Molecular Cloning of Endo-β-galactosidase C and Its Application in Removing α-Galactosyl Xenoantigen from Blood Vessels in the Pig Kidney

Galα1-3Gal is the major xenoantigenic epitope responsible for hyperacute rejection upon pig to human xenotransplantation. Endo-β-galactosidase C from Clostridium perfringens destroys the antigenic epitope by cleaving the β-galactosidic linkage in the Galα1-3Galβ1-4GlcNAc structure. Based on partial peptide sequences of the enzyme, we molecularly cloned the enzyme gene, which encodes a protein with a predicted molecular mass of about 93 kDa. The deduced protein sequence of the enzyme has limited homology in the C-terminal half with endo-β-galactosidase from Flavobacterium keratolyticus and β-1,3-glucanases. The enzyme expressed in Escherichia coli removed the α-galactosyl epitope nearly completely from pig erythrocytes and from pig aortic endothelial cells. The enzyme-treated endothelial cells in culture were greatly reduced in cell surface antigens, which were recognized by IgM, IgG, or IgA in human sera, and became much less susceptible to complement-mediated cytotoxicity caused by human sera. When the pig kidney was perfused with the enzyme, the vascular endothelial cells became virtually devoid of the α-galactosyl epitope, with concomitant decrease in binding to IgM in human plasma. These results demonstrated that the recombinant endo-β-galactosidase C is a valuable aid in xenotransplantation.

Xenotransplantation, especially from pig to human, has been considered as means to overcome the limitations in the number of donor organs available for transplantation surgery (1). However, the occurrence of hyperacute rejection whereby vascular endothelial cells are destroyed by an antibody- and complement-dependent mechanism, remains an obstacle to xenotransplantation. As reported here, we cloned the gene for this enzyme, produced the recombinant enzyme, and applied it to digest out the α-galactosyl antigen from pig blood vessels in the kidney.

EXPERIMENTAL PROCEDURES

Partial Peptide Sequence of Endo-β-galactosidase C—Endo-β-galactosidase C was purified from culture fluid of C. perfringens as described previously (18). The enzyme preparation obtained from 18 liters of culture medium was subjected to SDS-polyacrylamide gel electrophoresis using an 8% gel according to Laemmli (19). After light staining with Coomassie Brilliant Blue, bands were excised, and proteins in the gel were digested with trypsin as described by Rosenfeld et al. (20). The resulting peptides were separated on a reverse phase column (Monitor C 18, 1 × 150 mm) with a linear gradient of acetonitrile and 0.1% trifluoroacetic acid at a flow rate of 50 μl/min using a MAGIC 2002 (Michrom Bio Resources, Inc.). Amino acid sequences of the resulting peptides were determined with a PE Applied Biosystems protein sequencer model 494A.

Several approaches have been proposed to eliminate the Galα1-3Gal antigenic epitope from pigs or mice (2). Removal of the α-1,3-galactosyltransferase gene by gene targeting is a straightforward method but has so far been successful only in mice (7, 8). Introduction and overexpression of genes for glycosyltransferases (i.e. α-1,2-fucosyltransferase (9–12), N-acetylglucosaminyltransferase III (13), and α-2,3-sialytransferase (14)) results in decreased expression of Galα1-3Gal antigen by competition over the acceptor structure and other mechanisms. Furthermore, transgenic pigs with α-1,2-fucosyltransferase have been produced and have been reported to be reduced in the α-galactosyl antigen expression (9, 10, 12). Expression of the dominant negative form of α-1,3-galactosyltransferase has also been tried (2). However, by none of the methods has production of pig organs virtually devoid of the α-galactosyl antigen been achieved so far (2). As an alternative approach, α-galactosidase from the coffee bean was used to remove the α-galactosyl antigen from cultured pig endothelial cells. The enzyme-treated cells became resistant to complement-dependent lysis by human sera (15, 16). Although very recently the α-galactosidase has been applied to remove the α-galactosyl antigen from isolated femoral veins from the pig (17), this enzyme has not been applied to treat pig organs, probably because the acidic optimum pH of the enzyme makes it necessary to use enormous amounts of the enzyme.

