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Significant Downregulation of the Major Swine Xenoantigen by N-Acetylglucosaminyltransferase III Gene Transfection

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Introduction of the β -D-mannoside β -1,4-N-acetylglucosaminyltransferase III (GnT-III) gene into swine endothelial cells (SEC) reduced their susceptibility to normal human serum (NHS) in complement-mediated cell lysis and also suppressed the antigenicity to human natural antibodies as evidenced by flow cytometric analysis, as well as *Griffonia simplicifolia* 1 isoelectin (1B4 lectin) binding to the Gal α 1-3 Gal β 1-4 GlcNAc-R (the α -galactosyl epitope). Western blot analysis indicated that proteins smaller than 66 kDa had diminished reactivity to NHS and 1B4 lectin. GnT-III, a key enzyme involved in branch formation of N-linked sugars, was found to downregulate the expression of xenoantigen, suggesting that this approach may be of value in clinical xenotransplantation in the future. © 1997 Academic Press

Xenotransplantation offers a potential solution to the world-wide shortage of organs for transplantation. Since Galili, et al. reported that the α -galactosyl epitope is the major antigen in swine to human xenotransplantation, genetic approaches to modify the glycoantigen have been focused on the xenotransplantation study (1,2). One possible approach for the elimination of the α -galactosyl epitope from swine tissue is to dis-

rupt the swine galactosyl-transferase gene via homologous recombination (3,4). However, gene targeting by homologous recombination (3,4). However, gene targeting by homologous recombination in embryonic stem cells is not feasible because swine embryonic stem cells are not currently available.

Another strategy is to downregulate the α -galactosyl epitope by taking advantage of intracellular competition between the α 1,3 galactosyltransferase and the α 1,2 fucosyltransferase (α 1,2 FT) (5) for the common acceptor substrate in the trans Golgi stack and network, which are similar to other glycosyltransferases (6-8).

In this study, in addition to attempting to reduce the α -galactosyl epitope by competitive enzymes such as α 1,2 FT, we focused on the N-linked sugars which express a high level of antigenicity. We modified this antigenicity, using GnT-III (9,10). Our strategy to diminish the antigenicity of swine cells is directed, not only at the α -galactosyl epitope, but to other unknown epitopes, but to other unknown epitopes, as well.

METHODS

Endothelial cell cultures. A SEC line, MYP30, was cultured in DMEM containing 10% FBS with L-glutamine and penicillin/streptomycin (Gibco/BRL) (11).

Establishment of GnT-III and α 1,2FT transfectants. The cDNA of a human GnT-III, Act 5, which contains the entire coding sequence and deleted at the 5' noncoding region, was subcloned into the site of pCXN2 (12); the β -actin promoter and cytomegalovirus enhancer, with a neomycin-resistant gene. cDNA of human α 1,2 FT, a gift from Dr. John B. Lowe (University of Michigan), was also subcloned into pCXN2. The plasmids were separately transformed into *Escherichia coli* C600 and amplified using standard techniques. The cDNAs were introduced into MYP-30 by lipid-mediated DNA transfection with lipofectamine (LIPOFECTAMINE Reagent, GIBCO/BRL). Transfected MYP-30 was maintained in complete medium for several days in an atmosphere of humidified 5% CO₂ at 37°C. The

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Abbreviations used: GnT-III, β -D-mannoside β -1,4-N-acetylglucosaminyltransferase III; SEC, swine endothelial cells; NHS, normal human serum; 1B4, *Griffonia simplicifolia* 1; the α -galactosyl epitope, the Gal α 1-3 Gal β 1-4 GlcNAc-R; α 1,2 FT, α 1,2 fucosyltransferase; GnT-IV, α -D-mannoside β -1,4-N-acetylglucosaminyltransferase IV; GnT-V, α -D-mannoside β -1,6 N-acetylglucosaminyltransferase V; Gal-T, β -1,4 N-galactosyltransferase; UEA-1, *Ulex europaeus* agglutinin; the H-substance, fucosylated N-acetyl lactosamine; LDH, lactate dehydrogenase; GlcNAc, N-acetylglucosamine.

cells were then transferred to complete medium containing 1.0 mg/ml G418 (GIBCO/BRL) for selection (13). The expression of plasmids was confirmed by flow cytometry as described below.

