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_J Immunol_ 1999; 162:5256-5262; ;
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It has previously been demonstrated that xenograft rejection in rodents is dependent on CD4⁺ T cells. However, because of the lack of an appropriate in vivo model, little is known about the cellular basis of human T cell-mediated rejection of xenografts. In this study, we have evaluated the ability of human T cells to mediate rejection of porcine skin grafts in a novel in vivo experimental system using immunodeficient mice as recipients. Recombinase-activating gene-1-deficient mice (R⁻) lacking mature B and T cells were grafted with porcine skin and received human lymphocytes stimulated in vitro with irradiated porcine PBMC. Skin grafts on mice given either unseparated, activated human lymphocytes, or NK cell-depleted lymphocyte populations were rejected within 18 days after adoptive cell transfer. In contrast, skin grafts on mice given T cell-depleted human lymphocytes or saline showed no gross or histologic evidence of rejection up to 100 days after adoptive transfer. Purified CD4⁺ T cells were also able to mediate rejection of porcine skin grafts. These data suggest that human CD4⁺ T cells are sufficient to induce rejection of xenografts. Thus, strategies directed toward CD4⁺ T cells may effectively prevent cellular rejection of porcine xenografts in humans. The Journal of Immunology, 1999, 162: 5256–5262.

While much attention has been focused on humoral rejection of discordant xenografts, the nature of the human cellular immune response to xenografts has been studied in much less detail (1, 2). Most of these studies have relied on in vitro models of xenograft rejection (2). In contrast, much less is known about human or primate cellular immune responses to xenogeneic tissues in vivo. Because miniature swine are the most likely donor for clinical xenotransplantation (3), it is critically important to determine the mechanisms of the human antiporcine cellular immune response. The human T cell response to xenogeneic targets appears to be more potent than the response to allogeneic targets (4–6). In vitro studies have revealed that the human cellular response involves T cell proliferation and lysis of porcine targets by human T and NK cells (7–16). In addition, porcine endothelial cells are capable of directly activating human T cells to proliferate or lyse porcine target cells (6, 7, 17). However, while such studies suggest that T and NK cells are possible effector cells of human antiporcine immunity, the specific roles of these cells in xenograft rejection in vivo have not been determined with certainty. Furthermore, while CD4⁺ and CD8⁺ T cells participate in allograft rejection, xenograft rejection in rodents appears to be dependent on CD4⁺ T cells (2, 18–21). Little is known about the relative importance of CD4⁺ and CD8⁺ T cells in human antiporcine immunity.

We have developed a model to study rejection of porcine skin grafts mediated by human lymphocytes in vivo using immunodeficient mice (2). In this model, recombinase-activating gene-1 (rag-1)-deficient mice (R⁻ mice) are used as recipients of porcine skin grafts. R⁻ mice lack mature B and T cells (23), and accept porcine skin grafts indefinitely without further immunosuppression. To construct a model of the human immune system in R⁻ mice, human PBMC stimulated in vitro with irradiated porcine PBMC were used to reconstitute R⁻ mice (Hu-R⁻). R⁻ mice engrafted with porcine skin and reconstituted with human PBMC rejected porcine skin grafts (22). Thus, Hu-R⁻ mice represent a practical small animal model in which the human antiporcine cellular immune response can be studied in vivo. Using the Hu-R⁻ mouse model of porcine skin graft rejection, we have examined the requirement for human CD4⁺ T cells in rejection of porcine skin xenografts.

Materials and Methods

Animals

Mice. Homozygous R⁻ mice on a mixed (129/Sv × C57BL/6) background were obtained from The Jackson Laboratory (Bar Harbor, ME). All mice were housed in viral Ab-free microisolator conditions in autoclaved cages and maintained on irradiated feed and autoclaved acidified drinking water.

Pigs. Porcine blood and skin were obtained from miniature swine inbred for the appropriate SLA haplotype from the Massachusetts General Hospital herd housed at the Tufts University School of Veterinary Medicine. The immunogenetics of this herd and the intra-SLA recombinant haplotypes available have been described previously (24–26).

