American Journal of Pathology, Vol. 140, No. 5, May 1992

Hemagglutination and Graft-versus-Host Disease in the Severe Combined Immunodeficiency Mouse Lymphoproliferative Disease Model

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In the course of evaluating the severe combined immunodeficiency mouse-human peripheral blood lymphocyte (SCID-PBL) model of lymphoproliferative disease, we noted hemagglutination occurring in peripheral blood smears of mice with serum human immunoglobulin levels greater than 1.0 mg/ml. The hemagglutinating process was mediated by human anti-mouse red cell antibodies of the IgM class, peaked at five to seven weeks post-transfer of 5 to 7 × 10^7 human PBL and was generally self limiting. However, death resulted in some mice when serum immunoglobulin levels were greater than 3.0 mg/ml. The most severely affected mice had hemagglutination induced congestion of liver, lungs and spleen. Several mice also had lesions consistent with graft-versus-host disease (GVHD) including focal hepatic necrosis and destruction of mouse splenic hematopoietic elements. The lesions associated with hemagglutination and GVHD in SCID-PBL mice are distinct from those associated with EBV-induced lymphoproliferation. Recognition of these pathologic processes are required for a thorough understanding of the SCID-PBL model. (Am J Pathol 1992, 140:1187–1194)

The CB.17 scid/scid (SCID) mouse, originally described by Bosma et al,[1] is homozygous for a mutation that disallows generation of functionally rearranged immunoglobulin and T cell receptor genes.[1–8] The lack of mature T and B lymphocytes results in a severe combined immunodeficiency phenotype.[9–11] Because the SCID phenotype allows engraftment of xenogeneic tissues these mice are being extensively utilized as models to study the *in vivo* biology of normal, virus infected and malignant human cells.[12–20]

Short term engraftment of mature human T and B lymphocytes can be demonstrated in the SCID mouse following intraperitoneal transfer of human peripheral blood mononuclear cells from EBV seronegative donors.[13,14] When peripheral blood lymphocytes (PBL) are transferred from Epstein–Barr virus (EBV) seropositive human donors, EBV-induced lymphoproliferative disease occurs in the SCID mice. Although originally proposed as a model of EBV-induced lymphoma, we and others have demonstrated that this model of lymphomagenesis more closely resembles EBV-induced lymphoproliferation that frequently occurs in immunocompromised patients in the absence of specific cytogenetic changes.[21–24]

Although graft-versus-host disease (GVHD) would be expected to occur following xenografting in the SCID-PBL model, Mosier et al[13,14] and Cannon et al[23] reported that GVHD was minimal or nonexistent. In contrast, Bankert et al[25] reported that GVHD-related processes had occurred in this model system and were likely responsible for the inability of the SCID-PBL mice to generate primary antibody responses to protein antigens. They also reported that the SCID-PBL mice had human IgM bound to the surface of erythrocytes as demonstrated by immunofluorescence or agglutination with anti-human IgM.[25] In the course of evaluating the lymphoproliferative disease model we also noted hemagglutination and GVHD-related lesions in a high percentage of SCID-PBL mice engrafted with PBL from normal human donors.[22] The extent of the hemagglutinating disease was directly related to the level of human immunoglobulin in

Supported by Public Health Service CA 30196 and CA 36727 from the National Cancer Institute, Department of Health and Human Services, the State of Nebraska Department of Health, LB506, and the Lymphoproliferative Research Fund.

Accepted for publication December 13, 1991.

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the SCID-PBL mouse serum. We report here the characteristics and lesions associated with hemagglutination and GVHD in the SCID-PBL model.

Materials and Methods

Donors

After receiving informed consent, approximately 200 ml of heparinized blood was obtained from normal, EBV seropositive or seronegative human donors. All normal donors were healthy laboratory workers who were not involved with this project and therefore did not handle the SCID-PBL mice, cells, or tissues. These precautions were taken to circumvent accidental inoculation of autologous EBV-infected lymphoid cells into laboratory staff. Peripheral blood mononuclear cells (PBL) were isolated by Ficoll-hypaque density gradient centrifugation, washed with phosphate buffered saline (PBS) and counted. Cells were resuspended in sterile PBS at 5–7 $\times$ 10^7 cells/ml prior to transfer to the SCID mice. All procedures were carried out with approval of the Institutional Review Board of the University of Nebraska Medical Center (IRB# 270–89).