Enzyme activity. The enzyme activities of GnT-III, α -3-D-mannoside β -1,4-N-acetylglucosaminyltransferase IV (GnT-IV), α -6-D-mannoside β -1,6-N-acetylglucosaminyltransferase V (GnT-V), and β -1,4-N-galactosyltransferase (Gal-T) in each cell line were first assayed by HPLC as described in earlier reports (14,15). These were determined from the fluorescence intensities, using a pyridylaminated bi-antennary sugar chain as a substrate.

Reagents. Ulex europaeus agglutinin (UEA-1) or 1B₄ which binds fucosylated N-acetyl lactosamine (the H-substance) or the α -galactosyl epitope, respectively were obtained from Honen Co. Ltd. (Tokyo, Japan) (16-18).

Lactate dehydrogenase (LDH) assay. This assay was performed using a Kyokuto MTX "LDH" kit according to the manufacturer's recommended protocol. The transfected cells were plated at 2×10^4 per well in a 96-well tray 1 day prior to the assay. Fifteen hours later, the plates were incubated with 20% or 40% NHS, diluted in PBS, for 2 hr at 37°C and the released LDH was measured. The spontaneous release of LDH activity from target cells was below 5% of the maximal release of LDH activity, as determined by the addition of 2% NP40 (13).

Flow cytometry. H-substance was detected by indirect immunofluorescence using a monoclonal antibody specific for the H-epitope in order to confirm the effect of α 1,2 FT. Parental SEC and transfectants were incubated with various dilutions of NHS at 4 °C for 1 h, washed and then incubated with 1.25 mg of FITC-conjugated anti-human Ig (Cappl) as a second antibody for 1 hr at 4 °C. Stained cells were analyzed with an EPICS-Profile II flow cytometer. The direct fluorescence of cell-surface carbohydrate epitopes was also examined with a FITC-conjugated 1B₄ lectin.

Western blotting. Parent and transfected cell lysates (3 μ g) were subjected to 12% SDS/PAGE under reducing conditions using the methods of Laemmli (19) and then transferred electrophoretically onto a nitrocellulose membrane (Schleicher & Schuell). The membrane was blocked in PBS containing 3% BSA and incubated for 1 hr with 10% normal human pooled serum. After washing the blots were incubated with horse radish peroxidase-avidin complex (Vector) and developed using an ECL detection system (Amersham).

Lectin blots. Parent and transfected cell products were also tested by lectin blot analysis, using 1B₄. The cell lysate (3 μ g) was subjected to 12% SDS/PAGE under reducing conditions and then transferred electrophoretically onto a nitrocellulose membrane (Schleicher & Schuell). The blots were blocked in PBS containing 3% BSA and incubated for 30 min with biotinylated 1B₄ 10 μ g/ml.

RESULTS

GnT-III activity of parental SEC and transfected SEC. An expression vector, Act-5, which carried a human GnT-III cDNA under the control of the β -actin gene promoter in the pCXN2 plasmid was prepared as described (20). Transfection was performed using lipofectamine. Four positive clones were established, and the enzyme activities of the GnT-III, GnT-IV, GnT-V, and Gal-T of these clones were measured using fluorescence-labeled sugar chains as substrates. Parental SEC showed no detectable GnT-III activity but clearly showed GnT-V and Gal-T activities. In four positive clones, GnT-III activity was clearly elevated, while in-

