Target Cell Susceptibility to Lysis by Human Natural Killer Cells Is Augmented by α(1,3)-Galactosyltransferase and Reduced by α(1,2)-Fucosyltransferase*

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Susceptibility of porcine endothelial cells to human natural killer (NK) cell lysis was found to reflect surface expression of ligands containing Gal α(1,3)GlcNAc, the principal antigen on porcine endothelium recognized by xenoreactive human antibodies. Genetically modifying expression of this epitope on porcine endothelium by transfection with the α(1,2)-fucosyltransferase gene reduced susceptibility to human NK lysis. These results indicate that surface carbohydrate remodeling profoundly affects target cell susceptibility to NK lysis, and suggest that successful transgenic strategies to limit xenograft rejection by NK cells and xenoreactive antibodies will need to incorporate carbohydrate remodeling.

The severe shortage of human organs has focused recent investigation into cross-species transplantation. Pigs are an appropriate donor source, because their organs have similar physiology and size to human organs, they can be bred in large numbers, and they are relatively free of pathogens capable of causing infection in humans. However, porcine xenografts transplanted into primate recipients undergo hyperacute rejection within minutes to hours of engraftment. The process is mediated by host complement and preformed IgM antibodies directed against Gal α(1,3)Gal epitopes present in various cell surface structures on porcine endothelium (1–6). In contrast to pigs, humans and Old World monkeys do not express Gal α(1,3)Gal in their tissues, because the gene encoding the α(1,3)-galactosyltransferase, which links a terminal galactose residue to Gal β(1,4)GlcNAc oligosaccharide backbone structures, is inactive in these species (7,8).

Anti-Gal α(1,3)Gal antibodies develop in humans and higher primates within the first months of life, in parallel with the colonization of the gastrointestinal tract with bacteria containing α(1,3)-linked galactose residues in their cell walls (9,10). Consequently, there exists a window period in which these IgM antibodies are not present in neonatal primates (11). The absence of preformed IgM anti-Gal α(1,3)Gal antibodies in neonatal primates enables porcine cardiac xenografts transplanted heterotopically into unmedicated newborn baboons to survive beyond the hyperacute period (12); making this an appropriate model for studying the subsequent immunological barriers to xenotransplantation. In these recipients, a second primate anti-pig immunological response occurs after 3–4 days, resulting in graft loss accompanied by dense xenograft infiltration with natural killer (NK)1 cells, macrophages, and deposition of induced IgG antibodies (13–17). Because similar findings have been demonstrated in guinea pig-to-rat cardiac xenotransplantation in which the recipients were treated with cobra venom factor to inactivate the host complement system (18), these observations suggest that a T cell-independent delayed rejection process, mediated largely by NK cells, occurs in widely disparate transplant combinations, including pig to primate.

NK cell lysis is regulated by a balance of intracellular signals transmitted via stimulatory and inhibitory cell surface receptors after specific binding to their respective target cell ligands (19,20). Inhibitory receptors on NK cells have carbohydrate binding domains with specificity for target cell glycoprotein ligands encoded by certain major histocompatibility complex (MHC) class I genes (21, 22). Stimulatory receptors on NK cells also have carbohydrate binding domains within C-type lectin structures; however, their target cell glycoprotein ligands have not been well-defined (23, 24). Recent evidence suggests that NK cells and a subset of B cells may belong to an innate immunological system designed to combat frequently encountered carbohydrate antigens, such as those in the cell walls of bacterial pathogens (25–27). Carbohydrate antigens can induce T cell-independent B cell antibody responses and can directly stimulate NK cells, without previous antigen sensitization or MHC restriction, to initiate lysis and to produce IFN-γ. Co-stimulatory signals provided by the NK cells, together with the effects of NK cell-derived IFN-γ on B cell differentiation, isotype switching, and immunoglobulin secretion, ultimately result in augmentation of the IgG humoral response against the T cell-independent antigen (28–30). Because the T cell-independent process of delayed xenograft rejection involves NK cells and IgG antibodies, and the principal antigen on porcine endothelium recognized by xenoreactive human antibodies is the carbohydrate epitope Gal α(1,3)Gal, we addressed the possibility that receptors on human NK cells may also react with ligands containing terminal Gal α(1,3)Gal residues, leading to augmented natural cytotoxicity as well as IgG humoral activity against porcine endothelium.

