J. Biochem. 126, 1067-1073 (1999)

Regulation of Natural Killer Cell-Mediated Swine Endothelial Cell Lysis through Genetic Remodeling of a Glycoantigen[1]

Shuji Miyagawa,*,[2] Rie Nakai,* Mako Yamada,* Masahiro Tanemura,* Yoshitaka Ikeda,† Naoyuki Taniguchi,† and Ryota Shirakura*

**Division of Organ Transplantation, Biomedical Research Center, Osaka University Graduate School of Medicine, and †the Department of Biochemistry, Osaka University Medical School, 2-2 Yamadaoka, Suita, Osaka 565-0871*

Received June 30, 1999; accepted September 24, 1999

The effect of remodeling of a glycoantigen such as the α-Gal epitope, Galα1,3Galβ1, 4GlcNAc-R, by the introduction of glycosyltransferase genes on natural killer (NK) cell-mediated direct cytotoxicity was investigated using human peripheral blood mononuclear cells (PBMC) or an NK-like cell line, YT cells, as an effector, and swine endothelial cells (SEC) as a target. Several SEC transfectants were established by transfection with the genes for β1,4-*N*-acetylglucosaminyltransferase III, α2,3-sialyltransferase and α1,2-fucosyltransferase. These transfections led to dramatic reductions in both direct and indirect NK cell-mediated cytotoxicity, by 72-94% in the case of PBMC and 27-72% in that of YT cells, in addition to an effective reduction in xenoantigenicity, which is substantially caused by the α-Gal epitope, to human natural antibodies. The NK cell-mediated direct cytotoxicity was remarkably blocked by an anti-α-Gal epitope monoclonal antibody or GSI lectin which preferentially binds to the epitope. Furthermore, treatment of the parental cells with α-galactosidase resulted in a significant reduction in cytotoxicity. These results suggest that the α-Gal epitope is involved not only in hyperacute rejection and acute vascular rejection, but also in NK cell-mediated direct cytotoxicity. Thus, the genetic remodeling of the α-Gal epitope and probably other glycoantigens as well can be expected to represent a new approach for overcoming not only indirect but also direct immunity to xenografts.

Key words: α-Gal epitope, glycosyltransferase, natural killer cell, remodeling of glycoantigen, swine endothelial cell.

The fact that a worldwide shortage of donor organs exists has led to a revived interest in xenotransplantation. The expression of complement regulatory proteins, such as MCP (*CD46*), DAF (CD55), and CD59, in transgenic pigs has been shown to be very protective against hyperacute rejection in xenografts (*1-7*). Trials directed at overcoming hyperacute rejection *via* the modification of the glycoantigen using gene technology are also currentry underway (*8, 9*).

While trials directed at the prevention of hyperacute rejection have been carried out in many institutes, the inhibition of complement in models of xenotransplantation has been shown to be a delayed form of rejection, which is characterized by endothelial cell activation, and mononuclear cell infiltration by natural killer (NK) cells and monocytes. In addition, evidence has accumulated which indicates that the NK cell-mediated cellular response plays a role in xenograft rejection (*10, 11*).

One possible strategy for inhibiting NK cell activity toward swine endothelial cells (SEC) is to express a human MHC class I antigen, such as HLA-G (*12-14*), on SEC. NK cells are capable of lysing cells in which the surface expression of MHC class I molecules is absent or altered. MHC class I molecules on SEC provide signals for the inhibition of NK cell-mediated cytotoxicity *via* interaction with NK inhibitory receptors. An alternative strategy would be to express the Fas ligand on SEC, which would induce apoptosis in NK cells on exposure to the ligand. Fas represents a cell surface receptor which is widely distributed in tissues, and Fas-induced apoptosis appears to be a very potent process for killing Fas-expressing cells (*15*).

On the other hand, it has been reported that, in the case of interaction between NK cells and target cells, the potential target molecules for NK cells are not only proteins, but also carbohydrate determinants. Inverardi *et al.* recently noted that oligosaccharide ligands, including

[1] This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan, and the Program for the Promotion of Basic Research Activities for Innovative Biosciences.

[2] To whom correspondence should be addressed. Tel: +81-6-6879-3062, Fax: +81-6-6879-3069, E-mail: miyagawa@orgtrp.med.osaka-u.ac.jp

Abbreviations: NK, natural killer; PBMC, peripheral blood mononuclear cells; SEC, swine endothelial cell; the α-Gal epitope, the Galα1-3Galβ1-4GlcNAc-R; GSI, *Griffonia simplicifolia* I; α1, 3GT, α1,3galactosyltransferase; GnT-III, β-D-mannoside β-1,4-*N*-acetylglucosaminyltransferase III; α2,3ST, α2,3sialyltransferase; GlcNAc, *N*-acetylglucosamine; α1,2FT, α1,2fucosyltransferase; ADCC, antibody dependent cell-mediated cytotoxicity; D-MEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; NHS, normal human serum; FITC, fluorescein isothiocyanate; LDH, lactate dehydrogenase.

Galα1,3Galβ1,4GlcNAc-R (α-Gal epitope), a major xenoantigen, appear to play a role in antibody-independent adhesion to and the destruction of xenogenic endothelial cells by human unstimulated NK lymphocytes (*16*). This suggests that an oligosaccharide could be a target through which NK cell-mediated direct cytotoxicity is inhibited.