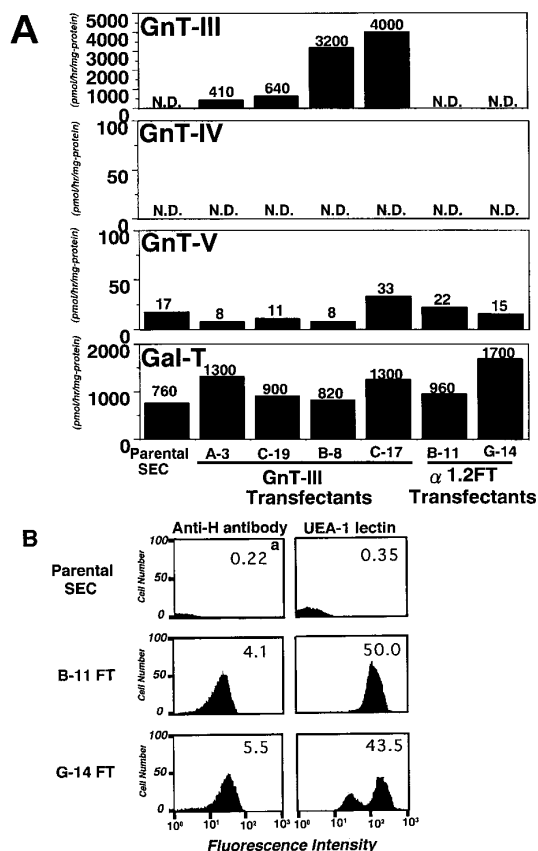


FIG. 1. Profiles of transfectants. (A) GnT-III, GnT-IV, GnT-V, and Gal-T activities of parental SEC and transfectants. These enzyme activities were measured using fluorescence-labeled sugar chains as substrates. (B) Flow cytometric profiles of α 1,2 FT transfectants. The H-substance synthesized by α 1,2 FT was measured using an anti-H monoclonal antibody and UEA-1 lectin, rather than the usual HPLC procedure. H-epitope expression is clearly increased in both clones, compared with a parental SEC line. a, the mean fluorescence value of each clone.

roduction of the GnT-III gene did not decrease the intrinsic activity of Gal-T (Fig. 1A). Among these, the relative low expression clone, A-3, and the high expression clone, C-17 were used for this study.

Fluorescence histograms of α 1,2 FT transfected SEC. The cDNA of a 1,2FT was inserted into the pCXN2 plasmid in a manner similar to that described above, and transfection was also performed in the same manner. Two positive clones were established as controls for comparison with GnT-III transfectants. Instead of measuring the α 1,2 FT enzyme activity by HPLC, the H-substance synthesized by α 1,2 FT was measured using UEA-1 lectin and anti-H monoclonal antibody by flow cytometry. The H-epitope and the UEA-1-epitope levels were increased in both clones, compared to the parental SEC (Fig. 1B).

LDH assay of GnT-III or α 1,2 FT transfected SEC. In initial experiments, the amelioration of complement-

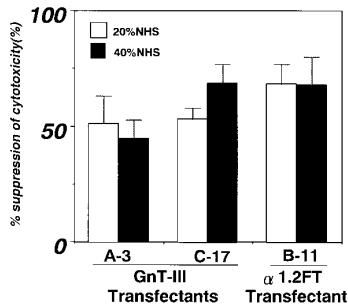


FIG. 2. LDH assay of GnT-III or α 1,2 FT transfected SEC. Amelioration of complement-mediated lysis by the transfectants and control parental SEC cells was estimated by 20 or 40% NHS, which is a source of natural antibodies and complement. The percent inhibition of complement-mediated lysis by GnT-III or the α 1,2 FT transfectants is shown. The control parental SEC lysis by 20 or 40% NHS is 23.1 ± 8.5 or $34.1 \pm 7.5\%$, respectively. Each value is expressed as the mean $\pm$ SD of four to six experiments.

mediated lysis by the SEC transfectants and the control parental SEC cells was assessed using 20% or 40% NHS which constitutes a source of natural antibody and complement. The percent inhibition of complement-mediated lysis by GnT-III or α 1,2 FT transfectants was estimated. The control parental SEC lysis by 20% or 40% NHS was found to be $23.1 \pm 8.5\%$ or $34.1 \pm 7.5\%$, respectively. These data are shown in Figure 2, with each value expressed as mean $\pm$ SD of four to six experiments.

The results of the GnT-III transfectants A-3 and C-17 suggest that GnT-III is quite effective in reducing the xenoepitope level of SEC. Approximately 70% inhibition of xenoantigenicity was observed in the high expression SEC transfectant C-17.

Fluorescence histograms of parental SEC, GnT-III transfected SEC and α 1,2 FT transfected SEC stained with NHS and 1B4 lectin. The effects of the increased GnT-III activity in transfected SEC were assessed by the susceptibility of the modified SEC to human natural antibody and 1B4 lectin. Typical flow cytometric histogram of these transfect cells are shown in Figure 3. Parental SEC (control) and stable transfectants were treated with NHS and anti-human Ig (Fig. 3A) or 1B4 lectin (Fig. 3B).