Endo-β-galactosidase C, an endo-β-galactosidase found in the culture fluid of Clostridium perfringens, cleaves the Galβ1-4GlcNAc linkage in the Galα1-3Galβ1-4GlcNAc structure, releasing Galα1-3Gal disaccharide (18). Due to its neutral optimum pH (18), the enzyme is expected to be valuable as an aid for xenotransplantation. As reported here, we cloned the gene for this enzyme, produced the recombinant enzyme, and applied it to digest out the α-galactosyl antigen from pig blood vessels in the kidney.
Cloning of the Endo-β-galactosidase C Gene—PCR was performed using the sense primer 5'-GAGYARAAAGTAYTG-3' (based on the amino acid sequence of peptide 1) and antisense primer 5'-TCYTCTT-NARITCUNCrCCTC-3' (based on the amino acid sequence of peptide IV) and genomic DNA of the bacteria as the template, after denaturation at 94 °C for 1 min, 45 cycles of 94 °C for 30 s, 55 °C for 2 min, and 72 °C for 2 min. The PCR product, which corresponded to a partial genomic sequence of the enzyme gene, was subcloned into the pGEM-T Easy Vector (Promega). The 5'- and 3'-ends of the DNA were extended by HindIII (9) cassette, using 5'-TGATACCGTGGTAGTA-GAATGCCCACCT-3', 5'-ATITATCTATTGATGATGTTTACG-3', 5'-AGATGATGATTAGCCTACGGTATTAAGCC-3', 5'-AGATGATGATTAGCCTACGGTATTAAGCC-3', and 5'-AGATGATGATTAGCCTACGGTATTAAGCC-3', as primers. Cassettes PCR was performed with 30 cycles of 94 °C for 30 s, 55 °C for 2 min, and 72 °C for 1 min. Full-length DNA was obtained by PCR using two primers, 5'-GACCGCGAATTCTAAGATATTCTCAGATTAAAGATTTAGTA-GTA-3' and 5'-GGGTGCCTGAGTTTTATATTTATTTTGTTAAGTTA-CTTATAGC-3', which were designed according to the information of the 5'- and 3'-DNA sequences obtained by cassette PCR with genomic DNA as a template. PCR was performed after denaturation at 94 °C for 1 min and 30 cycles of 94 °C for 30 s, 55 °C for 2 min, and 72 °C for 1 min.

**DNA Sequence Analysis**—DNA sequencing was performed by the dyeoxy chain termination method (21), using an ABI Prism 377 Sequencer. The sequence of the 5'-side was confirmed by 5'-rapid amplification of cDNA ends using terminal transferase (Roche Molecular Biochemicals); NotI-dT18 primer (Amersham Pharmacia Biotech); primers 5'-TGACTTGGTAGTCTACCT-3', 5'-TGACTTTGCTTGTTAAGGTAT- CATAGATTGCGCCACT-3', and 5'-GTTGTTATTACTATAGTGATAG- TATTAAGCC-3'; and total RNA from C. perfringens as a template.

**Assay of Endo-β-galactosidase C**—The enzymatic reaction was performed in 10 μl of 50 mm phosphate buffer, pH 7.2, containing 20 μg of Galal1–3Galβ1–4GlcNAc2–3Galβ1–4Glc (Galbiochem). After 30 min at 37 °C, the reaction was stopped by adding 20 μl of ethanol, and after desalting with Dowex 50W × 2 H+ form and 1 × 2 acetate form, one-tenth of the hydrolysate was spotted to a Silica Gel 60 TLC plate (Merck). The plate was developed in n-propyl alcohol/ethyl acetate/H2O (7:2:1), and sugar spots were revealed by spraying with orcinol-H2SO4 reagent heating (92). The amounts of released products were determined using a densitometer, Gel Doc 1000 (Bio-Rad). Under these assay conditions, enzymatic activity was proportional to the amount of the enzyme required to hydrolyze 1 μmol of the substrate per min.