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1 The abbreviations used are: NK, natural killer; MHC, major histocompatibility complex; PAEC, pig aortic endothelial cell; HUVEC, human umbilical vein endothelial cell; PBMC, peripheral blood mononuclear cell; IL, interleukin; FITC, fluorescein isothiocyanate.
**Gal α(1,3)Gal Expression and Susceptibility to NK Lysis**

**EXPERIMENTAL PROCEDURES**

**Preparation of Target Cells**

*Pig Aortic Endothelial Cells (PAECs)—* Fresh pig aortas were treated for 1 h with 0.5% collagenase (Type IV, Sigma), lightly washed with Hank’s solution, and gently nacked with a plastic cell scraper. The liberated endothelial cells were added to tissue culture vessels in RPMI 1640 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Life Technologies, Inc.) and 1% penicillin-streptomycin (Life Technologies). The cells were grown to confluence and then transferred to T25 flasks (Becton Dickinson, Franklin Lakes, NJ) in fresh medium.

*Human Umbilical Vein Endothelial Cells (HUVECs)—* HUVECs were purchased from the American Type Culture Collection (Rockville, MD; cell line CRL-1730), transferred to T25 flasks with fresh medium, and grown to confluence. COS Cells—COS-7 cells were purchased from the American Type Culture Collection (cell line CRL-1651), transferred to T25 flasks, and grown to confluence. All cells were used between the third and seventh passages.

**Preparation of Effector Cells**

*Peripheral Blood Mononuclear Cells (PBMCs)—* Human PBMCs were isolated from heparinized whole blood using Isopaque-Ficoll (Gallard Schlesinger Co., Carle Place, NY) and suspended at a concentration of 2.5 × 10^6 cells/ml in augmented RPMI 1640 medium. Depending on the assay condition, the cells were cultured for 12–14 h with or without the addition of 1000 units/ml recombinant human interleukin 2 (IL-2; Peprotech, Rocky Hill, NJ) before being used in functional assays.

**Cytotoxicity Assay**

Details of the cytotoxicity assay have been extensively described elsewhere (32). Briefly, target cells (2–10^5 cells/well) were seeded in flat bottom 96-well plates (Becton Dickinson) and grown to confluence at 37 °C and 5% CO2 overnight. The monolayers were washed with Hanks’ solution and labeled with 51Cr and used in standard NK lytic assays. Treatment of the porcine endothelium with α-galactosidase was specific for Gal α(1,3)Gal epitopes and did not affect other carbohydrate epitopes (data not shown). Furthermore, treatment with β-galactosidase did not remove Gal α(1,3)Gal epitopes or other carbohydrate epitopes serving as an appropriate negative control.

**Enzymatic Treatment of PAECs**

Porcine endothelium was treated with α-galactosidase at concentrations shown to reduce antibody-directed complement lysis of porcine endothelium (32). Briefly, PAEC monolayers were treated with either α-galactosidase isolated from the Green coffee bean (Sigma) or β-galactosidase isolated from Escherichia coli bacteria (Sigma) for 4 h at pH 6.0 or 7.3, respectively. The monolayers were then extensively washed with Hank’s solution and labeled with 51Cr and used in standard NK lytic assays. Treatment of the porcine endothelium with α-galactosidase was specific for Gal α(1,3)Gal epitopes and did not affect other carbohydrate epitopes (data not shown). Furthermore, treatment with β-galactosidase did not remove Gal α(1,3)Gal epitopes or other carbohydrate epitopes serving as an appropriate negative control.

**Inhibition of NK Lysis by Soluble Oligosaccharides**

All oligosaccharide derivatives were obtained from Dextra Laboratories (Reading, UK). Purified NK cells were incubated with a 10^-5 M concentration of each oligosaccharide derivative at 37 °C for 60 min before being used in standard lytic assays.

**Transfection of COS-7 Cells**

COS-7 cells were grown to confluence in RPMI 1640 medium. Details of the CD8 plasmid containing the murine α(1,3)-galactosyltransferase gene have been previously described (4). Briefly, vector DNA (7–10 µg/1 × 10^6 targeted cells) was added to Optimem culture medium (Life Technologies) with 5% LipofectAMINE (Life Technologies) and brought to a final concentration of 5–10 µg/ml. The DNA was incubated for 20 min at room temperature and then diluted to a final volume of 7–10 ml. The transfection media were added to 1 × 10^6 COS cells and incubated overnight at 37 °C with 5% CO2. The following morning the transfection media were aspirated, and the cells were cultured for 48 h before use.