Abbreviations used in this paper: R⁻ mice; recombinase-activating gene-1 (rag-1)-deficient mice; Hu, human; SLA, swine lymphocyte Ag; H&E, hematoxylin and eosin.
mAbs specific for the following human surface markers were used for FACS: CD3 (UCHT1; PharMingen, San Diego, CA), CD4 (RPA-T4; PharMingen), CD8 (RPA-T8; PharMingen), CD14 (M5E2; PharMingen), CD19 (B43; PharMingen), CD25 (M-A251; PharMingen), CD45 (HI30; PharMingen), and CD56 (MY31; Becton Dickinson, Mountain View, CA). All Abs were directly labeled with FITC or phycoerythrin. Lymphocyte purifications were performed using the following IG1 mouse anti-human mAbs: CD4 (B-F5; Biosource, Camarillo, CA), CD8 (HIT8a; PharMingen), CD20 (L27; Becton Dickinson), CD16 (3G8; PharMingen), and CD56 (B159; PharMingen). ImmuNohisChemistry was performed with biotinylated Abs to human CD3 (polyclonal rabbit anti-human; Dako), CD20, CD56, CD25 (M-A251; PharMingen), CD19 (B43; Dako), and CD68 (M5E2; Dako). CD45 (T299/33; Boehringer Mannheim, Indianapolis, IN), CD56 (123c3; Accurate, Westbury, NY), IgG (fluorescein rabbit anti-human; Dako), IgM (fluorescein rabbit anti-human; Dako), and perforin (6G9; PharMingen).

**Lymphocyte purification**

Human lymphocytes were isolated from buffy coats or byproducts of platelet pheresis from the Massachusetts General Hospital Blood Donor Center. Porcine lymphocytes were isolated from heparinized whole blood. PBMC were purified as described previously (22).

**In vitro stimulation of human PBMC**

Human lymphocytes were stimulated in vitro for 6 days with an equal amount of irradiated (2500 cGy) porcine PBMC SLA matched to the porcine skin donor. Stimulations were performed in RPMI 1640 media supplemented with 5% heat-inactivated pooled human AB serum (N.A.B.I., Miami, FL), 25 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 50 U/ml penicillin, and 50 μg/ml streptomycin in T75 flasks (Costar, Cambridge, MA) at 37°C in 6% CO₂. Following stimulation, cells were harvested and analyzed by cell surface staining and flow cytometry, as described previously (22).

**Human lymphocyte subset purification**

Human lymphocyte subsets were purified by magnetic bead negative selection using the MACS system (Miltenyi Biotec, Sunnyvale, CA), as described previously (16). Stimulated cells were stained with saturating concentrations of mouse IgG1 anti-human Abs directed against cell surface markers. The cells were then washed, resuspended in HBSS containing 0.5% BSA, and incubated with saturating concentrations of rat anti-mouse IgG1 Ab conjugated to superparamagnetic microbeads (Miltenyi). The cells were then passed over a C-type MACS separation column, and the nonmagnetic fraction was collected. Purities of collected cell populations were assessed by cell surface staining and flow cytometry. Abs used for staining following purification were not blocked by Abs used for magnetic bead depletion (data not shown).

**Skin grafting**

Skin grafting was performed and evaluated, as described previously (22). The following graded scale was used to score skin graft survival: 0 = skin grafts intact, soft, and white; 1 = soft with slight redness; 2 = mild induration with slight to severe redness; 3 = moderate induration, areas of scab formation, severe redness; 4 = severe induration, diffuse scab formation, obvious necrosis. Grade 3 is the minimal score at which grafts were considered to be rejected. Skin grafts receiving a grade 3 or higher did not spontaneously recover after initial injury (T.F. and J.I., unpublished observation). Split-thickness skin was used in all experiments.

**Adoptive transfer of human lymphocytes**

Two to three weeks following skin grafting, human lymphocytes were transferred into R⁻ mice by i.p. injection, as described (22). Before adoptive transfer, R⁻ mice were preconditioned to increase the efficiency of human lymphocyte engraftment with a modified version of our previous protocol (27): mice were injected i.p. with 50 μl of antiasialo GM1 polyclonal rabbit antisemur (Wako, Richmond, VA) 1 day before adoptive transfer to deplete NK cells, and given 600 cGy whole body irradiation 6 h before adoptive transfer. The dose of antiasialo GM1 given was based on the manufacturer’s specification and our own empirical analysis of the amount of antisera needed to deplete NK1.1-positive cells from the blood of treated mice (28). Only mice bearing well-healed skin grafts were used as recipients of human cells. Each mouse received cells from a single human donor, and multiple donors were used in each experiment.

