Human natural "anti-Gal" antibodies are specifically directed to Galα1-3Galβ1-4GlcNAc residues expressed on non-primate mammal and new world monkey cells. We investigated the relative involvement of purified IgG and IgM anti-Gal as xenoreactive natural antibodies (XNA). IgG and IgM were isolated from human plasma, and anti-Gal antibodies were purified by affinity chromatography on a Synsorb-14 column (Chembliomed, Edmonton, Alberta, Canada). Anti-Gal of both IgM and IgG classes represent the bulk of human XNA that bind to porcine platelets in enzyme-linked immunosorbent assay (ELISA). On immunoblots, normal human sera, as well as purified IgM and IgG fractions, reacted with 115-, 125-, 135-, 150-, 180-, 210-, and 240-kD pig platelet glycoproteins, whereas purified anti-Gal antibodies of both IgM and IgG classes mainly bound to 135-, 150-, 180-, and 210-kD glycoproteins. A low reactivity was observed in ELISA with anti-Gal free IgM and IgG, indicating that xenobodies are not solely directed to galactosyl epitopes. These antibodies revealed bands of 115, 125, and 240 kD. α-Galactosidase treatment of porcine platelet glycoproteins (gps) enriched by affinity chromatography abrogated the reactivity of 135- and 210-kD proteins. N- and O-glycosidase treatments demonstrated that α-galactosyl residues are located on the O-glycans of the 135-kD component. Finally, glycoproteins of 90 and 135 kD were identified by amino acid sequencing as the pig analogs of the human glycoproteins Ila and IIb, respectively, whereas the 240-kD component was identified as the porcine fibrinogen, using a new murine monoclonal antibody (NaM147-7B6; IgG1) specific for its β-chain.

**Characterization of Porcine Platelet Glycoproteins Recognized by Human Natural "Anti-Gal" Antibodies**

By Karen Thibaudeau, Luis Borche, Jean-Paul Souliillou, and Dominique Blanchard

XENOREACTIVE natural antibodies (XNA) constitute the main obstacle to the use of pig-to-human tissue xenografts. Complement-activating IgM xenobodies represent the major factor implicated in hyperacute rejection, which occurs within minutes after vascularized graft reperfusion. Several studies, including those from our group, have shown that human natural antibodies are mostly directed against Galα1-3Gal epitopes, termed α-galactosyl epitopes herein, carried by both glycoproteins (gps) and glycolipids. These so-called "anti-Gal" antibodies are present in human sera, as well as in sera of apes and old world monkeys. They have been estimated to contribute approximately 1% of circulating IgG in humans. They are probably produced throughout life as a polyclonal response to antigenic stimulation by gastrointestinal bacteria. Their major targets on porcine endothelial cells and platelets are gps of both IgM and IgG classes mainly bound to 135-, 150-, 180-, and 210-kD glycoproteins. A low reactivity was observed in ELISA with anti-Gal free IgM and IgG, indicating that xenobodies are not solely directed to galactosyl epitopes. These antibodies revealed bands of 115, 125, and 240 kD. α-Galactosidase treatment of porcine platelet glycoproteins (gps) enriched by affinity chromatography abrogated the reactivity of 135- and 210-kD proteins. N- and O-glycosidase treatments demonstrated that α-galactosyl residues are located on the O-glycans of the 135-kD component. Finally, glycoproteins of 90 and 135 kD were identified by amino acid sequencing as the pig analogs of the human glycoproteins Ila and IIb, respectively, whereas the 240-kD component was identified as the porcine fibrinogen, using a new murine monoclonal antibody (NaM147-7B6; IgG1) specific for its β-chain.

**MATERIALS AND METHODS**

**Human sera, IgG, and IgM preparations.** Normal human sera samples were obtained from 15 healthy blood donors (Centre Régional de Transfusion Sanguine, Nantes, France). The reactivity was first investigated with a previously described enzyme-linked immunosorbent assay (ELISA) using porcine platelets. Since large individual variations not correlated with the donor ABO blood group were previously reported, sera with elevated optical density (OD) values were selected, pooled, and stored at −80°C until use. IgM and IgG were purified from human plasma using sequential ammonium sulfate and polyethylene glycol precipitations. Final protein pellets were dissolved in 10 mmol/L sodium phosphate and 150 mmol/L NaCl, pH 7.4 (phosphate-buffered saline [PBS]), containing 0.01% NaN₃. Igs were quantified by nephelometry, with purity monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. Each fraction was further purified by affinity on a protein G column (protein G-Sepharose; Sigma, St Quentin-Fallavier, France).

Anti-Gal antibodies were obtained by affinity chromatography on a Synsorb-14 column (silica beads conjugated with a synthetic disaccharide, Galα1-3Galβ-R) purchased from Chembliomed (Edmonton, Alberta, Canada). Briefly, Igs were diluted in PBS and loaded onto the column. After extensive washings with PBS, anti-Gal antibodies were eluted with 2% NH₄OH and quickly neutralized with 1 mol/L KH₂PO₄. The nonretained fraction was freed of anti-Gal by successive depletions. Fractions were quantified by OD read-

52x357

53x401

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ings (OD 280 nm) and extensively dialyzed against PBS containing 0.01% NaN₃. The biologic activity of fractions was measured by agglutination of rabbit (RBC).