**Transfection of Porcine Endothelial Cells**

The porcine endothelial cell line was cotransfected with the pig α(1,2)-fucosyltransferase cDNA in the expression vector pCDNA-1 (pHT plasmid; Ref. 33) and the pCDNA-1-neo plasmid (Invitrogen, Carlsbad, CA) using a standard calcium phosphate technique with 20 µg of pHT plasmid and 1 µg of pCDNA-1-neo plasmid. Cells were selected for stable integration of transfected DNA by selection in media containing G418, cloned using limiting dilution, and maintained in media containing G418.

**Flow Cytometry**

5 × 10^5 of appropriate target cells were washed and resuspended in phosphate-buffered saline with 0.1% bovine serum albumin. Fluorescein isothiocyanate (FITC)-conjugated lectin (2 µg/ml), IB4, or Ulex europaeus agglutinin, type I (Sigma) was added to each cell suspension and incubated for 45 min at 4 °C. The cells were washed and fixed in 1% paraformaldehyde (Sigma). Mean channel fluorescence was measured in a FACScan flow cytometer (Becton Dickinson).

**RESULTS**

**Susceptibility of Porcine Endothelium to Human NK Lysis Correlates with Expression of Gal α(1,3)-Gal**—Two endothelial cell lines with opposing α(1,3)-galactosyltransferase activities and surface expression of terminal Gal α(1,3)Gal residues were selected as initial targets for lysis by human NK cells: PAECs and HUVECs (Fig. 1a). Human NK lysis of xenogeneic porcine endothelium was 2–4-fold greater than that of allogeneic human endothelium (Fig. 1b), consistent with the possibility that expression of Gal α(1,3)Gal increases susceptibility of xenogeneic endothelium to lysis by human NK cells. To more directly examine the role of the terminal Gal α(1,3)Gal structure in the heightened susceptibility of xenogeneic porcine endothelium to human NK lysis, inhibition experiments were performed using the plant lectin IB4, which specifically binds to this structure (34). NK lysis of porcine endothelium was markedly reduced in the presence of IB4 in a concentration-dependent manner (Fig. 1c). The next set of experiments sought to identify the terminal α(1,3)-linked galactose residue within the Gal α(1,3)Gal structure as an essential component of porcine ligands involved in...
results extend these observations and show that the increased
by Soluble Gal
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are constitutively expressed.

NK lysis was inhibited by a mean of 35% accompanying a 44%
gene encoding the
Derivatives—
Transfection of porcine endothelium with the
serum after transfection with
bility to complement-mediated lysis in the presence of human
shown to reduce the level of Gal
a
NK lysis of PAECs and HUVECs by 1.5- and 1.7-fold, respectively, suggesting that the effects of cytokine activation are not target cell-dependent.

FIG. 1. a, expression of Gal α(1,3)Gal on PAECs and HUVECs. Flow cytometric analysis of PAECs and HUVECs was performed using the FITC-conjugated lectin IB4, which specifically binds to Gal α(1,3)Gal. b, specific NK lysis of PAECs and HUVECs targets. Standard 51Cr release lytic assays were performed using human PBMCs as effector cells with PAEC or HUVEC targets. Freshly isolated PBMCs demonstrated significantly
increased NK lysis of PAECs compared with HUVECs, which was augmented after activation of the PBMCs with IL-2. IL-2 activation increased NK lysis of PAECs and HUVECs by 1.5- and 1.7-fold, respectively, suggesting that the effects of cytokine activation are not target cell-dependent.

c, inhibition of NK lysis of porcine endothelium by IB4. IL-2-activated human PBMCs were used as effector cells against PAEC targets incubated with increasing concentrations of the lectin IB4. d, reduction of Gal α(1,3)Gal on PAECs after treatment with α-galactosidase. PAECs were treated with 0.2–20 units/ml α-galactosidase, and expression of Gal α(1,3)Gal was measured by direct flow cytometry using FITC-conjugated IB4. 
reduction in NK lysis of PAEC targets after treatment with α-galactosidase. IL-2-activated human PBMCs were used as effector cells against α-galactosidase-treated PAEC targets. f, specificity of α-galactosidase treatment of PAECs for reduction in NK lysis. IL-2-activated human PBMCs were used as effector cells against a- or β-galactosidase (gal'ase)-treated PAEC targets.