**Histology and immunohistochemistry**

Mice were sacrificed at the time points specified, and skin grafts were harvested for histologic analysis, as described (22). One-half of each graft was used to prepare 3–4 μm paraffin-embedded sections, and the other half was frozen in O.C.T. compound and used to prepare frozen sections (29). Briefly, 4-μm paraffin-embedded sections were stained with hematoxylin and eosin (H&E). Immunohistochemical staining was performed on both paraffin-embedded (CD3, CD45, CD20, CD56, Ig) and frozen (CD4, CD8, perforin) tissue sections. Slides were blocked using an avidin-biotin blocking kit (Vector Laboratories, Burlingame, CA) before staining with Abs. After staining, slides were developed with avidin-conjugated horseradish peroxidase, using the Vectastain ABC kit (Vector Laboratories). Biotinylated isotype-matched Abs were used as negative controls for all staining experiments. For all histology experiments, tissue sections prepared from grafts harvested from at least two mice per group were analyzed, and in most cases sections of grafts from three mice per group were examined. Representative sections are shown.

**Detection of human lymphocytes in reconstituted R⁻ mice**

At the time points indicated, the presence of human cells was analyzed in peritoneum, peripheral blood, and spleen of reconstituted R⁻ mice. Peripheral blood was collected by retro-orbital bleeding of anesthetized mice in the presence of EDTA to prevent clotting. Cell suspensions were prepared from spleen and peritoneal lavage, as described previously (27). In all cases, RBCs were lysed in ammonium-chloride-potassium buffer. Cell surface staining was performed, as described previously (22, 27).

**Results**

**Human T cells are required for rejection of porcine skin grafts in Hu⁻ mice**

We have demonstrated previously that human PBMC stimulated in vitro with irradiated porcine PBMC are able to mediate rejection of porcine skin grafts when transferred into R⁻ mice (22). We set out to examine the contribution of T cells and NK cells to rejection in this model. After in vitro stimulation with irradiated porcine PBMC, T cells or NK cells were depleted by magnetic bead separation. Unseparated or depleted human PBMC populations were then transferred into R⁻ mice bearing healed porcine skin grafts. Porcine PBMC used for in vitro stimulation and skin grafts were SLA matched in all experiments. Mice received either 10⁸ unseparated human PBMC. T-cell-depleted human PBMC (<4% T cells detectable by FACS; data not shown), or NK cell-depleted human PBMC (<1% NK cells). The number of T or NK cell-depleted human PBMC administered was adjusted to match the absolute number of the corresponding lymphocyte populations in mice receiving unseparated human PBMC. Typically, recipients of T-cell-depleted PBMC were given a total of 1–2 × 10⁶ cells, while recipients of NK cell-depleted PBMC were given 8–9 × 10⁷ cells. By day 15 following adoptive transfer, all skin grafts on mice receiving unseparated lymphocytes or NK cell-depleted lymphocytes were rejected based on visual scoring (Fig. 1). In contrast, skin grafts on mice receiving T-cell-depleted PBMC or saline showed no evidence of rejection and remained intact for the length of the experiment. Grafts on the animals in the T-cell-depleted and saline control groups that were not sacrificed for histologic analysis survived more than 60 days without evidence of rejection, at which time the experiment was terminated. At day 18, H&E staining of tissue sections from mice receiving unseparated human PBMC (Fig. 2A) or NK cell-depleted human PBMC (Fig. 2B) revealed a dense perivascular mononuclear cell infiltrate within the dermal layer of the grafts. The majority of dermal vessels were destroyed and exhibited significant cellular infiltration and fibrin deposition. Signs of acute tissue damage such as focal hemorrhage and red cell extravasation were also apparent. We did not observe any significant difference in rejection mediated by unseparated and NK cell-depleted human PBMC. In contrast, grafts harvested from mice receiving T-cell-depleted human PBMC (Fig. 2C) showed no
signs of tissue damage based on H&E staining, and were indistinguishable from grafts harvested from control mice receiving saline (Fig. 2D). These data strongly suggest that human T cells are required for porcine skin graft rejection in Hu-R2 mice.