Mice

C.B-17 SCID breeding trios were originally obtained from McLaughlin Research Institute (Great Falls, MT). Animals utilized for these studies were bred and maintained in a pathogen free environment within a Class-II laminar flow safety hood (Baker, Stanford, ME) in micro-isolator cages at the Animal Resource Facility, University of Nebraska Medical Center. All mice were screened for production of endogenous immunoglobulin (i.e., "leaky" phenotype) by assaying for serum immunoglobulin by radial immunodiffusion (Chemicon, El Segundo CA) at 6 weeks of age. Mice without detectable serum immunoglobulin were utilized as recipients. As prophylaxis against *Pneumocystis carinii* pneumonia, mice were maintained on trimethoprim-sulfamethoxazole (0.32 g/ml trimethoprim and 1.6 g/ml sulfamethoxazole) (Biocraft, Elmwood, NJ) in their drinking water for three of every seven days. The remainder of the week the mice were provided with sterile, acidified water.

PBL Transfer, Monitoring, and Tissue Processing

Mice were inoculated with 5–7 $\times$ 10^7 PBL by intraperitoneal injection. Twenty eight of the 32 mice were engrafted with PBL from EBV seronegative donors and were secondarily inoculated with B95–8 EBV at six weeks post transfer of human PBL as described previously.[21,22] The remaining 4 mice were engrafted with PBL from EBV seropositive donors. At 3 weeks post transfer of PBL and weekly thereafter approximately 0.1 to 0.2 ml of blood was obtained from each mouse by retro-orbital sinus plexus puncture. Serum was assayed by radial immunodiffusion for human immunoglobulin to assess engraftment and peripheral blood smears were made and evaluated for hemagglutination. The degree of hemagglutination was retrospectively assigned a relative value of 1 to 3+ by the following criteria: negative, no evidence of hemagglutination; 1+, mild hemagglutination with occasional small aggregates of 3–4 red cells each; 2+, marked hemagglutination with multiple small and large, multicellular aggregates; and 3+, characterized as 2+ hemagglutination or severe anemia with physical signs of disease.

The mice were monitored three times weekly for changes in physical appearance such as respiratory distress, organomegaly, ruffled fur, decreased mobility, inability to eat, diarrhea, or weight loss. Mice with evidence of hemagglutination on peripheral smear and physical signs of distress were euthanized and autopsied. At necropsy the mice were examined for gross evidence of organomegaly, tissue necrosis or lymphoproliferative lesions. The liver, spleen, heart, lungs, and kidneys as well as grossly visible lymphoproliferative lesions were removed, fixed in 10% buffered formalin and processed for routine tissue sections and microscopic evaluation. The tissues were dehydrated and embedded in paraffin, cut at 5 μm and stained with hematoxylin and eosin. All protocols were carried out in accordance with the guidelines of the Animal Review Committee, University of Nebraska Medical Center (ARC# 89-104-02).

Immunohistochemical Analysis

Immunohistochemical examination was performed on blocks of tissues which were snap frozen in liquid nitrogen or fixed in formalin. The latter were stained with antibodies to a T cell related antigen CD45RO (UCHL-1), a B cell related antigen CD20 (L26), and HLA-DR (LN3) by the avidin-biotin-peroxidase complex (ABC) method.[32,33] Background staining was determined by substituting purified mouse immunoglobulin as primary antibody followed by ABC (negative control). Biotinylated secondary antibodies and ABC were purchased from Vector (Burlingame, CA). Following reaction with 3,3′-diaminobenzidine the sections were counterstained with hematoxylin and examined with the light microscope.