**Mass Spectrometry**—Mass spectrometry was performed using a triple quadrupole mass spectrometer (API 300, Perkin-Elmer) equipped with an electrospray ion source. The sample was directly infused into a quadrupole mass spectrometer, API 300 (Perkin-Elmer) equipped with an electrospray ion source. The sample was directly infused into the instrument at 3.5 μl/min. The instrument was operated in positive ion mode, using a capillary temperature of 200 °C, a corona needle voltage of 3500 V, and a nebulizing gas pressure of 40 psig. The compounds were separated using a Xterra 3.5 μm C18, 100 × 2.1 mm column. The Xterra column was equilibrated in 99.5% water and 0.5% formic acid to maintain the performance of the column. The compounds were eluted with a linear gradient of 0 to 100% CH3CN-water containing 0.1% formic acid over 30 min. The flow rate was 0.2 ml/min. The mass spectrometer was operated in positive ion mode, using a capillary temperature of 200 °C, a corona needle voltage of 3500 V, and a nebulizing gas pressure of 40 psig. The compounds were separated using a Xterra 3.5 μm C18, 100 × 2.1 mm column. The Xterra column was equilibrated in 99.5% water and 0.5% formic acid to maintain the performance of the column. The compounds were eluted with a linear gradient of 0 to 100% CH3CN-water containing 0.1% formic acid over 30 min. The flow rate was 0.2 ml/min.

**Other Biochemical Analyses**—The amount of protein in the purified enzyme was determined using a Micro BCA protein assay reagent kit (Fierce), with bovine serum albumin as a standard. Possible contamination of other glycosidases was determined by incubating respective p-nitrophenyl glycosides in 0.2 ml of reaction mixture with 29.4 μl of 25 or 50% normal human sera diluted in 400 μl of PBS/BSA, and then incubated with 3 mg/ml dimethyl sulfoxide dihydrochloride in 0.1 M Na2CO3 containing 0.15 M NaCl and 0.1 M EDTA at 37 °C for 20 min to prevent agglutination. Erythrocytes were washed with PBS/BSA and suspended in PBS/BSA to make a 1% suspension. Aliquots of 50 μl of the erythrocyte suspension thus prepared were centrifuged at 1000 × g for 10 min, and the precipitated erythrocytes were suspended in 50 μl of PBS/BSA to which 20 million units of recombinant endo-β-galactosidase C in 4 μl of PBS was added, and the reaction was continued at 37 °C for 30 min.

**Production of Recombinant Endo-β-galactosidase C**—The coding sequence of the enzyme was ligated into the expression vector pFLAG-Shift1D (Sigma), which produces a gene product that can be secreted into the periplasm. The recombinant gene was transformed into Escherichia coli BL-21 cells, which were cultured at 37 °C in 2 liters of LB medium containing 50 μg/ml of ampicillin and 0.4% of glucose and were induced to express the enzyme by adding 0.1 ml isopropyl β-D-thiogalactopyranoside to the culture medium. After 30 min, the bacterial cells were collected by centrifugation at 5000 × g for 10 min and washed with 300 ml of Tris-HCl, pH 8.0, twice, and then 300 ml of 0.5 M sucrose, 30 ml Tris-HCl, pH 8.0, and 1 ml EDTA at room temperature. The cells collected by centrifugation were immediately suspended in 250 ml of ice-cold distilled water to release the enzyme from the periplasm. The supernatant containing the released enzyme was collected by centrifugation at 3500 × g for 10 min at 4 °C and purified by chromatography on columns of Sephadex G-200 (2.7 × 100 cm) and DEAE-Sephadex A-25 (1.0 × 10 cm) as described previously (18). The purified fraction contained about 700 μg of protein. When large amounts of the enzyme were required, this purification procedure was repeated.