In this study, the effects of transfection of several glycosyltransferase genes into SEC on the NK cell-mediated direct SEC lysis and the resulting remodeling of oligosaccharides (*17*) were examined, in order to elucidate the involvement of oligosaccharides in this lysis. Furthermore, the issue of whether or not the specific structure of the oligosaccharide contributes to NK cell-mediated direct cytotoxicity was also examined using an anti-α-Gal epitope antibody and the epitope-specific lectin *Griffonia simplicifolia* I (GSI).

MATERIALS AND METHODS

Cell Culture—A SEC line, MYP30 (*18*), was cultured in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), L-glutamine and penicillin/streptomycin (Gibco/BRL). K562 cells, which were obtained from the American Type Culture Collection (Rockville, MD), were cultured in RPMI1640 supplemented with 10% heat-inactivated FBS and penicillin/streptomycin.

Effector Cells—Fresh whole blood obtained from healthy volunteers was diluted 1:1 in phosphate-buffered saline (PBS), overlaid on Lymphoprep (Pharmacia), and then centrifuged at 1,500 rpm for 30 min. Peripheral blood mononuclear cells (PBMC) were sampled from the monolayer and washed twice in PBS. The NK-like cell line, YT cells, kindly provided by Drs. Junji Yodoi and Keisuke Teshigawara (University of Kyoto, Kyoto) (*19, 20*), was maintained in RPMI1640 medium supplemented with 10% heat-inactivated FBS and penicillin/streptomycin.

Construction of Plasmids—cDNAs for mouse α2,3ST (ST3Gal III) and the human α1,2FT were gifts from Dr. Shuichi Tsuji (RIKEN, Saitama), and Dr. John B. Lowe (University of Michigan), respectively. The cDNAs for human GnT-III (*21*), mouse α2,3ST (ST3Gal III) (*22*), and human α1,2 FT were subcloned into the site of pCXN2 (*23*) in which the transcription of the inserted cDNA is driven by a β-actin promoter and a cytomegalovirus enhancer. A neomycin-resistant gene contained in the vector permitted the selection of the transfectants by the antibiotics G418. The resulting plasmids were purified by CsCl-gradient ultracentrifugation and then used for transfection.

Transfection Experiments—The purified plasmids (20 μg) were introduced into MYP-30 by lipid-mediated DNA transfection using a LIPOFECTAMINE™ Reagent (GIBCO/BRL). The transfected MYP-30 cells were maintained in complete medium for several days under an atmosphere of humidified 5% CO_2 at 37°C. The cells were then transferred to the complete medium containing 0.4 mg/ml G418 (GIBCO/BRL) for selection (*18*). Expression of the glycosyltransferases was confirmed by activity assays, as described below.

Glycosyltransferase Assays—The glycosyltransferase activities in each cell line were assayed using fluorescence-labeled oligosaccharides as the acceptor substrates with a reversed-phase HPLC system equipped with a fluorescence detector (*24-26*). For the enzyme activity assays, cells were washed twice with PBS and then centrifuged at 1,500×*g* for 10 min. The pelleted cells were resuspended in 100 μl of PBS and then lysed by sonication. The resulting lysates were then assayed for activity. In the activity assays for α2,3ST, α1,2FT, and α1,3GT, the common acceptor substrate, pyridylaminated lacto-*N*-neotetraose (LNnT-PA; Seikagaku Kogyo) was used. To determine these enzyme activities, the cell lysates were incubated at 37°C for 3 h in the reaction mixtures. The mixtures used were 50 mM cacodylate buffer, 10 mM $MnCl_2$, 0.23% Triton X-100, 5 mM CMP-sialic acid as the donor substrate and 10 μM LNnT-PA as the acceptor, pH 6.8, for α2,3ST (*27*); 13 mM potassium phosphate buffer, 0.1% Triton X-100, 13 mM phenyl-β-D-galactoside, 5 mM ATP, 1 mM GDP-fucose, and 10 μM LNnT-PA, pH 6.1, for α1,2FT (*28*); and 10 mM HEPES buffer, 10 mM $MnCl_2$, 33 mM NaCl, 3 mM KCl, 20 mM UDP-Gal, and 10 μM LNnT-PA, pH 7.2, for α1,3GT (*28*). When the activity of GnT-III was assayed, the cell lysates were reacted with 20 mM UDP-GlcNAc and 20 μM the pyridylaminated agalacto biantennary oligosaccharide [GlcNAcβ1-2Manα1-6(GlcNAcβ1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc-PA] in the presence of 63 mM MES, 10 mM $MnCl_2$, 200 mM GlcNAc, and 0.5% Triton X-100, pH 6.25 (*24*). The assay mixtures were incubated at 37°C for 3 h, and then the reactions were terminated by boiling for 5 min, followed by centrifugation of the samples at 12,000×*g* for 5 min. The resulting supernatants were injected onto a reversed phase HPLC equipped with a TSKgel column, ODS 80TM (4.6×250 mm). The product and the substrate were isocratically separated with 20 mM ammonium acetate buffer (pH 4.0) containing 0.01% *n*-butanol. The fluorescence of the column eluate was detected with a fluorescence detector (Shimadzu, model RF-10AXL) at excitation and emission wavelengths of 320 and 400 nm, respectively.

Protein Determination—Protein concentrations were determined with a BCA protein assay kit (Pierce) using bovine serum albumin as a standard.