Flow Cytometric Analysis

Erythrocytes obtained from mice with 3+ hemagglutination were evaluated by flow cytometric analysis for

class of surface bound human immunoglobulin by modification of previously described techniques.[26] Approximately 0.2 ml peripheral blood collected in sodium heparin was diluted in PBS with 5% fetal bovine serum (Gibco, Grand Island, NY) and .01% sodium azide to a concentration of 10^6 erythrocytes/ 0.1 ml and aliquoted at 10^6 cells/tube. Individual red cell aliquots were incubated for 30 minutes at 4°C with saturating concentrations of fluorescein (FITC) conjugated goat anti-human IgG, IgM and IgA heavy chains and kappa and lambda light chains (Tago, Burlingame, CA). Background immunofluorescence was determined by staining an aliquot of cells with FITC conjugated goat anti-mouse IgG. Cells were analyzed on a three decade log fluorescence scale for percent positivity and mean channel fluorescence following background subtraction. All assays were performed on a Coulter EPICS C flow cytometer (Coulter, Hialeah, FL) using Coulter software.

Results

Hemagglutination

Of 32 mice receiving 5–7 × 10^7 human PBL by intraperitoneal injection, 23/32 (71%) showed evidence of hemagglutination on peripheral smear (Fig. 1A-C). The degree of hemagglutination was variable from mouse to mouse at the same initial cell dose of PBL but was directly related to the concentration of human immunoglobulin in the serum (Fig. 2). Nine of 32 mice showed no evidence of hemagglutination in peripheral blood smears and all had human immunoglobulin levels less than 1.0 mg/ml. Fourteen mice which had either 1+ or 2+ hemagglutination with no physical signs of distress had immunoglobulin levels between 1.0 and 3.0 mg/ml. Nine mice were severely affected with 2+ hemagglutination or severe anemia on peripheral smear and physical signs of distress (i.e., 3+ hemagglutination). Seven of these nine mice (78%) had immunoglobulin levels greater than 3.0 mg/ml.

The hemagglutinating disease process peaked at 5 to 7 weeks post-transfer of 5–7 × 10^7 human PBL and was generally self limiting. The timing and appearance of more severe hemagglutination was heralded by earlier development of measurable serum levels of human immunoglobulin. Of the 32 mice engrafted, three died of the disease and six were euthanized with 3+ disease. Physically the mice presented with features of GVHD including wasting and diarrhea. One of the mice which had died during the peak of hemagglutination developed cyanosis and necrosis of the digits.

Gross and Microscopic Pathology

Gross and microscopic examination was performed on the six mice euthanized for 3+ disease. Grossly, there

