hu serum. The concentrations of human IgG present in the serum of BNX-hu ($n = 6$) or SCID-hu mice ($n = 6$) 6 wk after reconstitution were determined by a human IgG-specific ELISA as described in Materials and Methods. The serum concentration of human IgG for each mouse is shown as well as the mean for the group.

ysis in the spleens or lymph nodes of the BNX-hu mice. Thymic transplantation also corrected the T cell-dependent B cell defect in BNX mice. This was evidenced by the normalization of serum IgG subclass levels in BNX-hu mice and the ability of KLH-immunized BNX-hu mice to mount an antigen-specific antibody response to KLH.

The absence of comparable reconstitution in age-matched BNX mice housed together with the BNX-hu mice for the entire period of the experiment indicated that the restoration of the murine immune system was due to the implanted tissue and not to environmental factors. As reported after transplantation of adult thymus into untreated SCID mice (32), we did not detect significant numbers of CD4- or CD8-positive mouse cells in the SCID-hu hu-thy/liv implant.

The regional clustering of the double-positive murine T cells in the BNX-hu hu-thy/liv implant suggested that mouse pre-T cells from the bone marrow homed to the hu-thy/liv implant and subsequently differentiated. It is unlikely that this reflects deposition of circulating double-positive T cells that had differentiated extrathymically, because while >75% of the mouse T cell population in the implant consisted of double-positive lymphocytes, no double-positive mouse T cells were detected in the peripheral blood, spleen, lymph nodes, or thymic rudiments of the BNX-hu mice. The detection of TCR- β^- mouse T cells in adult human thymic tissue engrafted in anti-asialo GM-1-treated SCID mice led Barry et al. (32) to suggest that mouse pre-T cells could home to the human thymus. However, since mouse T cells double positive for CD4 and CD8 were not demonstrated in the thymic implant, the possibility that the mouse T cells present in the human thymus were mediating graft rejection and not maturing in the thymic graft could not be excluded. This contrasts with our observation of mouse thymocytes at different stages of differentiation in the hu-thy/liv implant in BNX-hu mice. The mechanism by which mouse pre-T cells home to the human thymus is unknown, but it may be related to the ability of mouse thymocytes to bind to human thymic epithelial cells reported by Barry et al. (32). Colonization of the human thymus implanted into SCID-hu mice with mouse Ia⁺ dendritic cells has been reported (19), and was observed by us in the SCID-hu and BNX-hu hu-thy/liv implants. An intriguing possibility is that the mouse dendritic cells that colonize the implant may play a role in the homing and maturation of the mouse T cells in the BNX-hu thymic implant.

After implantation, the degree of peripheral reconstitution of the BNX-hu mice with CD4- or CD8-expressing mouse lymphocytes varied. Whereas the percentage of CD4⁺ lymphocytes in BNX-hu mouse lymph nodes (34%) was comparable to that of C.B-17 mouse lymph nodes (40.5%), the percentage of CD8⁺ cells in the BNX-hu mouse lymph nodes (8.9%) was lower than that of the C.B-17 mouse lymph node (25.5%). Lymph nodes of nude mice implanted with rat thymus exhibited preferential reconstitution of the mouse CD4-positive lymphocyte population compared with the mouse CD8-positive lymphocyte population, and this was felt to reflect an impairment in the positive selection of CD8-

positive cells against class I rat MHC molecules (33). It is possible that the differential distribution of mouse CD4 and CD8 cells in BNX-hu mice may reflect the localization of T cell populations that underwent positive selection for self-MHC restriction extrathymically and then underwent further thymus-dependent differentiation and negative selection intrathymically (34).

The fraction of the human T cells and the concentration of human IgG detected in the peripheral blood of the BNX-hu mice was significantly less than that observed in the SCID-hu mice. Although maturation of human thymocytes in the hu-thy/liv graft was not affected by the reconstituted BNX-hu mouse immune system, peripheral engraftment of human lymphocytes might have been inhibited. This may be either a result of the production of natural antibodies by the reconstituted BNX-hu immune system that can inhibit engraftment of xenografts as reported for the rat \rightarrow mouse species combination (35), or may be the effect of other murine myeloid cells (6).

The presence of both human and mouse double- and single-positive thymocytes in the hu-thy/liv implant at relative percentages comparable to normal human thymus (29) and normal murine thymus (16) >3 mo after implantation suggested that active thymic maturation of both lineages was occurring. This contrasts with rat thymic tissue implanted in nude mice, where, although the rat thymus became populated with large numbers of murine lymphocytes, few rat lymphoid cells were observed (36). The most likely explanation for this difference is that the human fetal liver coimplanted adjacent to the thymus provided a source of human pre-T cells to undergo maturation in the hu-thy/liv implant. This is compatible with the recent observation of Namikawa et al. (13) that long-term human T lymphopoiesis occurred much more frequently when hu-thy/liv were coimplanted in the same SCID-hu mice than when human thymus alone was implanted. Similarly, Frey et al. (12) demonstrated that mouse thymus implanted in SCID mice atrophied after 4–5 wk unless it was replenished with stem cells from cotransplanted normal mouse bone marrow stem cells.

Examination of the function of human and mouse lymphocytes that matured together in the thymus should prove useful in understanding the process of positive and negative selection in the thymic environment. In addition, maturation of murine T cells in human thymus may permit a unique strategy to evaluate the effects of T cell maturation in an HIV-infected thymic environment. Because murine cells are not susceptible to HIV infection, the consequences of HIV infection of human thymic cells on mouse T cell maturation can be studied independently of the influence of their infection with HIV. The presentation of HIV antigens to murine T cells undergoing maturation in the thymus may result in the negative selection of T cells expressing antigen receptors that recognize HIV antigens. This may have implications in understanding the effect of congenital HIV infection on maturation and clonal deletion of uninfected T cells in the thymus (37).

BNX-hu mice with a reconstituted autologous immune

system that are tolerant to the continued presence of human T cells may prove useful as a new model for the evaluation of vaccines for human-specific pathogens. Although the elicited immune response in the BNX-hu model is that of mouse origin and not of human origin, nevertheless, the BNX-hu mice mounted a significant systemic murine IgG and T cell response after immunization with KLH (Figs. 5 and 6). Since human thymic implants in BNX-hu mice could be productively infected with HIV, as described for SCID-hu mice (38),

BNX-hu mice may be useful in the evaluation of the capacity of HIV vaccines to generate in vivo murine protective immune responses. After immunization with an HIV antigen, the ability of the resultant murine immune response to protect the thymic implant from HIV infection could be assessed. Coimplantation of other human fetal tissue with the hu-thy/liv should also permit the use of this model for the investigation of vaccines directed against other human-specific pathogens.

We thank C. Rapelje for assistance in the flow cytometry, A. Watford for assistance in animal care, and F. Lilly and J. Berman for critical reading of the manuscript.

This work was supported by the National Institutes of Health (research grants AI-20671 and AI-27741).

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Received for publication 25 June 1992 and in revised form 19 November 1992.

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