**Human CD4+ T cells are sufficient to induce porcine skin graft rejection in vivo**

Since human T cells were critical for rejection in the Hu-R model, we set out to determine the relative importance of CD4+ and CD8+ T cells to the rejection process. R2 mice bearing healed skin grafts received either 10⁶ unseparated human PBMC, purified CD4+ T cells (greater than 97% pure by FACS; data not shown), CD4+ T cell-depleted human PBMC (less than 0.5% CD4+ T cells detectable), or saline. In all experiments, human PBMC were stimulated in vitro with irradiated porcine PBMC before cell separation. The number of cells transferred in each group was adjusted to match the absolute number of the corresponding cell types transferred into mice receiving unseparated human PBMC. Typically, mice receiving purified CD4+ T cells or CD4+ T cell-depleted PBMC were given 4–6 × 10⁷ cells.

Skin grafts on mice receiving unseparated human PBMC or purified CD4+ T cells were all rejected within 13 days, according to clinical scoring (Fig. 3). In contrast, skin grafts on mice receiving CD4+ T cell-depleted human PBMC (comprising CD8+ T cells, B cells, and NK cells) or saline buffer showed no gross evidence of clinical rejection and survived until the animals were sacrificed (up to 60 days for several animals). At day 18 after adoptive transfer, histologic examination of skin grafts harvested from mice receiving either unseparated human PBMC (Fig. 4A) or purified CD4+ T cells (Fig. 4B) showed evidence of moderate to severe perivascular mononuclear cell infiltration, edema at the dermal-epidermal junction, and damage to the epidermis. Vascular endothelium within the porcine dermis appeared swollen and disorganized. In contrast, skin grafts from mice receiving CD4+ T cell-depleted human PBMC (Fig. 4C) appeared normal upon histologic examination at day 18 and were identical to control skin grafts harvested from mice receiving saline (Fig. 4D). We did not observe a significant mononuclear cell infiltrate in grafts harvested from mice receiving CD4+ T cell-depleted human PBMC. These data indicate that human CD4+ T cells are sufficient to induce rejection of porcine skin xenografts in the Hu-R2 model.
FIGURE 4. H&E staining of porcine skin grafts harvested from R2mice reconstituted with human PBMC subpopulations (day 18, ×125). Representative grafts from recipients of unseparated PBMC (A) or purified CD4+ T cells (B) demonstrate extensive perivascular mononuclear infiltrates (arrowheads) consistent with acute cellular rejection. Focal hemorrhage in areas of endothelial destruction is also observed (arrows). Examination of grafts from recipients of CD4+ T cell-depleted PBMC (C) or saline (D) shows normal cellularity without evidence of infiltrate.

FIGURE 5. Expression of human lymphocyte lineage markers in rejected skin grafts (day 18, ×250). Immunostaining of serial sections of representative grafts from R2mice receiving unseparated PBMC revealed heavy perivascular infiltration by CD45+ human lymphocytes (A), comprised of approximately equal numbers of CD4+ (D) and CD8+ (F) cells. Immunostaining of serial sections of a representative graft from the recipient of purified CD4+ T cells shows similar heavy infiltration by CD45+ human lymphocytes (B), which all stain for CD4 (E). No CD8+ cells were observed in these grafts (H). There was no evidence of infiltration by CD45+ human lymphocytes in grafts from recipients of CD4+-depleted PBMC (C) or cell-free buffer (not shown), and therefore stains for human lymphocyte subsets were not performed. Staining for the human B cell surface Ag CD20 was negative in representative grafts from all groups (not shown).
CD4\(^+\) T cells infiltrate porcine skin grafts only in the presence of CD8\(^+\) T cells