**Removal of α-Galactosyl Antibodies from Living Cells**—Pig erythrocytes were collected by centrifugation (1000 × g) from citric acid-treated blood and washed twice with PBS(−) containing 0.2% bovine serum albumin (PBS/BSA). Then they were incubated with 3 mg/ml dimethyl sulfoxide dihydrochloride in 0.1 M Na2CO3 containing 0.15 M NaCl and 0.1 M EDTA at 37 °C for 20 min to prevent agglutination. Erythrocytes were washed with PBS/BSA twice and suspended in PBS/BSA to make a 1% suspension. Aliquots of 50 μl of the erythrocyte suspension thus prepared were centrifuged at 1000 × g, and the precipitated erythrocytes were suspended in 50 μl of PBS/BSA to which 20 million units of recombinant endo-β-galactosidase C in 4 μl of PBS was added, and the reaction was continued at 37 °C for 30 min.

**Pig aortic endothelial cells** were isolated from a pig as described previously (16). The cells were harvested with trypsin-EDTA (Life Technologies, Inc.) and washed twice with PBS/BSA and resuspended in PBS/BSA. Recombinant endo-β-galactosidase C (70 million units in 7 μl PBS/BSA) was added to the aortic endothelial cells (2 × 105 cells/50 μl) and the reaction was continued at 37 °C for 1 h.

**Assay of Complement-mediated Cytotoxicity**—Pig aortic endothelial cells were plated at 1 × 104 cells/well in a 96-well culture plate 1 day prior to the assay. After washing with PBS(−), the cells were incubated with 50 μl of Dulbecco’s modified Eagle medium containing 70 million units of recombinant endo-β-galactosidase C at 37 °C for 2 h. The control cells were incubated without the enzyme. The cells were washed twice and incubated with 50 μl of 25 or 50% normal human sera diluted with PBS(−) at 37 °C for 1 h. The human sera were pooled from 10 healthy volunteers. After incubation, the viability of the cells was determined by an assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Sigma) as described by Nagasaka et al. (24). The absorbance of each well was measured by an automatic plate reader (EAS340, SLT-Labinstruments, Austria) at 540 nm, and the
values were referred to as "cell viability in the presence of complement." In each assay, "cell viability in the absence of complement" was determined using the sera treated at 56 °C for 30 min to inactivate complement. Complement-mediated cytotoxicity was calculated from the following formula: percentage cytotoxicity = ((cell viability in the absence of complement - cell viability in the presence of complement) / cell viability in the absence of complement) × 100. Experiments were performed in triplicates.

**Fig. 2.** DNA sequence of endo-β-galactosidase C and its deduced protein sequence. Peptide sequences found in the tryptic digestion of the enzyme are underlined. DNA sequences extended by cassette PCR are nucleotides numbered 1–347 and 2298–3013.

**Fig. 3.** Comparison of protein sequences of endo-β-galactosidase C (Endo-GalC), endo-β-galactosidase from *F. keratolyticus* (Endo-Gal) (see Ref. 28; accession number AF083896) and β-1,3-glucanase from *Bacillus circulans* (Endo-Glc) (see Ref. 30; accession number M34503). Amino acids shared with endo-β-galactosidase C and one of the other enzymes are marked with asterisks, and amino acids conserved between all three enzymes are boxed.
fusión con o sin endo-$\beta$-galactosidasa C. Entonces los riñones se perfundieron dos veces con 100 ml de plasma humano fresco obtenido de tres voluntarios sanos. Para estudios histológicos, las muestras biopsiadas se fijaron con formalina y se hicieron con hematoxilina-eosina y periodic acid-Schiff. Las muestras fueron también inmediatamente enjuagadas en líquido de nitrógeno y se enjuagaron con FITC-BSA. El plasma humano was coleccionado, y los niveles de inmunoglobulinas restantes fueron analizados por FACS usando pig erythrocytes como descrito anteriormente.

RESULTADOS

Clonación y análisis estructural de endo-$\beta$-galactosidasa C— SDS-polyacrylamide gel electrophoresis de un endo-$\beta$-galactosidasa C preparación reveló varios bandas de proteína, entre los cuales un 50-kDa mayor banda y un 95-kDa banda (Fig. 1A) co-migrado con la actividad enzimática sobre DEAE-Sephadex A-25 column chromatography. Secuencia de análisis de péptidos tricéticos indicó que el 50-kDa banda era C. perfringens O proteín (26), y el 95-kDa banda era una proteína novedosa.