While control parental SEC reacted strongly with human natural antibodies in NHS, GnT-III or α 1,2 FT, transfected SEC showed a diminished reactivity. The percent reduction of xenoantigenicity to human antibodies was approximately 65 to 80% in the GnT-III transfectants, and 60 to 75% in the α 1,2 FT transfectants, as judged by the mean fluorescence intensity. The α -galactosyl epitope was approximately 50 to 70% downregulated both in GnT-III transfectants and α 1,2 FT transfectants. No specific differences in the downregulation of xenoantigenicity to human natural anti-

bodies or to the α -galactosyl epitope were detected between these two transfectants.

Western blot and lectin blot analysis. Western blotting was performed in order to analyze alterations in reactivity to human natural antibodies and 1B4 in GnT-III transfectants. Cell membrane glycoproteins from parental SEC and transfectants were separated by electrophoresis in a 12% polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was then incubated with 10% of NHS and stained. An evaluation of image analyzer profiles revealed that proteins of the GnT-III transfectants with molecular sizes under 66 kDa had a reduced reactivity to NHS, especially IgG, as compared to the parental SEC. Contrary to the difference in the function of GnT-III and α 1,2 FT enzymes, the difference between those two transfectants with respect to the reduction of xenoantigenicity to human natural antibodies (Fig. 4A,B) was negligible.

Lectin blotting of whole-cell lysates, obtained from

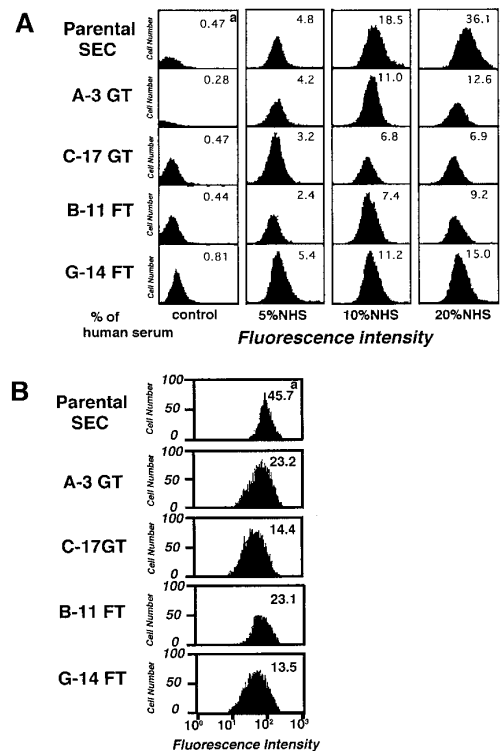


FIG. 3. Flow cytometric profiles of GnT-III transfected SEC stained with NHS and 1B4 lectin. Typical flow cytometric histograms for GnT-III or α 1,2 FT transfectants are shown. Parental SEC (control) and stable transfectants were treated with 5, 10, or 20% NHS as the first antibody and FITC-conjugated anti-human Ig as the second antibody (A) or FITC-conjugated 1B4 lectin (B). While control parental SEC reacted strongly with human natural antibodies in NHS, the xenoantigenicity to human natural antibody was reduced and the α -galactosyl epitope was also downregulated in both transfectants. a, the mean fluorescence value of each clone.