Skin grafts harvested from mice reconstituted with unseparated human PBMC, purified CD4\(^+\) T cells, or CD4\(^+\) T cell-depleted human PBMC were examined by immunohistochemistry to characterize human lymphocytes infiltrating the grafts. Staining of skin grafts harvested from mice receiving unseparated human PBMC (Fig. 5A) or purified CD4\(^+\) T cells (Fig. 5B) with Ab to human CD45 revealed a perivascular human lymphocyte infiltrate. Staining with Ab to human CD3 revealed a similar pattern of positive cells within the grafts, indicating that the majority of infiltrating lymphocytes were T cells (not shown). Staining of serial sections with Abs to human CD4 (Fig. 5D) and CD8 (Fig. 5F) revealed the presence of both T cell subsets within rejected grafts harvested from mice receiving unseparated human PBMC. CD8\(^+\) and CD4\(^+\) T cells were present in similar numbers. We did not detect B cells or human IgM and IgG deposition within rejected grafts from any mice (not shown). As expected, CD4\(^+\) but not CD8\(^+\) T cells were present in rejecting grafts harvested from mice receiving purified CD4\(^+\) T cells (Fig. 5, E and G). There was no difference observed in the degree of T cell infiltration in skin grafts harvested from mice receiving purified CD4\(^+\) T cells and unseparated human PBMC.

While T cells were detectable in mice receiving unseparated human PBMC or purified CD4\(^+\) T cells, we were unable to detect human cells infiltrating skin grafts from mice receiving CD4\(^+\) T cell-depleted human PBMC preparations (Fig. 5C). We therefore examined engraftment of human cells in mice receiving unseparated human PBMC, purified CD4\(^+\) T cells, and CD4\(^+\) T cell-depleted human PBMC by cell surface staining and flow cytometry (not shown). We were able to detect significant quantities of both CD4\(^+\) and CD8\(^+\) T cells in the blood, spleen, and peritoneal cavity of mice receiving unseparated human PBMC at 18 days after adoptive cell transfer. We detected CD4\(^+\) T cells, but not CD8\(^+\) T cells in R\(^-\) mice receiving purified CD4\(^+\) T cells. However, we were unable to detect human cells in mice receiving CD4\(^+\) T cell-depleted human PBMC. These data indicate that the presence of CD4\(^+\) T cells is required for continued survival of other human lymphocytes in the murine environment.

Perforin-mediated cytotoxicity does not appear to play a major role in CD4\(^+\) T cell-mediated xenograft rejection

To examine the mechanism by which T cells mediate rejection of porcine xenografts, tissue sections of rejected skin grafts harvested from mice receiving unseparated human PBMC or purified CD4\(^+\) T cells were stained with Abs specific for human perforin. All sections analyzed contained a significant mononuclear cell infiltrate consisting of CD45\(^-\), CD3\(^-\) T cells similar to that observed in Fig. 5. As shown in Fig. 6, while perforin-positive cells were readily observed in control tissue sections from rejected human heart allografts, perforin-positive cells were rare in tissue sections of grafts from mice receiving unseparated human PBMC or CD4\(^+\) T cells, even though these grafts contained significant numbers of T cells. No perforin-positive cells were observed in grafts harvested from control mice receiving saline (not shown).

Discussion

In this study, we examined the ability of human T cell subsets to reject porcine skin xenografts in R\(^-\) mice. Adoptive transfer of purified CD4\(^+\) T cells induced skin graft rejection that was indistinguishable from rejection mediated by unseparated human PBMC. In the absence of CD4\(^+\) T cells, the remaining human PBMC did not survive in the murine host and may consequently have failed to mediate graft rejection. Thus, in our model, CD4\(^+\) T cells are necessary and sufficient to induce porcine xenograft rejection. While it has been demonstrated previously in rodent immune systems that xenograft rejection is dependent on CD4\(^+\) T cells. As shown, few perforin-positive cells were observed in tissue sections of grafts from mice receiving unseparated human PBMC or purified CD4\(^+\) T cells (see arrows) even though staining of adjacent tissue sections for human CD45 demonstrated heavy infiltration of human lymphocytes. Perforin expression in a positive control tissue section harvested from a rejected human heart allograft autopsy specimen. Tissue sections prepared from grafts harvested from mice receiving CD4\(^+\) T cell-depleted human PBMC or saline contained no perforin-positive cells (not shown).