Flow Cytometry—Parental SEC and transfectants were incubated with 10% normal human serum (NHS) at 4°C for 1 h, washed and then incubated with 1.25 mg of fluorescein isothiocyanate (FITC)-conjugated anti-human Ig (Cappel) as a second antibody for 1 h at 4°C. The stained cells were analyzed with a FACSCalibure flow cytometer (Becton Dickinson). The cell-surface α-Gal epitope was also examined with a mouse mAb, M86 (a generous gift from Dr. Uri Galili, Philadelphia) (*29*), and FITC-conjugated anti-mouse IgM as a second antibody, or, alternatively, analyzed with an FITC-conjugated GSI lectin (Honen, Tokyo) which binds to the α-Gal epitope.

NK Cell-Mediated Cytotoxicity Assay—The parental, transfected cells, or mock cell were plated at 1×10^4 cells per well in a flat-bottomed gelatin-coated 96-well plate. Fifteen hours after plating the cells, the plates were incubated with effector cells, PBMC or YT cells, at various effector: target (E:T) ratios. An autologous human antibody purified on DEAE Sephacel was added to the wells in order to observe antibody-dependent cell-mediated cytotoxicity (ADCC). Each assay was performed in triplicate. After 4 h incubation at 37°C, the released lactate dehydrogenase (LDH) was measured using a Kyokuto MTX-"LDH" kit according to the manufacturer's recommended

protocol. The spontaneous release of LDH activity from effector cells and target cells was less than 10% and 5%, respectively, as compared to the maximal release obtained on sonication. The results are expressed as percentages of specific lysis (*30*).

Blocking Assay—YT cells were incubated with SEC or K562 in plates with or without 5 μl of M86 mAb, GSI lectin, anti-H mAb (*17*), or *Ulex europaeus* (UEA-1) (Honen, Tokyo) lectin as in the cytotoxicity assay as above (*31*).

α-Galactosidase Treatment—SEC (1×10^6) cells were treated without (control) or with 2 units of α-galactosidase (Sigma) for 2 h at pH 6.5, and then the α-Gal epitope was checked by FACS using GSI lectin. Subsequently, the treated SEC were subjected to the NK cell-mediated cytotoxicity assay, as described above.

Statistics—Data are presented as means$\pm$SEM. Student's *t* test was used to ascertain the significance of differences within groups. Differences were considered statistically significant when $p<0.05$.

RESULTS

An SEC clone which stably expresses either GnT-III, α2,3ST, or α1,2FT was established *via* transfection of each glycosyltransferase gene. The enzyme activities of these glycosyltransferases in these stable clones are listed in Table I. Neither GnT-III, α2,3ST nor α1,2FT activity was detectable in the parental SEC under the assay conditions used, whereas the transfectant cells exhibited sufficiently high activities of the introduced glycosyltransferase.

Flow cytometric analysis with NHS showed that the parental SEC react strongly with human natural antibodies (Fig. 1), as was reported previously, while decreased mean fluorescence intensities were observed in the cases of GnT-III-, α2,3ST-, and α1,2FT-transfected SEC clones. The percent reduction of xenoantigenicity to human natural antibodies was approximately 60% for all transfectants, as judged by the shifts in mean fluorescence intensity (Fig. 1A). By the use of a monoclonal antibody, M86, and GSI lectin, both of which are specific to the α-Gal epitope in the

TABLE I. **Profiles of transfectants.**[a]

Cells	Enzyme activity (pmol/h/mg protein)		
	GnT-III	α2,3ST	α1,2FT
Parental SEC	ND	ND	ND
GnT-III	1,850$\pm$506	ND	ND
α2,3ST	ND	172$\pm$32	ND
α1,2FT	ND	ND	193$\pm$32

[a]Enzyme activities were measured by HPLC, using fluorescent-labeled sugar chains as substrates. Each value is expressed as the mean$\pm$SEM for three independent experiments. ND: not detected.

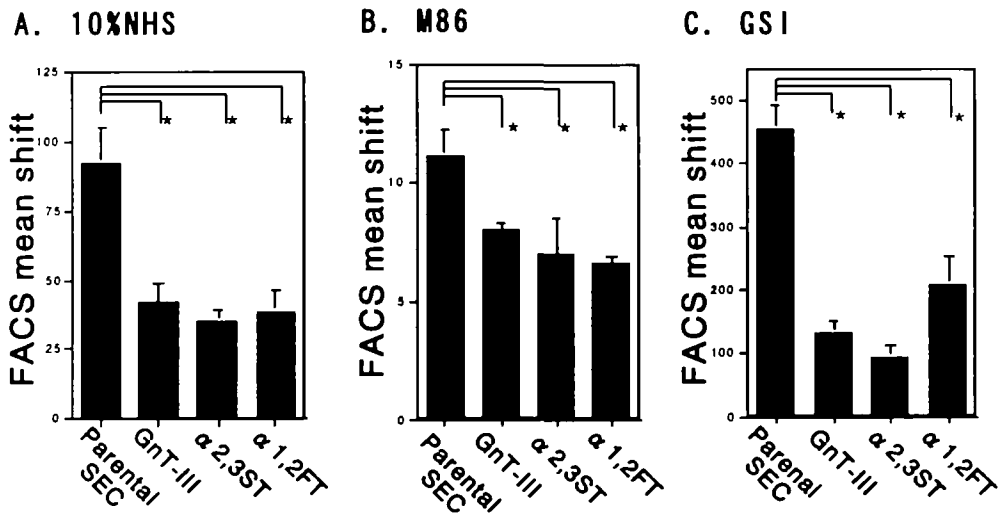

Fig. 1. **Xenoantigenicity of transfectants to NHS, M86, or GSI, as investigated by flow-cytometry.** (A) Parental SEC (control) and stable transfectants were treated with 10% NHS as the first antibody and FITC-conjugated anti-human Ig as the second antibody. (B and C) The reduction of the α-Gal epitope on the cell surface was analyzed, using the anti-α-Gal epitope mAb M86, or GSI lectin. Parental SEC (control) and stable transfectants were treated with FITC-conjugated anti-mouse IgM or GSI lectin. Each value is expressed as the mean$\pm$SEM for four to six independent experiments.