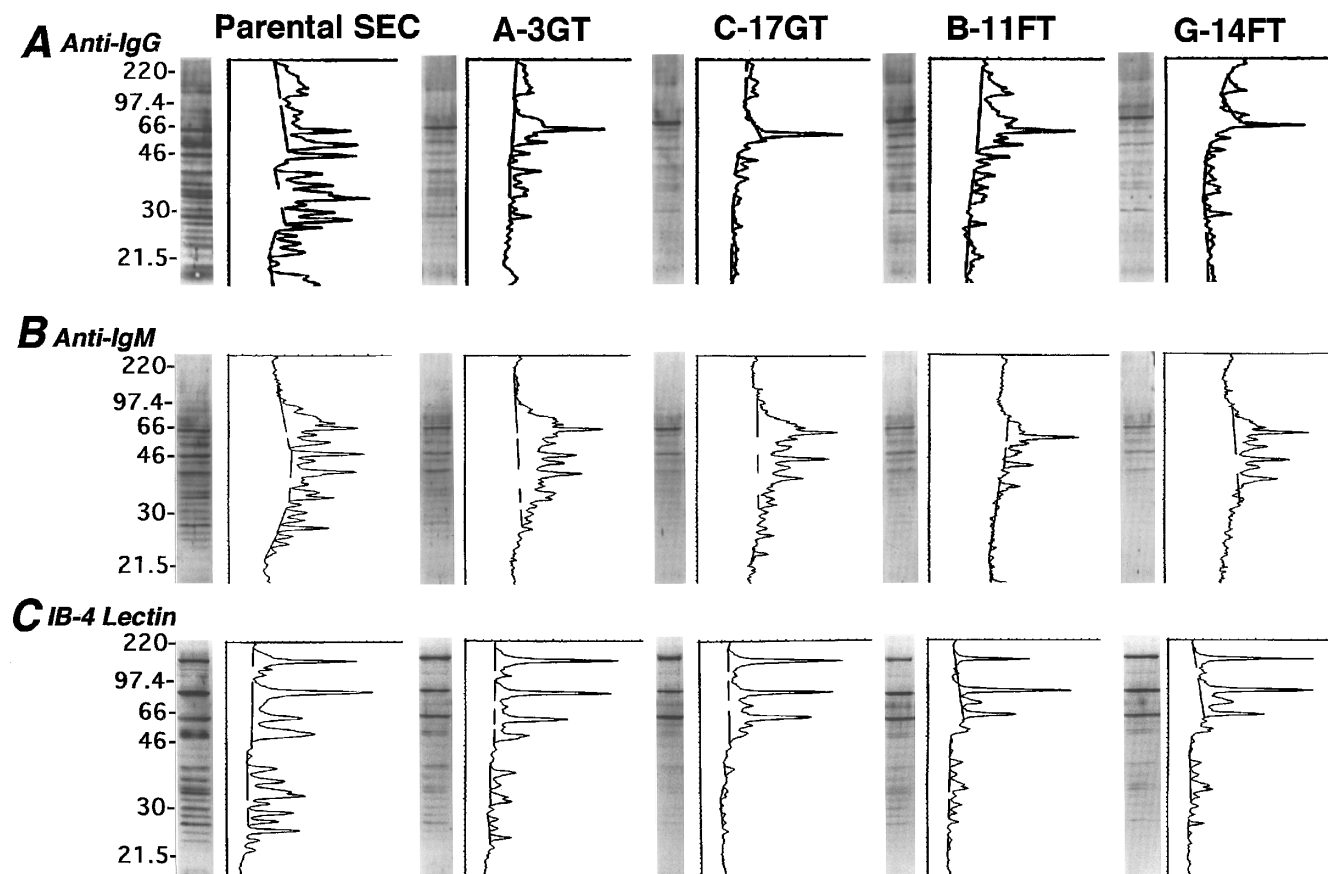


FIG. 4. Western blot and lectin blot analysis. Parental SEC and transfectant cell membrane glycoproteins (3 μ g) were separated by electrophoresis in a 12% polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was incubated for 1 hr with 10% of NHS and then stained. After washing, the blots were incubated with horse radish peroxidase-avidin complex and developed using an ECL detection system. Image analyzer profiles were used to evaluate a typical Western blot pattern of IgG (A) and IgM (B). The position markers are indicated at the bottom. Lectin blot analysis of whole-cell lysates from the parental SEC and transfectants was also performed. Cell lysates from SEC, GnT-III, and α 1,2 FT transfectants were separated on a SDS/PAGE and transferred onto a nitrocellulose membrane. The blots were probed with biotinylated 1B4 lectin (10 μ g/ml). The position markers are indicated (C).

the parental SEC cell and transfectants was also performed. Cell lysates from these cells were separated on a 12% SDS/PAGE and transferred onto a nitrocellulose membrane. The blots were probed with 1B4 lectin. Similar to the western blotting, observations proteins, especially those with molecular sizes under 66 kDa of both transfected cells reduced the reactivity to 1B4, as compared to the parental SEC (Fig. 4C).