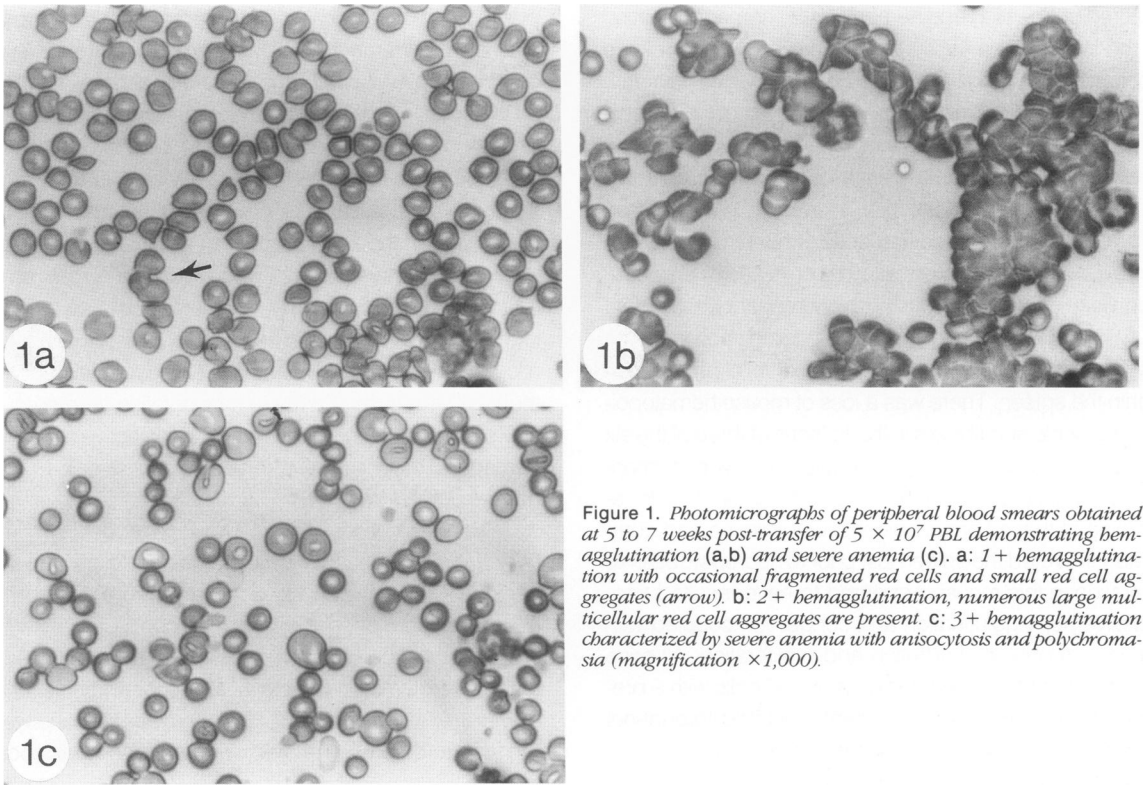

Figure 1. *Photomicrographs of peripheral blood smears obtained at 5 to 7 weeks post-transfer of 5 × 10^7 PBL demonstrating hemagglutination* (a,b) *and severe anemia* (c). a: *1+ hemagglutination with occasional fragmented red cells and small red cell aggregates (arrow).* b: *2+ hemagglutination, numerous large multicellular red cell aggregates are present.* c: *3+ hemagglutination characterized by severe anemia with anisocytosis and polychromasia (magnification ×1,000).*

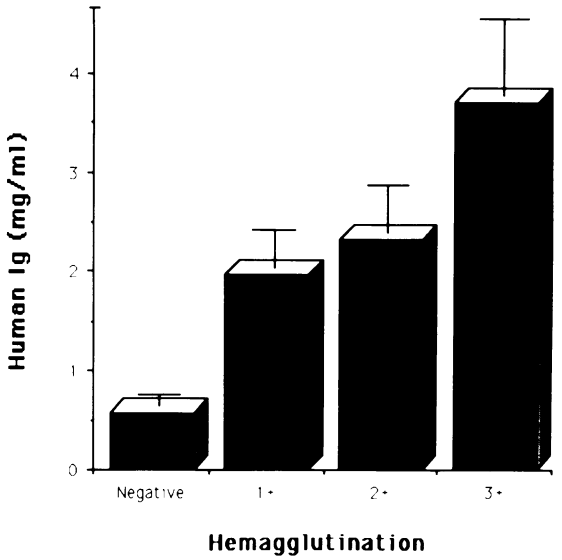

Figure 2. *Degree of hemagglutination on peripheral smear at 5–7 weeks postcell transfer plotted against human immunoglobulin levels in mouse serum. Hemagglutination was assessed as described under Methods. Immunoglobulin levels reflect the group mean values ± one standard deviation. The negative group represents the mean of three mice who had measurable levels of human immunoglobulin. The remaining six mice in this group did not develop measurable immunoglobulin levels in the time period studied. The number of animals in the remaining groups were: 1+ (n = 7), 2+ (n = 7), 3+ (n = 9).*

was mottling of the liver and spleen consistent with congestion in two of the six mice. This finding was confirmed on microscopic examination which demonstrated marked congestion and hemostasis by hemagglutination in the liver, spleen, and lungs. The livers of all six mice contained numerous iron laden macrophages with variable sinusoidal dilatation as a result of hemostasis. Focal areas of necrosis were evident in the livers of three mice (Fig. 3a). Periportal and perivascular cellular infiltrates were present in the livers of all six mice (Fig. 3b). These infiltrates consisted predominantly of human T cells with smaller numbers of B cells, plasma cells and mouse neutrophils (Figs. 4a-c). Grossly, splenomegaly was evident in two mice while microscopically all mice showed some degree of human lymphocyte infiltration and proliferation within the spleen. There was a loss of mouse hematopoietic elements and fibrosis in the spleens of three of the six mice (Figs. 3c and 3d). Immunohistochemistry demonstrated mouse stromal elements and human T cells remaining in these fibrotic spleens. One mouse had completely infarcted the spleen just prior to necropsy. Splenic infarction in this mouse appeared to have resulted from massive hemagglutination and congestion coupled with human lymphocyte infiltration and proliferation. All mice had small, focal aggregates of lymphoid cells with a predominant plasma cell component scattered throughout the visceral and parietal peritoneum (Fig. 3e).