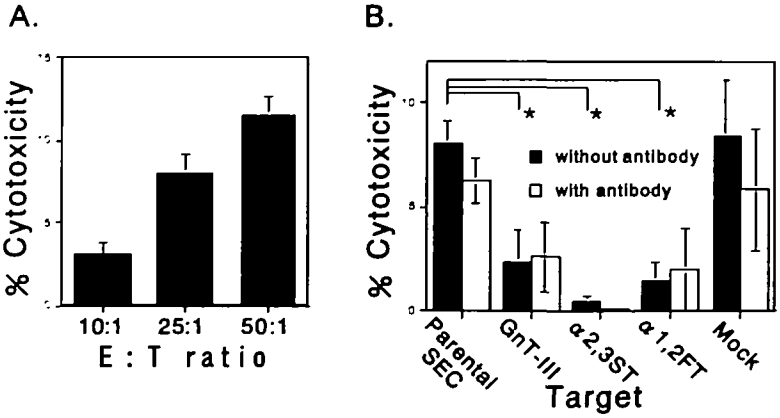

Fig. 2. **Direct SEC lysis by PBMC.** (A) PBMC isolated from six subjects were incubated with SEC for 4 h at different E:T ratios. The data are expressed as the means$\pm$SEM of the percentage of specific lysis. (B) PBMC isolated from several blood donors were incubated with SEC at an E:T ratio of 25:1, either with or without heat-inactivated autologous serum. The resulting cytolysis is expressed as the mean percentage of specific lysis$\pm$SEM for six to eight independent experiments.

analysis, it was found that expression of the epitope in the transfectants was also reduced in SEC as the result of transfection of the glycosyltransferase genes (Fig. 1, B and C). These results indicate that the introduction and expression of the α1,2FT gene, as well as the GnT-III (*8*) and α2,3ST (*28*) genes, whose effects on xenoantigenicity were examined in previous studies, effectively downregulate the xenoepitope(s), and this is consistent with a very recent report (*32*).

In order to examine the involvement of the oligosaccharides in cell-mediated cytotoxicity, assays for NK cell-mediated direct and antibody-dependent cytotoxicity were carried out using the GnT-III-, α2,3ST-, and α1,2FT-transfected SECs. As shown in Fig. 2A, when the ratio of PBMC, as effector cells, to the target cells, parental SEC, was varied in the assay, a clear dose-dependent increase in the cytotoxicity was observed, indicating that the SEC were sensitive to NK cell-mediated direct cytotoxicity. On the other hand, the cytotoxicity to the transfected SECs was remarkably decreased, compared to the parental cells and mock transfectants (Fig. 2B). Thus, it is clear that the transfectants are much more resistant to NK cell-cytotoxicity, suggesting that cell surface oligosaccharides play a role in the cytotoxicity against SEC. Since the addition of autologous antibodies to the assay resulted in no observable change in each cell line, even in the parental SEC, ADCC does not appear to play a significant role in this cytotoxicity. When an NK-like cell line, YT cells, was used as the effector in place of PBMC, a significant reduction in NK cell-mediated direct killing was also observed in these transfectants, even though the extent of the reduction was relatively modest (Fig. 3).

To examine the contribution of the α-Gal epitope to the NK cell-mediated cytotoxicity involving cell surface oligosaccharides of SEC, M86 mAb-, and GSI lectin-blocking assays on NK cell-mediated SEC direct cytotoxicity were carried out. The addition of M86 mAb F(ab')^2 or GSI lectin to the assay for NK cell-mediated cytotoxicity of the parental SEC led to a significant reduction in lysis, as shown in Fig. 4A. On the other hand, M86 mAb and 1B4 lectin had no effect on the cytotoxicity of the human cell line, K562 cells (Fig. 4B). These results suggest that the α-Gal epitope plays a major role as a xenoantigen in NK cell-mediated cytotoxicity. Even when PBMC obtained from two independent healthy donors were used as the effector in this blocking assay, essentially the same or similar effects of the blocking agents were observed (Fig. 5). Therefore, this provides support for the general involvement of the α-Gal epitope in this cytotoxicity.

In order to further verify the role of the α-Gal epitope in

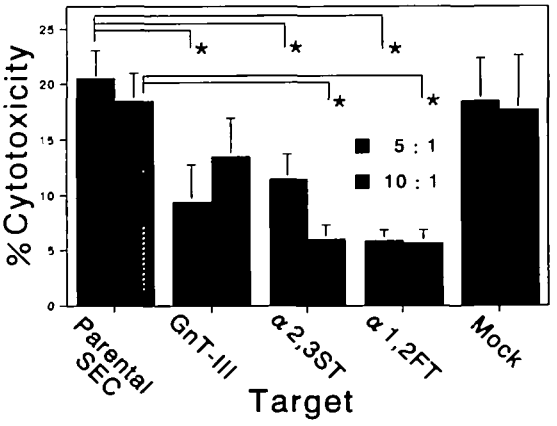

Fig. 3. **Direct SEC lysis by the NK cell line, YT cells.** YT cells were incubated with SEC at E:T ratios of 10:1 or 5:1. The resulting cytolysis is expressed as the mean percentage of specific lysis ± SEM for six to eight independent experiments.