Basado en las secuencias de cuatro péptidos tricéticos derivados de la 95-kDa banda (Fig. 2), PCR primers fueron diseñados, y PCR usando genómica DNA de C. perfringens como un template produjo una DNA de 1946 base pares. Cassette PCR fue performed to obtain los 5' y 3' secuencias. El composite DNA encendió un open reading frame de 2535 bp correspondiendo a un protein de 845 amino acids (Fig. 2). Upstream desde la putativo translacional inicación site, there was a typical Shine-Dalgarno sequence, AGGAGG (nucleotides 231–236) (27). The deduced protein sequence contained all of the peptide sequence identificado en la 95-kDa protein (Fig. 2). In all but one caso amino acidos next to the N terminus of the identificado péptidos fueron amino acidos o lysine, which was consistent with the fact that the péptidos were derivados por trypsin digestión. In one case (peptide I, Fig. 2), el proximal amino acid was alanine. We confirmed the correctness of the sequencias by 5'-rapid amplification of cDNA ends. Therefore, we inferred that peptide I of the 95-kDa protein was derived from the N-terminal portion of the 95-kDa protein, which was expected to be generated by proteolitic processing of the nascent protein. This was confirmed by N-terminal sequence analysis; the purified recombinant enzyme described en la siguiente sección había el N-terminal sequence DENEYVL, which is the N-terminal sequence of peptide I. Furthermore, upstream the peptide amino acids (amino acids 1–34, Fig. 2) had a cluster of hidrofobic amino acids typically found in a signal sequence (28). This putativo signal sequence might be involved in secretion of the enzyme. Endo-$\beta$-galactosidasa from Flavobacterium keratolyticus is known to have a signal sequence (29). The molecular mass of the protein devoid of the putativo signal sequence was 93 kDa and was in good agreement with the observed molecular mass of the protein, 95 kDa.

A distinct region in the C-terminal half of the enzyme showed protein sequence homology to endo-$\beta$-galactosidase purified from F. keratolyticus (29) and $\beta$-1,3-glucanases purified from various sources (30, 31) (Fig. 3).

Expression of Recombinant Endo-$\beta$-galactosidase C—The enzime was expresado en E. coli como una periplasmic enzyme under el control del ompA gene. The recombinant enzyme was purified by Sephadex G-200 and DEAE-Sephadex A-25 column chromatography. A major band with apparent molecular mass of 95 kDa y a closely related minor band de 97 kDa were found in el purified preparation (Fig. 1B). As mentioned above, el major band corresponded to the protein devoid of the putativo signal sequence. From the molecular mass difference, the minor band appeared to correspond a nascent protein with a signal sequence.

The recombinant enzyme hydrolyzed a pentasaccharide, Ga1α–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc (20 $\mu$g) was hydrolysed with 4 milliliters of the enzyme at 37 °C for 30 min in 10 $\mu$l de 30 m NaCl. After stopping the reaction with 20 $\mu$l de ethanol, el reaction mixture was mixed with 1.5 volumes de 83% acetonitrile containing 7 m ammonium acetate. Mass spectrometry fue performed as described under “Experimental Procedures.” A, before enzymatic digestion. Ethanol was added before mixing the enzyme and the substrate. B, after enzymatic digestion.

![Fig. 4. TLC of the products of endo-$\beta$-galactosidase C digestion.](image)

![Fig. 5. Identification of the products of endo-$\beta$-galactosidase C digestion by electrospray mass spectrometry.](image)
former corresponded to the mass of GlcNAcβ1–3Galβ1–4Glc plus Na⁺, and the latter to that of Galα1–3Gal plus Na⁺. These observations established that the cloned enzyme was endo-β-

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FIG. 6. Decreased binding of GS-IB4 and human immunoglobulins to pig cells treated with recombinant endo-β-galactosidase C. A and C, erythrocytes. B and D, aortic endothelial cells. Enzymatic treatment and FACS analysis of binding to FITC-GS-IB4 or to immunoglobulins in human sera were performed as described under “Experimental Procedures.” Thick lines, after enzymatic treatment; thin broken lines, before enzymatic treatment.