Flow Cytometric Analysis

Flow cytometric analysis for cell surface bound human immunoglobulin was performed on red cells obtained from four mice with 3+ hemagglutinating disease (Fig. 5). These mice represented three separate donors including two EBV seronegatives and one EBV seropositive. After background subtraction, all four specimens demonstrated bound immunoglobulin to be IgM with variable amounts of IgG. We had difficulty demonstrating light chains at the same percent positivity as surface bound IgM however, those demonstrable were predominantly of the kappa class. To determine if circulating, pre-formed anti-mouse red cell antibodies were detectable in the sera of the three donors, we screened donor serum by flow cytometry on erythrocytes obtained from a naive SCID mouse. In none of the three donors could we definitively identify circulating anti-mouse red cell antibodies of IgG, IgM or IgA heavy chain class (not shown).

Discussion

In 1954 Barnes and Loutit[27] reported a secondary disease occurring in mice which had received an allogeneic spleen graft following lethal irradiation. This secondary disease (the primary disease being radiation sickness) was characterized by severe wasting, diarrhea, and skin lesions. A few years later Billingham and Brent described a runting syndrome occurring in newborn mice transplanted with parent strain spleen cells.[28] This runting syndrome was characterized by severe growth retardation, diarrhea, hyperplasia of the lymphatic system followed by hypoplasia, skin lesions, and focal necrosis of the liver. These phenomena were thought to be the result of an immmunologic attack of the graft on the host. Further transplantation studies using parental/F_1 mouse systems clearly established the concept of graft-versus-host disease and that this process was based on a reaction of engrafted, immunologically competent cells against an immunodeficient host.[29] The pathology associated with allogeneic GVHD in humans became well defined with the advent of bone marrow transplantation therapy.[30] In acute GVHD the hallmark lesion is selective epithelial damage of target organs including the skin, liver and gastrointestinal tract.

Initial descriptions of the SCID-PBL model[13,14,23] reported that obvious GVHD did not occur following intraperitoneal transfer of up to 5×10^7 mature human peripheral blood mononuclear cells. In contrast, Bankert et al[25] observed GVHD-related lesions in skin and liver of the SCID-PBL mice following intraperitoneal transfer of as few as 1×10^7 human PBL. GVHD-related lesions were reported to be present at necropsy fourteen weeks post-