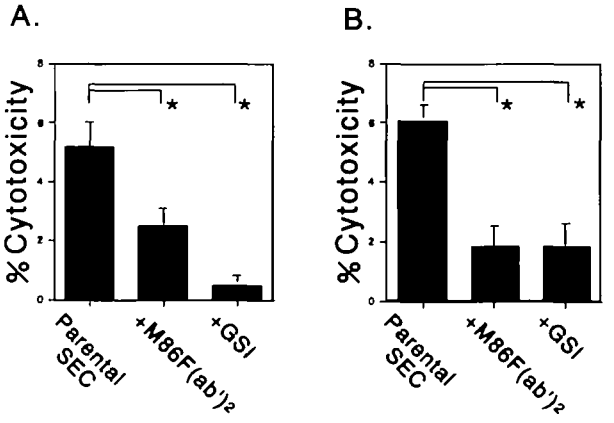

Fig. 5. **Effect of M86 mAb and GSI lectin blocking in PBMC-mediated direct cytotoxicity.** PBMC cells from two volunteers, A and B, were incubated with SEC at an E:T ratio of 25:1, and then NK cell-mediated direct cytotoxicity was measured with M86 F(ab')^2 and GSI lectin. The resulting cytolysis is expressed as the mean percentage of specific lysis ± SEM for six to eight independent experiments.

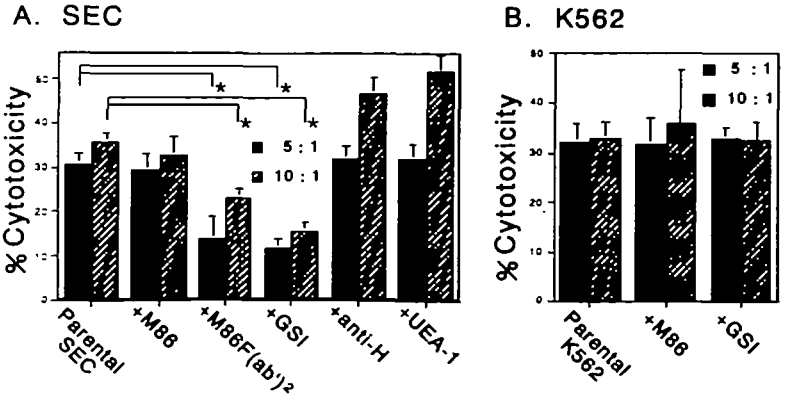

Fig. 4. **Effect of M86 mAb or GSI lectin blocking in YT cell-mediated direct cytotoxicity.** YT cells were incubated with SEC (A) or K562 (B) at E:T ratios of 10:1 or 5:1 for 4 h with saturating amounts of the following antibodies or lectin: nothing, M86, M86 F(ab')^2, GSI lectin, anti-H, and UAE-1 lectin. In this experiment, the cytotoxicity against SEC is expressed as the mean percentage of specific lysis ± SEM for six to eight independent experiments.

TABLE II. **Profiles of α-galactosidase treated SEC.**[a]

Cells	Mean fluorescence intensity
Control SEC	396±42
α-Galactosidase-treated SEC	275±41

[a]SEC were treated with 2 units of α-galactosidase for 2 h at pH 6.5. The expression levels of the α-Gal epitope were then determined by flow cytometry using GSI lectin. Each value is expressed as the mean±SEM for three to four independent experiments.

NK cell-mediated direct killing, SEC whose α-Gal epitope had been decreased by treatment with α-galactosidase were subjected to the assay for the NK cell-mediated cytotoxicity. As evidenced by a decrease in the fluorescence intensity observed on FACS with GSI lectin, the α-Gal epitope was found to be decreased in the α-galactosidase-treated SEC (Table II). The NK cell-mediated killing assay showed that the cytotoxicity toward the treated SEC was significantly reduced, as compared to that toward the control, non-treated cells. These and the results of the above blocking assays suggest that human NK cells exhibit direct cytotoxicity against SEC, and that this cytotoxicity is, at least in part, dependent on the α-Gal epitope on SEC (Fig. 6).

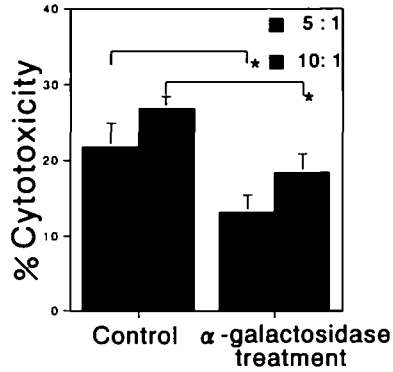

Fig. 6. **Effect of removal of α-Gal epitopes on SEC by treatment with α-galactosidase.** α-Galactosidase treated or untreated SEC were used as target cells, and NK cell-mediated direct killing was assessed. The resulting cytolysis is expressed as the mean percentage of specific lysis±SEM for six to eight independent experiments.

DISCUSSION

Increasing evidence has accumulated suggesting that NK cells play an important role in swine to human xenogenic cytotoxicity, whereas the molecular mechanism by which oligosaccharides are recognized by NK cells remains controversial (*31*). For instance, it has been reported that the sensitivity to NK cells is increased by treatment of cells with sialidase, suggesting that the content of surface sialic acid is inversely correlated with the sensitivity to NK cell cytotoxicity (*33*). These investigations also revealed that rat NKR-P1 contains a lectin-like domain with an order of preference of GalNAc>GlcNAc>Fuc>Gal>Man (*34*). In addition, it has been reported that the presence of high mannose-type glycans is correlated with NK cell susceptibility (*35*). It has also been reported that the remodeling of oligosaccharides in GnT-III-transfected K562 cells led to a decrease in the susceptibility to NK cell cytotoxicity (*36*). On the contrary, another report demonstrated that sialic acid α2,6-linked to the *N*-linked chains of target cell glycoproteins did not play a major role in recognition of the target by human NK cells (*37*).