DISCUSSION

In the present study, we examined the amelioration of antigenicity of N-linked oligosaccharides by the over-expression of GnT-III, with the goal of providing a permanent solution for overcoming hyperacute rejection in clinical xenotransplantation.

In general, the regulation of sugar chain biosynthesis is under the control of glycosyltransferases, the sub-

strate specificities of the enzymes, and the localization of the enzymes in tissues and organelles. GnT-III catalyzes the branching of N-linked oligosaccharides to produce a bisecting N-acetylglucosamine (GlcNAc) residue. The attachment of a bisecting GlcNAc inhibits the further processing of oligosaccharides by other glycosyltransferases. That is, once a bisecting GlcNAc residue is added to the core mannose by GnT-III, a competitive enzyme; GnT-IV and GnT-V are not able to form any further tri-structure in the Golgi stack (20-22).

In this study, the introduction of the GnT-III gene did not decrease the intrinsic activity of Gal-T, but decreased the level of xenoantigen such as, for example, the α -galactosyl epitope. Clearly, further studies will be required, in order to understand and fully explain this effect. However, not only a reduction in the number of branches formed, including β 1,6 branches (22) occurs, but it is also thought that the entire N-linked oligosac-

charides are remodeled, as a result of the overexpression of GnT-III (23,24).

On the other hand, this bisecting GlcNAc structure has been found in glycoproteins from a variety of tissues. It is well known that GnT-III is abundant in brain and kidney tissues (25-26). Therefore, the products produced as a result of the overexpression of GnT-III cannot constitute a new epitope to human natural antibodies.

In the present study, the GnT-III activity of each clone was accurately measured by HPLC. GnT-III transfectants, C-17 and B-8, may play a major role in the remodeling of the antigenicity of SEC to human natural antibodies. Using these high expression transfectants, this new enzymatic carbohydrate remodelling approach was investigated. As expected, the downregulation of xenoantigenicity was quite obvious in these GnT-III transfectants, as evidenced by the LDH assay. In addition, no specific differences were detected between the GnT-III transfectants and the α 1,2 FT transfectants that showed a high level of enzymatic activity (4, 6) in flow cytometric analysis.

Therefore, a Western blot of the GnT-III transfectants was examined, in order to investigate this more fully. The difference in the reactivity of IgM and IgG antibodies to SEC is shown in Fig 4. GnT-III is more effective in reducing the xenoepitope anti-human IgG than is IgM. Interestingly, the Western blotting pattern for IgG resembles that for 1B4. This may relate to the evidence reported by Galili et al. (1, 2), who reported that the anti-Gal antibody constitutes approximately 1% of circulating IgG in humans. With respect to each blotting pattern of the GnT-III transfectants, N-linked sugars exist on proteins of all sizes. Therefore, all blotting bands in each blotting were diminished. Furthermore, the proteins with molecular mass of under 66 kDa might easily lose N-linked sugars as a result of GnT-III.

We speculate that the Western blotting and lectin blotting patterns for the GnT-III transfectants would be different from those of the α 1,2 FT transfectants. While GnT-III reduced the levels of, not only the α -galactosyl but also other epitopes in N-linked sugars, the α 1,2 FT reduced the α -galactosyl epitope, not only in N-linked sugars, but also in O-linked sugars and glycolipids. However, in this study, little difference was detected between these two transfectants either with respect to the reduction pattern of xenoantigenicity to human natural antibodies (Fig. 4A) or in the 1B4 lectin blot (Fig. 4B). That is, entire proteins, especially those having molecular masses less than 66kDa had reduced reactivity to both human natural antibodies and 1B4 lectin. It is possible that the Western blot and lectin blot of these quite different transfectants showed a similar pattern is purely accidental. Another possibility is that these two overexpressed genes might act mainly

against the same xeno-epitope on N-linked sugars. Further experiments will be required to examine these possibilities. Moreover, the possibility of synergism between these two different glycosyltransferases, cannot be excluded.

In conclusion, GnT-III was overexpressed to reduce the antigenicity of swine xenoantigen to humans and the effect was assessed using GnT-III transfected SEC cells. This is the first report which demonstrates that the introduction of a glycosyltransferase besides those competing with the α 1,3 galactosyltransferase results in significant suppression of xenoantigen.

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