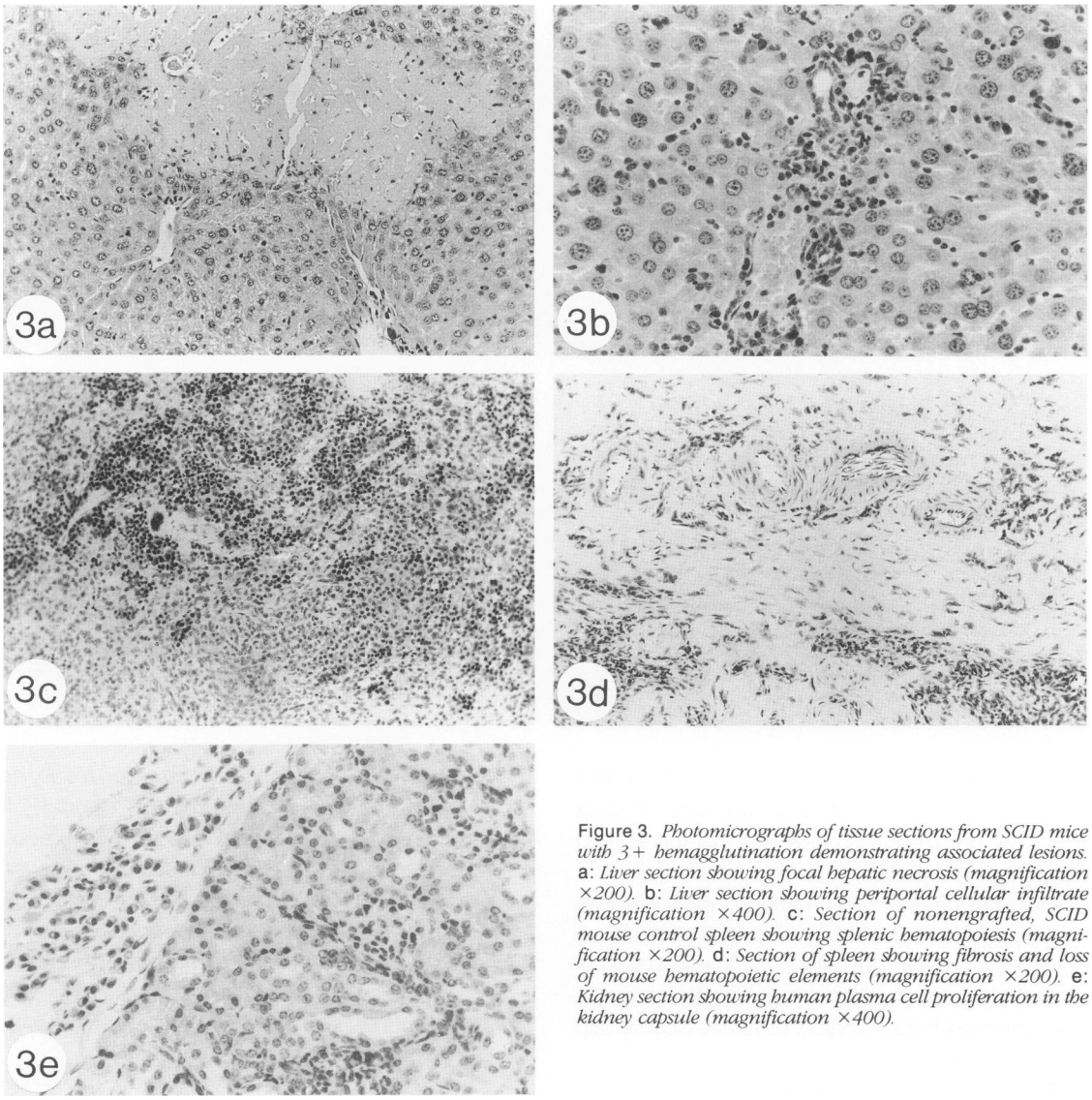

Figure 3. *Photomicrographs of tissue sections from SCID mice with 3+ hemagglutination demonstrating associated lesions.* a: *Liver section showing focal hepatic necrosis (magnification ×200).* b: *Liver section showing periportal cellular infiltrate (magnification ×400).* c: *Section of nonengrafted, SCID mouse control spleen showing splenic hematopoiesis (magnification ×200).* d: *Section of spleen showing fibrosis and loss of mouse hematopoietic elements (magnification ×200).* e: *Kidney section showing human plasma cell proliferation in the kidney capsule (magnification ×400).*

transfer of PBL, concurrently with extensive lymphoproliferative disease. When 1×10^8 PBL were transferred, the mice exhibited clinical symptoms of GVHD at 3–4 weeks post-transplantation and all mice were dead by six weeks. Intermediate cell doses clearly demonstrated that the timing of GVHD was dose related. They also had noted that the mouse erythrocytes had human IgM bound to the plasma membranes.

Consistent with Bankert et al[25] we have also found that significant GVHD occurred in SCID-PBL mice. In the course of our evaluation of the model we noted a hemagglutinating disease process in many animals with evidence of B cell engraftment.[22] Affected mice all had serum immunoglobulin levels greater than 1.0 mg/ml while unaffected mice had levels less than 1.0 mg/ml. Flow cytometric evaluation of erythrocytes obtained from affected mice demonstrated human IgM and small amounts of human IgG bound to the red cell surface. These results confirm Bankert's earlier observation.[25]