We previously reported that the introduction of GnT-III cDNA to SEC resulted in significant suppression of xenoantigen, especially the α-Gal epitope, and that α2,3ST and α1,2FT were also very effective in the downregulation of the α-Gal epitope (*17, 28*). That is, these pathways for decreasing the antigenicity of SEC were incidentally effective in the regulation of antibody-dependent NK cell-mediated killing. The mechanism by which the introduction of the GnT-III gene significantly suppresses xenoantigens is not fully understood, but this suppression could, in part, be due to the inhibition of further branching and/or a lack of maturation of the processing through the addition of a bisecting GlcNAc, which is catalyzed by the enzyme (*38*). It is clear that further studies are required to elucidate the mechanism in terms of the regulation of oligosaccharide biosynthesis, and such studies are now in progress. On the other hand, in the case of α2,3ST or α1,2FT, the xenoantigenicity would be expected to be reduced as the result of a decrease in the α-Gal epitope content, since both α2,3ST and α1,2FT compete with α1,3GT for the terminal β-Gal residue of the common acceptor substrate.

One of the main purposes of this study was to determine whether or not the remodeling of glycoantigens, especially the α-Gal epitope, by these glycosyltransferases affects the susceptibility of SEC to NK-mediated direct lysis in the absence of an antibody-mediated reaction. As expected, the data herein demonstrate the possibility of downregulation of NK cell-mediated direct killing as a result of the transfection of the glycosyltransferases.

We initially developed a 4 h cytotoxicity assay with SEC as target cells in order to evaluate the relative importance of direct, as well as antibody-dependent xenogeneic anti-SEC cytotoxicity in different subjects. The evidence conflicts somewhat with previous reports (*31*). Namely, the addition of autologous serum or an anti-α-Gal antibody to these effector cells failed to induce an increase in cytotoxicity. These effects were found to be exerted by not only PBMC but also YT cells (data not shown). To explain the paradoxical decrease in ADCC, one possible explanation is that the anti-α-Gal antibody downregulated the direct NK cell-mediated cytotoxicity by masking the α-Gal epitope in spite of the effect induced by the ADCC reaction. In addition, the YT cells used in this study showed a weak level of CD16 expression (data not shown). These experiments suggest that the intricate relation between oligosaccharides on SEC includes the α-Gal epitope and NK-mediated cytotoxicity.

Therefore, the following experiments were carried out in order to focus on the relation between NK cell-mediated direct cytotoxicity and the α-Gal epitope. Blocking the α-Gal epitope with an anti-α-Gal mAb M86 or GSI lectin diminished the direct reactivity of NK cells against SEC in the cytotoxicity assay with both the YT clone and PBMC from volunteers. A correlation between α-Gal expression and NK cell-mediated direct killing of SEC was revealed. mAb M86 exhibits high specificity to the α-Gal epitope, but relative low affinity (*30*), while the GSI lectin has been a popular probe for determining the α-Gal epitope in many studies. Moreover, the amelioration of NK cell-mediated

direct killing by the downregulation of the α-Gal epitope was determined using α-galactosidase. The data obtained in this study also demonstrate the importance of the α-Gal epitope in NK cell-mediated direct cytotoxicity.

Finally, human NK cells contain CD94-NKG2A/B receptors, which have an extracellular domain which contains a carbohydrate recognition domain, C-type lectin (*39*). Although many researchers have investigated these molecules, neither the role of oligosaccharide recognition in the function of the receptor nor the ligand of the lectin domain is known. However, the possibility exists that human NK cells are able to recognize not-self oligosaccharide epitopes, such as the α-Gal epitope, *via* this receptor. Additionally, since naive human cells do not express the α-Gal epitope on the membrane, the human NK cell will not use the α-Gal epitope as a usual ligand in the cell-cell interaction of the immuno-system. However, it may be possible for the human NK cell to recognize the α-Gal epitope on the cells from the outside world.

In conclusion, NK cells are able to contribute to cellular rejection in swine to human xenotransplantation. However, the results presented here clearly show that the modification of glycosyl epitopes such as the α-Gal epitope can lead to effective therapeutic approaches for overcoming NK cell-mediated rejection.

The authors wish to thank Dr. Milton S. Feather for editing the manuscript, and Drs S. Koyota and M. Koma for the valuable discussions.