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FIG. 7. Effects of endo-β-galactosidase C treatment on susceptibility to complement-mediated cytotoxicity by human sera. Pig aortic endothelial cells with or without endo-β-galactosidase C treatment were incubated with 25 or 50% human sera in the presence or absence of complement. Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and complement-mediated cytotoxicity was calculated as described under “Experimental Procedures.” Gray bars, without enzymatic treatment; black bars, with enzymatic treatment. Average values from triplicate assays are shown with S.E. values.

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FIG. 8. Removal of α-galactosyl epitope from blood vessels in the kidney by endo-β-galactosidase C digestion. Tissue sections were stained by FITC-GS-IB4. Time for exposure to take photographs was set to be identical. Photographs show the area of the juxtamedullary cortex, in which GS-IB4-positive sites are mostly peritubular capillary. A, without treatment (incubation with University of Wisconsin solution). B, with treatment (incubation with University of Wisconsin solution containing endo-β-galactosidase C). Bar, 20 μm.

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Endo-β-galactosidase C for Xenoantigen Removal
perfringens (18). Furthermore, at 4 °C the enzyme exhibited 40% of the activity observed at 37 °C.

**Action of Recombinant Endo-β-galactosidase C on Pig Erythrocytes and Pig Endothelial Cells**—After treatment with recombinant endo-β-galactosidase C, more than 99% of the α-galactosyl epitope, which was recognized by FITC-GS-IB4, was removed from pig erythrocytes (Fig. 6A). Similarly, 98% of the α-galactosyl epitope could be enzymatically removed from pig aortic endothelial cells (Fig. 6B). Binding of IgM to the treated erythrocytes was reduced to 25% of that in untreated cells (Fig. 6A); in endothelial cells, the reduction was to less than 23% (Fig. 6B). Binding of IgA and IgG in human sera was also reduced to 21 and 33%, respectively, in the case of enzymatically treated endothelial cells (Fig. 6B), while less reduction was observed in treated erythrocytes for IgG (Fig. 6A).

Furthermore, using antibodies against human IgG subclasses, we found that binding to IgG1, IgG2, or IgG3 was markedly reduced in the treated cells (Fig. 6C and D), while no changes in binding to IgG2 were detected. The cells treated with endo-β-galactosidase C became much less susceptible to complement-mediated cytoxicity caused by human sera (Fig. 7).

**Enzymatic Removal of the Xenoantigen from the Luminal Surface of Vascular Endothelial Cells in the Pig Kidney**—We then examined the effects of the enzymatic digestion of the α-galactosyl epitope on intact blood vessels of the pig kidney. The pig kidney was perfused with the enzyme solution, and the reaction was performed at 4 °C, since lower temperature is preferable for preservation of the tissue, and endo-β-galactosidase C effectively worked even at low temperature. Staining with FITC-GS-IB4, verified that the α-galactosyl epitope facing the lumen of the blood vessels was virtually removed by the enzymatic treatment at 4 °C for 4 h (Fig. 8). No histological change was observed in the enzymatically treated kidneys.

With this powerful method of removing α-galactosyl epitopes in situ, we investigated whether binding of immunoglobulins in human plasma was decreased in enzymatically treated blood vessels as in the case of aortic endothelial cells or erythrocytes. Although quantitative analysis is possible for dissociated cells using FACS, direct measurement of bound immunoglobulins is not easy in intact blood vessels. Therefore, we measured the decrease in binding activity of immunoglobulins to pig erythrocytes after perfusion into the kidney. To avoid the effects of variation between pigs, we used two kidneys from the same animal; one kidney was treated with endo-β-galactosidase C, and the other was treated with the medium without the enzyme. In three independent experiments, enzymatically treated kidneys bound significantly lower amounts of IgM than the kidney treated only with the medium. On the average, 31% of binding activity of IgM remained in human plasma after perfusion into the pig kidney without the enzyme treatment (Fig. 9). However, 64% of the binding remained after perfusion into the kidney with the enzyme treatment (Fig. 9). This difference was statistically significant. The result demonstrated that large amounts of IgM-binding antigenic epitope were eliminated after endo-β-galactosidase C digestion. However, the ability to absorb IgG and IgA antibodies was less reduced by enzymatic treatment, and the differences were not statistically significant (Fig. 9).