trigging human NK cell lysis. Enzymatic treatment of porcine endothelium with α-galactosidase reduced NK lysis in a concentration-dependent manner, which correlated with the level of reduced Gal α(1,3)Gal expression (Fig. 1, a and c). At the highest concentration of α-galactosidase used, 20 units/ml, NK lysis was inhibited by a mean of 35% accompanying a 44% reduction in cell surface expression of Gal α(1,3)Gal. This inhibition of NK lysis was specific to cleavage of terminal α(1,3)-linked galactose residues, because enzymatic treatment with β-galactosidase had no effect (Fig. 1f).

Expression of Gal α(1,3)-Gal in COS Cells Increases Susceptibility to NK Lysis—To directly demonstrate the effect of surface expression of α(1,3)-linked galactose residues on susceptibility to NK lysis, COS cells were transfected with the murine α(1,3)-galactosyltransferase gene (Fig. 2a). These cells do not normally express Gal α(1,3)Gal epitopes and acquire susceptibility to complement-mediated lysis in the presence of human serum after transfection with α(1,3)-galactosyltransferase (4). In the present study, COS cells transfected with α(1,3)-galactosyltransferase, but not the vector alone, showed increased susceptibility to lysis by human NK cells at every effector: target ratio tested (Fig. 2b). A recent study using similarly transfected COS cells demonstrated enhanced adhesion of human NK cells to COS cells expressing Gal α(1,3)Gal (35). Our results extend these observations and show that the increased binding of NK cells to terminal Gal α(1,3)Gal residues expressed by ligands on cells transfected with α(1,3)-galactosyltransferase leads to activation of NK cell stimulatory receptors and causes increased target cell lysis. Moreover, because augmented NK lysis of α(1,3)-galactosyltransferase transfected cells was observed for both NK cells at rest and after cytokine activation (Fig. 2c), these findings suggest that the stimulatory NK cell receptors that bind ligands containing Gal α(1,3)Gal are constitutively expressed.

Inhibition of Human NK Cell Lysis of Porcine Endothelium by Soluble Gal α(1,3)-Gal or Fuc α(1,2)-Gal Oligosaccharide Derivatives—Transfection of porcine endothelium with the gene encoding the α(1,2)-fucosyltransferase enzyme has been shown to reduce the level of Gal α(1,3)Gal expression (36). The α(1,2)-fucosyltransferase competes with α(1,3)-galactosyltrans-
ferase for the acceptor substrate Gal β(1,4)GlcNAc and diverts synthesis of Gal α(1,3)Gal β(1,4)GlcNAc to Fuc α(1,2)Gal β(1,4)GlcNAc (“H substance” or blood type O phenotype). To compare the effect of terminal Gal α(1,3)Gal or Fuc α(1,2)Gal residues on NK lysis of porcine endothelium, human NK cells were incubated with two pairs of soluble oligosaccharides, each pair consisting of the tetrasaccharide backbone and its appropriate derivative after glycosyltransferase catalysis (Fig. 3, a–d). The type I tetrasaccharide lacto-N-tetra inhibited NK lysis by 2.1-fold higher levels than the type II tetrasaccharide lacto-N-neo-tetra (Fig. 3e), suggesting that carbohydrate binding structures on human NK cells may have a preference for ligands containing type I structures. Addition of a terminal Gal α(1,3)Gal residue inhibited specific NK lysis of porcine endothelium by 3.3-fold higher levels than the lacto-N-neo-tetra backbone structure (Fig. 3c), consistent with our previous data that ligands containing Gal α(1,3)Gal are bound by receptors on human NK cells. The addition of a terminal Fuc α(1,2)Gal residue also increased inhibition of NK lysis of porcine endothelium by levels 2.5-fold higher than the lacto-N-tetra backbone structure (Fig. 3c). Thus, human NK cells can bind both Gal α(1,3)Gal and Fuc α(1,2)Gal residues.