REFERENCES

1. Seya, T., Turner, J.R., and Atkinson, J.P. (1986) Purification and characterization of a membrane protein (gp45-70) that is a cofactor for cleavage of C3b and C4b. *J. Exp. Med.* **163**, 837-855
2. Nicholson-Weller, A., Burge, J., Fearon, D.T., Weller P.F., and Austen, K.F. (1982) Isolation of a human erythrocyte membrane glycoprotein with decay-accelerating activity for C3 convertase of the complement system. *J. Immunol.* **129**, 184-189
3. Sugita, Y., Nakano, Y., and Tomita, M. (1988) Isolation from human erythrocytes of a new membrane protein which inhibits the formation of complement transmembrane channels. *J. Biochem.* **104**, 633-637
4. Miyagawa, S., Hirose, H., Shirakura, R., Naka, Y., Nakata, S., Kawashima, Y., Seya, T., Matsumoto, M., Uenaka, A., and Kitamura, H. (1988) The mechanism of discordant xenograft rejection. *Transplantation* **46**, 825-830
5. Rosengard, A.M., Cary, N.R.B., Langford, G.A., Tucker, A.W., Wallwork, J., and White, D.J.G. (1995) Tissue expression of human complement inhibitor, dacay-accelerating factor, in transgenic pigs. *Transplantation* **59**, 1325-1333
6. McCurry, K.R., Kooyman, D.L., Alvarado, C.G., Cotterell, A.H., Martin, M.J., Logan, J.S., and Platt, J.L. (1995) Human complement regulatory proteins protect swine-to-primate cardiac xenografts from humoral injury. *Nature Med.* **5**, 423-427
7. Fodor, W.L., Williams, B.L., Matis, L.A., Madri, J.A., Rollins, S.A., Knight, J.W., Velander, W., and Squinto, S.P. (1994) Expression of functional human complement inhibitor in a transgenic pig as a model for the prevention of xenogenic hyperacute organ rejection. *Proc. Natl. Acad. Sci. USA* **91**, 11153-11157
8. Tanemura, M., Miyagawa, S., Ihara, Y., Nishikawa, A., Suzuki, M., Yamamura, K., Matsuda, H., Shirakura, R., and Taniguchi, N. (1997) Suppression of xenoantigen Gal α(1,3)Gal by *N*-acetylglucosaminyltransferase III (GnT-III) in transgenic mice. *Transplant. Proc.* **29**, 895-896
9. Koike, C., Kannagi, R., Takuma, Y., Akutsu, F., Hayashi, S., Hiraiwa, N., Kadomatsu, K., Muramatsu, T., Yamakawa, H., Nagui, T., Kobayashi, S., Okada, H., Nakashima, I., Uchida K., Yokoyama, I., and Takagi, H. (1996) Introduction of α(1,2)-fucosyltransferase and its effect on α-Gal epitopes in transgenic pig. *Xenotransplant* **3**, 81-86
10. Lin, Y., Vandeputte, M., and Waer, M. (1997) Natural killer cell- and macrophage-mediated rejection of concordant xenografts in the absence of T and B cell responses. *J. Immunol.* **158**, 5658-5667
11. Lin, Y., Goebels, J., Xia, G., Ji, P., Vandeputte, M., and Waer, M. (1998) Induction of specific transplantation tolerance across xenogenic barriers in the T-independent immune compartment. *Nature Med.* **4**, 173-180
12. Seebach, J.D., Comrack, C., Germana, S., LeGuern, C., Sachs, D.H., and DerSimonian, H. (1997) HLA-Cw3 expression on porcine endothelial cells protects against xenogenic cytotoxicity mediated by a subset of human NK cells. *J. Immunol.* **159**, 3655-3661
13. Pazmany, L., Mandelboim, O., Vales-Gomez, M., Davis, D.M., Reyburn, H.T., and Strominger, J.L. (1996) Protection from natural killer cell-mediated lysis by HLA-G expression on target cells. *Science* **274**, 792-795
14. Sasaki, H., Xu, X., Smith, D.M., Howard, T., and Mohanakumar, T. (1999) HLA-G expression protects porcine endothelial cells against natural killer cell-mediated xenogenic cytotoxicity. *Transplantation* **67**, 31-37
15. Fujiwara, I., Nakajima, H., Yamagishi, H., Matsuda, T., Mitzuta, N., and Oka, T. (1998) The molecular mechanism of apoptosis induced by xenogeneic cytotoxicity. *Xenotransplantation* **5**, 50-56
16. Inverardi, L., Clissi, B., Stolzer, A.L., Bender, J.R., Sandrin, M.S., and Pardi, R. (1997) Human natural killer lymphocytes directly recognize evolutionarily conserved oligosaccharide ligands expressed by xenogenic tissues. *Transplantation* **63**, 1318-1330
17. Tanemura, M., Miyagawa, S., Ihara, Y., Matsuda, H., Shirakura, R., and Taniguchi, N. (1997) Significant downregulation of the major swine xenoantigen by *N*-acetylglucosaminyltransferase III gene transfection. *Biochem. Biophys. Res. Commun.* **235**, 359-364
18. Miyagawa, S., Shirakura, R., Iwata, K., Nakata, S., Matsumiya, G., Izutani, H., Matsuda, H., Terado, A., Matsumoto, M., Nagasawa, S., and Seya, T. (1994) Effects of transfected complement regulatory proteins, MCP, DAF, MCP/DAF hybrid, on complement-mediated swine endothelial cell lysis. *Transplantation* **58**, 834-840
19. Yodoi, J., Teshigawara, K., Nikaido, T., Fukui, K., Noma, T., Honjo, T., Takigawa, M., Sasaki, M., Minato, N., Tsudo, M., Uchiyama, T., and Maeda, M. (1985) TCGF (IL-2)-receptor inducing factor(s). I. Regulation of IL-2 receptor on natural killer-like cell line (YT cells). *J. Immunol.* **134**, 1623-1630
20. Yamabe, T., Takakura, K., Sugie, K., Kitaoka, Y., Takeda, S., Okubo, Y., Teshigawara, K., Yodoi, J., and Hori, T. (1997) Induction of the 2B9 antigen/dipeptidyl peptidase IV/CD26 on human natural killer cells by IL-2, IL-12 or IL-15. *Immunology* **91**, 151-158
21. Ihara, Y., Nishikawa, A., Tohma, T., Soejima, H., Niikawa, N., and Taniguchi, N. (1993) cDNA cloning, expression, and chromosomal localization of human *N*-acetylglucosaminyltransferase III (GnT-III). *J. Biochem.* **113**, 692-698
22. Kono, M., Ohyama, Y., Lee, Y.C., Hamamoto, T., Kojima, N., and Tsuji, T. (1997) Mouse β-galactoside α2,3-sialyltransferases: comparison of *in vitro* substrate specificities and tissue specific expression. *Glycobiology* **7**, 469-479
23. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193-200
24. Nishikawa, A., Fujii, S., Sugiyama, T., and Taniguchi, N. (1988) A method for the determination of *N*-acetylglucosaminyltransferase III activity in rat tissues involving HPLC. *Anal. Biochem.* **170**, 349-354
25. Hase, S., Ibuki, T., and Ikenaka, T. (1984) Reexamination of the pyridylamination used for fluorescence labeling of oligosacchar-