**DISCUSSION**

Although the principal aim of the present investigation was to reduce α-galactosyl xenogenic determinants in blood vessels in situ using an enzyme, the results presented here raised other interesting points. First, the protein sequence of endo-β-galactosidase C shares some amino acids in the more C-terminally located region with endo-β-galactosidase from F. keratolyticus (29) and β-1,3-glucanases (30, 31). Although the

![Fig. 9. Decreased absorption of immunoglobulins in human plasma to blood vessels of pig kidney after treatment with endo-β-galactosidase C. The right kidney from a pig was treated with endo-β-galactosidase C, while the left kidney was treated only with the medium as described under “Experimental Procedures.” Then, 100 ml of human plasma was perfused into the kidneys under gravity. The perfused plasma samples were reacted with pig erythrocytes, followed by reaction with FITC-conjugated antibodies to human immunoglobulins. Stained erythrocytes were analyzed by FACS. The results are expressed as percentages of the mean fluorescence intensity relative to that obtained using the plasma before perfusion. White bars, before perfusion; gray bars, after perfusion with the medium; black bars, after perfusion with the enzyme. Average values from three independent experiments are shown with S.E. values. Results were statistically analyzed by Student’s t test (*, p < 0.05).](http://www.jbc.org/)

**Flavobacterium endo-β-galactosidase and β-1,3-glucanases act on polysaccharides to release oligosaccharides of various sizes, endo-β-galactosidase C acts on carbohydrate moieties of glycoproteins and glycolipids and releases only a disaccharide (18). Nevertheless, the similarity in structure of these enzymes suggested that there are similarities in the mechanisms of action between endo-β-galactosidase C and the other endoglycosidases mentioned above.**

Second, endo-β-galactosidase C, at the partially purified stage, has been proved useful for the structural analysis of glycoconjugates such as poly-N-acetyllactosamine in Ehrlich carcinoma cells (32) and teratocarcinoma glycopeptides (33). The newly available recombinant enzyme is of a high order of purity and lacks proteases and is expected to be broadly applicable in studies of various glycoconjugates because of the widespread occurrence of the Galβ1→4GlcNAc structure.

The most interesting finding of this study was that after perfusion with recombinant endo-β-galactosidase C, the pig vascular endothelial cells in the kidney became virtually devoid of the α-galactosyl antigen with concomitant loss of a significant portion of the IgM-binding xenoantigen, which is known to be primarily responsible for hyperacute rejection in pig to primate transplantation (34). The recombinant enzyme could act at physiological pH and even at a low temperature. Thus, the enzyme is expected to be a valuable aid in xenotransplantation. It will be especially helpful in removing residual α-galactosyl antigen in transgenic pigs, which express transgenes of appropriate glycosyltransferases and have reduced α-galactosyl antigen levels. The reappearance of the antigenic activity after the endoglycosidase digestion may be prevented if the enzyme is present in the bloodstream. Production of transgenic pigs with the endo-β-galactosidase C gene should also be considered. Transfection of the enzyme gene mediated by liposome or virus vector might be also helpful. In these gene manipulation experiments, endo-β-galactosidase C is more suitable than α-galactosidase, because levels of protein expression required will be less in case of the endoglycosidase than α-galactosidase. For nearly complete removal of the α-galactosyl antigen from isolated pig blood vessels, 20 units/ml (2 mg/ml) of α-galacto-
sidase is required (17), while 0.45 units/ml (0.043 mg/ml) of endo-\(\beta\)-galactosidase C was sufficient for the removal from blood vessels in the kidney. Finally, it might be appropriate to remind that xenografts initially protected from hyperacute rejection for a certain period might become resistant to rejection even in the presence of xenoantibodies, by a mechanism called accommodation (35).

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