Expression of H Substance in Porcine Endothelium Reduces Target Cell Susceptibility to NK Lysis—To investigate the effect of cell surface carbohydrate remodeling on susceptibility to human NK lysis, porcine endothelial cells were transfected with α(1,2)-fucosyltransferase cDNA, and lines were derived that demonstrated stable expression but widely divergent levels of the H substance (Fig. 4, a and b). Surface expression of Gal α(1,3)Gal β(1,4)Glc was inversely proportional to that of Fuc α(1,2)Gal β(1,4)Glc, reflecting the degree of competition for Gal β(1,4)Glc substrate by the glycosyltransferases. Reduction in surface expression of Gal α(1,3)Gal significantly reduced susceptibility of porcine endothelial cells to lysis by human NK cells (Fig. 4c). Although lytic susceptibility decreased in direct parallel with reduction in surface levels of Gal α(1,3)Gal, human NK lysis could not be reduced by >55% even with >80% reduction of Gal α(1,3)Gal expression. This level of human NK lysis approaches that seen with allogeneic endothelium.
Because the process of delayed xenograft rejection involves NK cells and inducible IgG antibodies, we addressed the possibility that human NK cells may also react with ligands containing terminal Gal\(^{a}(1,3)\)Gal residues—the principal antigen on porcine endothelium recognized by xenoreactive antibodies. Enzymatic treatment of porcine endothelium with \(\alpha\)-galactosidase reduced human NK lysis of porcine endothelium, which specifically correlated with the level of Gal\(^{a}(1,3)\)Gal expression. Furthermore, transfecting a primate cell line with the murine \(\alpha(1,3)\)-galactosyltransferase gene increased the susceptibility to NK lysis of COS cells transfected with \(\alpha(1,3)\)-galactosyltransferase cDNA. Flow cytometric analysis performed on COS cells transfected with either a plasmid containing the murine \(\alpha(1,3)\)-galactosyltransferase gene or the plasmid alone. After \(\alpha(1,3)\)-galactosyltransferase transfection, 28.2% of COS cells expressed Gal\(^{a}(1,3)\)Gal; in contrast, COS cells transfected with control vector failed to demonstrate any surface expression of Gal\(^{a}(1,3)\)Gal. b and c, COS cells transfected with the \(\alpha(1,3)\)-galactosyltransferase gene increased susceptibility to NK lysis, b, human PBMCs were activated with IL-2 and used as effector cells against transfected COS cell targets. COS cells transfected with \(\alpha(1,3)\)-galactosyltransferase showed increased susceptibility to NK lysis compared with COS cells transfected with the control vector. c, freshly isolated and IL-2-activated purified human NK cells were used as effector cells. Lysis of \(\alpha(1,3)\)-galactosyltransferase-transfected COS cells was greater than COS cells transfected with the control vector, which was also observed after NK activation with IL-2. The relative increase in NK lysis of COS cells transfected with \(\alpha(1,3)\)-galactosyltransferase was \(\sim 30\%\) irrespective of whether the NK cells were resting or IL-2-activated.
Gal α(1,3)Gal Expression and Susceptibility to NK Lysis

With the development of transgenic pig organs resistant to complement-mediated hyperacute rejection, the subsequent immunological barrier confronted by these genetically modified xenografts on transplantation into primate recipients will be that comprising NK cells and macrophages. In this report, we have shown that primate NK cells react prominently with the same principal xenogenit on porcine endothelium that is recognized by naturally occurring xenoreactive antibodies, confirming the relationship between NK cells and B cells within an innate compartment of the immune system that is T cell-independent. High level expression of α(1,2)-fucosyltransferase, which reduces binding of xenoreactive antibodies, protected porcine endothelium against lysis by human NK cells. Because the alternative transgenic strategy for overcoming complement-mediated hyperacute rejection is to induce expression of human complement inhibitory proteins to protect porcine endothelium against the effects of human complement, organs modified in this manner will continue to be susceptible to a process of delayed xenograft rejection mediated by NK cells and induced IgG antibodies reactive with ligands expressing Gal α(1,3)Gal epitopes. Our study suggests that successful transgenic strategies for pig-to-primate xenotransplantation will need to incorporate carbohydrate remodeling to limit xenograft rejection by a T cell-independent cellular and humoral process.

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