Coombs positive hemolytic anemia is frequently associated with acute GVHD in allogeneic mouse models[29] and has also been documented in humans with acute GVHD following bone marrow transplantation.[31] It seems likely that in the SCID-PBL xenograft model the anti-mouse red cell response is analogous. Hemagglutination was generally self-limited at the cell doses used for these studies. Thirteen of twenty two affected mice recovered and of these, only seven went on to develop lymphoproliferative disease. This finding indicates that despite the continued presence of mouse red cell antigen the specific B cell clone(s) either became tolerant or more likely attenuated. The fact that mice engrafted with PBL from EBV seronegative donors show only a low level, transient expression of serum immunoglobulin is consistent with

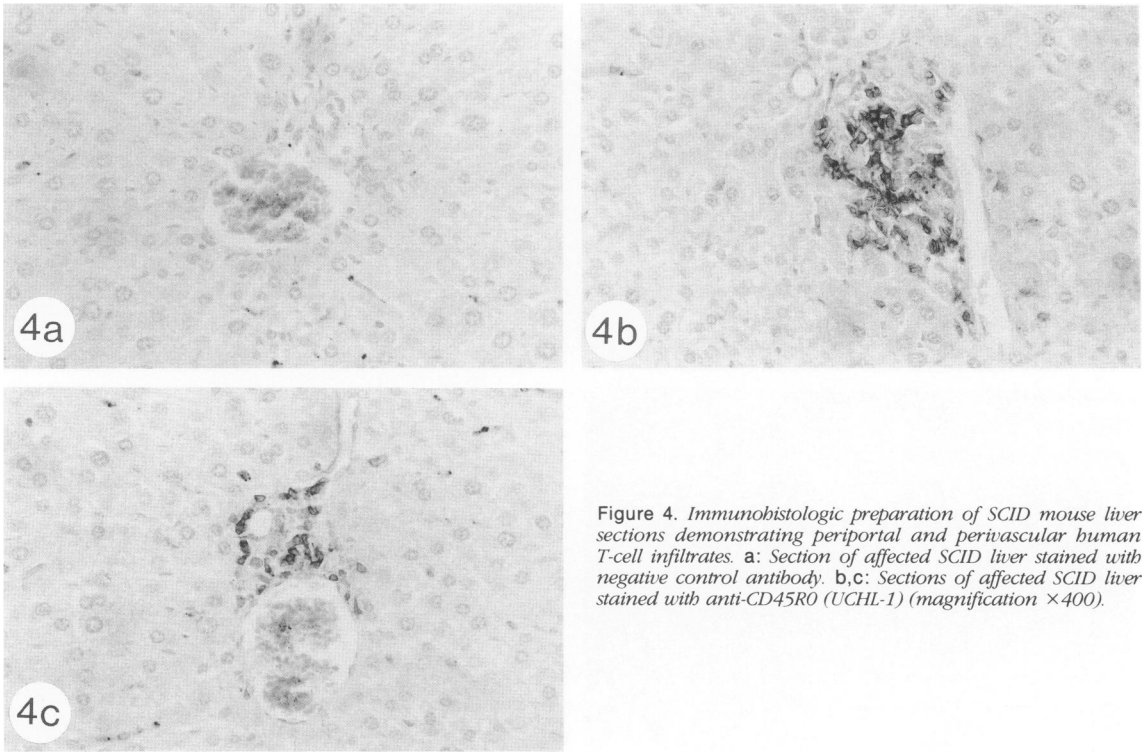

Figure 4. *Immunohistologic preparation of SCID mouse liver sections demonstrating periportal and perivascular human T-cell infiltrates.* a: *Section of affected SCID liver stained with negative control antibody.* b,c: *Sections of affected SCID liver stained with anti-CD45R0 (UCHL-1) (magnification ×400).*

the eventual loss of these clones.[13,14,21–23] Given that with our protocol hemagglutination was often evident prior to EBV inoculation also indicates that the anti-red cell response was not EBV driven.

The fact that the anti-red cell immunoglobulin was predominantly IgM is consistent with a primary anti-mouse response. It is however, possible, that the specificity of these antibodies for mouse red cells represented naturally occurring antibodies analogous to anti-blood group antigens in humans. We could not however, readily demonstrate circulating antibodies in donor serum by flow cytometry. The possibility that donors were previously sensitized to mouse antigens also seems unlikely since only one of the donors had previously worked with mice. Alternatively, these antibodies may represent silent, potentially autoreactive clones (i.e. with primary reactivity to human red cells) that are reactivated *in vivo* as part of the GVHD response. We did not directly test this possibility.