Immunohistochemistry revealed that after adoptive transfer of unseparated human PBMC, CD8\(^+\) T cells infiltrated porcine skin grafts in approximately equal numbers to CD4\(^+\) T cells. In the absence of CD4\(^+\) T cells, we failed to establish stable engraftment of CD8\(^+\) T cells. Similar results have been obtained by Murray et al. (31). It is possible that if CD8\(^+\) T cells were able to engraft in the absence of CD4\(^+\) T cells, then CD8\(^+\) cells could contribute to xenograft rejection. However, the presence of CD8\(^+\) T cells is clearly not required for xenograft rejection to occur. The fact that the presence of CD4\(^+\) T cells is required for continued CD8\(^+\) T cell survival in the murine host suggests that CD4\(^+\) T cells may secrete cytokines or other factors that are required for CD8\(^+\) T cell
survival. It is also possible that CD4\(^+\) T cells are required for recruitment of CD8\(^+\) T cells into rejecting xenografts, where stimulation by porcine Ag may allow the CD8\(^+\) T cells to survive.

The perivascular distribution of T cells suggests that porcine vascular endothelial cells are a primary target of rejection in this model. We and others have previously shown that dermal vascular endothelial cells within the skin grafts express SLA class II molecules (22, 32). It is therefore possible that CD8\(^+\) and CD4\(^+\) T cells may interact directly with porcine SLA class I and II Ags expressed on porcine vascular endothelium. Since it has been shown previously that the presence of Ag is required at the site of T cell-mediated pathology (33, 34), we suggest that direct recognition of SLA molecules on vascular endothelium may be required to retain T cells within the graft.

Previous studies have demonstrated that human NK cells are able to bind and damage vascular endothelium in perfused rat hearts (35). Human NK cells have also been shown to contribute significantly to antiporcine cytotoxicity in vitro (36), although the role of NK cells in porcine xenograft rejection has not been determined. Human PBMC depleted of NK cells before adoptive cell transfer were able to efficiently mediate rejection of porcine skin grafts. We did not observe any significant difference in rejection mediated by unseparated and NK cell-depleted human PBMC. It is important to note that we were able to detect human NK cells in the peritoneum of R\(^\text{+}\) mice receiving unseparated human PBMC (approximately 5% of human cells; not shown). These data suggest that rejection of porcine tissue by human cells is predominantly a T cell-mediated process, as has been observed in a similar model of human allograft rejection and pig xenograft rejection (31, 32, 37, 38).

Our data indicate that human T cell-mediated graft rejection in this model is unlikely to utilize perforin-dependent mechanisms. While perforin-positive cells were detectable in rejected grafts, these cells represented less than 1% of the total number of human graft-infiltrating cells. Based on these data, it appears that the number of cells expressing perforin is insufficient to account for the acute cellular xenograft rejection observed, although perforin appears to play a significant role in human allograft rejection (31). We suggest that production of cytokines by human CD4\(^+\) T cells, expression of Fas ligand (CD95L), or perhaps other cytolytic machinery may participate.

It is clear from several studies that Abs play a major role in both hyperacute as well as delayed xenograft rejection (39). The presence of xenoreactive Ab in other systems has made it difficult to examine cell-mediated rejection. In our model, we were unable to detect B cells or human Ig within rejected grafts. Thus, rejection in this model is strictly a cell-mediated process and does not involve Ig. The fact that we were able to observe acute xenograft rejection in the absence of Ab suggests that once the problem of humoral rejection of xenografts is overcome, cell-mediated rejection will be the next major barrier. The system we have developed provides a practical model to test strategies designed to prevent cell-mediated xenograft rejection. Our finding that human CD4\(^+\) T cells are necessary and sufficient to induce porcine xenograft rejection suggests that antirejection strategies should be directed toward CD4\(^+\) T cells.

Acknowledgments
We thank the Massachusetts General Hospital Blood Bank for their assistance in providing human lymphocytes, Scott Arn for assistance with procurement of porcine tissues and blood, and P. DellaPelle for help with immunohistochemistry. In addition, we thank H. Autschluss and D. K. C. Cooper for critical review of the manuscript.

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