ides and its application to glycoproteins. *J. Biochem.* **95**, 197-203
26. Kondo, A., Suzuki, J., Kuraya, N., Hase, S., Kato, I., and Ikenaka, T. (1990) Improved method for fluorescence labeling of sugar chains with sialic acid residues. *Agric. Biol. Chem.* **54**, 2169-2170
27. Sasaki, K., Watanabe, E., Kawashima, K., Sekine, S., Dohi, T., Oshima, M., Hanai, N., Nishi, T., and Hasegawa, M. (1993) Expression cloning of a novel Gal(1-3/1-4)GlcNAc α2,3-sialyltransferase using lectin resistance selection. *J. Biol. Chem.* **268**, 22782-22787
28. Tanemura, M., Miyagawa, S., Koyota, S., Koma, M., Matsuda, H., Tsuji, S., Shirakura, R., and Taniguchi, N. (1998) Reduction of the major swine xenoantigen, the α-galactosyl epitope by transfection of the α2,3sialyltransferase gene. *J. Biol. Chem.* **273**, 16421-16425
29. Galili, U., La Temple, D.C., and Radic, M.Z. (1998) A sensitivity assay for measuring α-Gal epitope expression on cells by a monoclonal anti-Gal antibody. *Transplantation* **65**, 1129-1132
30. Korzeniewski, C. and Callewaert, D.M. (1983) An enzyme-release assay for natural cytotoxicity. *J. Immunol. Methods* **64**, 313-320
31. Watier, H., Guillaumin, J.M., Vallee, I., Thibault, G., Gruel, Y., Lebranchu, Y., and Bardos, P. (1996) Human NK cell-mediated direct and IgG-dependent cytotoxicity against xenogenic porcine endothelial cells. *Transplant. Immunol.* **4**, 293-299
32. Artrip, J.H., Kwiatkowski, P., Michler, R.E., Wang, S.-F., Tugulea, S., Ankersmit, J., Chisholm, L., McKenzie, I.F.C., Sandrin, M.S., and Itescu, S. (1999) Target cell susceptibility to lysis by human natural killer cells is augmented by α(1,3)-galactosyltransferase and reduced by α(1,2)-fucosyltransferase. *J. Biol. Chem.* **274**, 10717-10722
33. Bezouska, K., Yuen, C., O'Brien, J., Child, R.A., Chai, W., Lawson, A.M., Drbal, K., Fiserova, A., Pospisil, M., and Feizi, T. (1994) Oligosaccharide ligands for NKR-P1 protein activate NK cells and cytotoxicity. *Nature* **372**, 150-157
34. Bezouska, K., Vlahas, G., Horvath, O., Jinochovas, G., Fiserova, A., Giorda, R., Chamber, W.H., Feizi, T., and Pospisil, M. (1994) Rat natural killer cell antigen, NKR-P1, related to C-type animal lectins is a carbohydrate-binding protein. *J. Biol. Chem.* **269**, 16945-16952
35. Ahrens, P.B. (1993) Role of target cell glycoproteins in sensitivity to natural killer cell lysis. *J. Biol. Chem.* **268**, 385-391
36. Yoshimura, M., Ihara, Y., Ohnishi, A., Ijuhin, N., Nishiura, T., Kanakura, Y., Matsuzawa, Y., and Taniguchi, N. (1996) Bisecting *N*-acetylglucosamine on K562 cells suppresses natural killer cytotoxicity and promoters spleen colonization. *Cancer Res.* **56**, 412-418
37. Dall'Olio, F., Mariani, E., Tarozzi, A., Meneghetti, A., Chiricolo, M., Lau, J.T.Y., and Facchini, A. (1997) Expression of β-galactoside α2,6-sialyltransferase does not alter the susceptibility of human colon cancer cells to NK-mediated cell lysis. *Glycobiology* **7**, 507-513
38. Taniguchi, N., Yoshimura, M., Miyoshi, E., Ihara, Y., Nishikawa, A., and Fujii, S. (1996) Remodeling of cell surface glycoproteins by *N*-acetylglucosaminyltransferase III gene transfection: modulation of metastatic potentials and down regulation of hepatitis B virus replication. *Glycobiology* **6**, 691-694
39. Lanier, L.L., Chang, C., and Phillips, J.H. (1994) Human NKR-P1A. A disulfide-linked homodimer of the C-type lectin superfamily expressed by a subset of NK and T lymphocytes. *J. Immunol.* **153**, 2417-2428