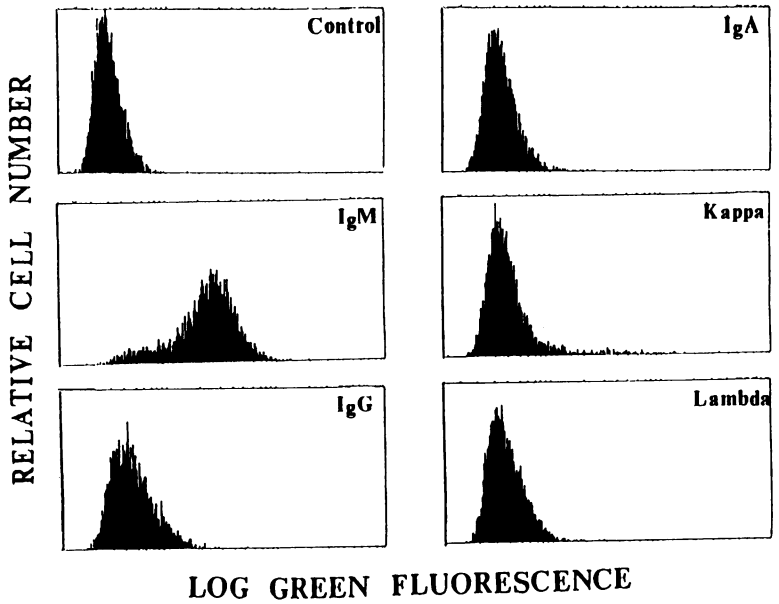

Figure 5. *Single parameter flow cytometric histograms of red cells from SCID mouse with 3+ hemagglutination. Control cells were stained with goat anti-mouse IgG FITC. Test cells were stained with goat anti-human Ig specific for heavy and light chains as indicated in the histograms and cells were analyzed on a three decade log fluorescence scale. After background subtraction, IgM = 96% positive, IgG = 24% positive, and kappa = 12% positive. IgA and lambda were negative.*

The transfer of EBV negative human PBL to SCID mice results in a peak of lymphocyte engraftment that is dependent on the initial cell dose.[13,14,25] Many of the measures of human lymphocyte engraftment such as rising serum immunoglobulin levels and splenic T cell proliferation may really be manifestations of an acute graft-versus-host response. We and others have previously documented GVHD-like lesions in the skin of SCID-PBL mice[22,25] however, these changes were modest in comparison to the skin and gut lesions characteristic of GVHD following bone marrow allografting.[30] It must be kept in mind that the SCID-PBL model system represents xenografting of tissues between widely divergent species. For this reason, the more classic GVHD lesions seen in the gut and skin following allografting may not predominate. The disparity in protein antigens would be expected to elicit a vigorous antibody response which was seen in the anti-red cell antibodies. We are not sure whether other specificities were elicited since we did not specifically look for them. The cellular arm of the GVH response would be dependent on the ability of human T cells to efficiently traffic to target tissues and to effectively engage mouse MHC antigens through the T cell receptor. Since mouse MHC class I and class II antigens would be expressed on hematopoietic elements in the SCID mouse spleen, these cells would represent good cellular targets. Loss of splenic hematopoiesis and subsequent fibrosis was documented in several mice with the most severe hemagglutinating disease. Further, the occurrence of focal hepatic necrosis originally described in mouse GVHD models[28,29] also appears to be a component of GVHD in the SCID-PBL model. We and others[24] have documented these lesions in the absence of obvious LPD indicating a process distinct etiologically from EBV-induced lymphoproliferation.

Despite the development of hemagglutination and acute GVHD in SCID mice engrafted with human PBL this model remains very useful for investigating EBV-induced lymphoproliferative disease. At cell doses between 1×10^7 and 5×10^7 human PBL, the majority of mice will develop lymphoproliferative disease and only a small fraction of animals will be lost to hemagglutination or acute GVHD. It is important, however, to distinguish the lesions associated with these processes. A more thorough understanding of the SCID-PBL model is needed to assure appropriate utilization and interpretation of experimental results.

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