Preparation of platelet lysates. Pig blood was collected at the local slaughterhouse. Platelets were obtained from platelet-rich plasma by centrifugation at 3,000 g for 20 minutes and washed three times in 10 mmol/L Tris hydrochloride and 150 mmol/L NaCl, pH 7.4 (TBS), containing 1 mmol/L EDTA. Cell extracts were prepared by mixing 1 vol platelet pellet with 3 vol TBS, pH 7.4, containing 1% Triton X-100, 1 mmol/L CaCl₂, and 10⁻⁵ mol/L leupeptin. After incubation for 45 minutes on ice and centrifugation at 30,000 g for 20 minutes at 4°C, the supernatant was collected and stored at −80°C until use. Protein content of the lysate was measured using the bicinchoninic acid assay as described by the manufacturer (Pierce, Rockford, IL).

ELISA on porcine platelets. Microtiter plates (Maxisorb; Nunc, Roskilde, Denmark) were coated with 7.5 × 10⁶ porcine platelets/well by overnight incubation at 4°C. Plates were then gently washed with TBS, pH 7.4, containing 0.05% Tween 20, and saturated with 2.5% bovine serum albumin in TBS for 1 hour at room temperature. Plates were incubated sequentially with human XNA for 90 minutes and with peroxidase-labeled secondary antibody (anti-human IgG, y-chain-specific, diluted 1/5,000, or anti-human IgM, p-chain-specific, Sigma; or N-glycanase (PGNase, 100 U/mL; Boehringer Mannheim), and 2 U N-glycanase (PGNase, 100 U/0.5 mL; Boehringer Mannheim). Reactions were stopped by adding 30 mmol/L Tris hydrochloride, 3 mmol/L EDTA, and 15% SDS. Control incubations were performed in the appropriate buffer without enzyme.

SDS-PAGE and immunoblotting. Proteins (~60 µg) were applied on an 8% separating polyacrylamide gel run according to the Laemmli method, in a Mini-gel cuvet (Pharmacia, St Quentin Yvelines, France). Porcine and human fibrinogen fractions (fraction 1; Sigma) were previously incubated with protein A–Sepharose to remove contaminant IgG. Proteins were electrophoretically transferred to nitrocellulose sheets (0.1 µm; Schleicher and Schuell, Cera-labo, Ecuquevilly, France) as previously described. Immunoblots were saturated with TBS, pH 7.4, containing 0.5% gelatin and 0.05% Tween 20 for 2 hours at room temperature, and sequentially incubated with human XNA and with alkaline phosphatase–labeled secondary antibodies (anti–human y- or µ-chain-specific, Sigma; or anti–human IgG(H + L), Bioalutic, Nantes, France). The immunoblots were then stained with the nitroblue tetrazolium procedure.

Production of murine monoclonal antibodies (MoAbs). Mice were injected intraperitoneally three times and boosted 4 days before the fusion with 30 µg pig platelet glycoproteins purified on LCH-Sepharose. Their spleen cells were fused with X63Ag 8.653 myeloma cells. Hybridomas were selected by ELISA and immunoblotting of supernatants using porcine platelets. Antibody-producing clones were isolated by two limiting-dilution procedures. Isotypes were determined by dot-blot analysis using rat MoAbs directed against mouse Ig classes (ISO 2; Sigma).

RESULTS

Reactivity of human anti-Gal antibodies with pig platelets. On a quantitative basis, approximately 1% of both IgM and IgG were retained on and eluted from the affinity Synsorb-14 column. This value is in good agreement with data reported by others. Purified IgM and IgG, as well as corresponding nonretained and eluted fractions from the Synsorb-14 affinity column, were first studied by ELISA. The antibodies retained on the Synsorb-14 column (referred to as anti-Gal antibodies) strongly bound to porcine platelets (Fig 1). However, the anti-Gal–free fractions, obtained after several passages on the column, showed a low but significant IgM reactivity. This data showed that the bulk of human XNA directed against porcine platelet antigens are IgM and IgG anti-Gal and that non–anti-Gal IgM carry residual unknown xenoreactive specificities. From ELISA performed with porcine platelets, the reactivity of IgG and IgM XNA not retained on the Synsorb column was approximately 20% and 25% of the reactivity of total fractions.

Identification of xenoreagents by immunoblotting. Immunoblots prepared with whole platelet lysates and probed with normal human sera (NHS) had platelet components of 115, 125, 135, 180, and 210 kD. Grossly similar patterns were obtained using IgG or IgM (total fractions), but the intensity of each band varied depending on Ig type (Fig 2). In particular, bands of the 115/135-kD triplet were stained with the same intensity by IgM, whereas the 135-kD molecule was the predominant component stained by IgG. The 210-kD...
Fig 1. Biologic activity of IgM and IgG separated by Synsorb-14 affinity chromatography. Total Ig (x), anti-Gal (■), and flow-through Synsorb (□) fractions were analyzed by porcine platelet ELISA. Bound lgs were revealed with peroxidase-labeled anti-human IgM and anti-human IgG.

band appeared clearly as a doublet. Additional components at approximately 150 kD or larger than 240 kD were also revealed with both IgM and IgG as weak bands (150 kD) or strong bands (>240 kD; the molecular weight could not be precisely defined, because the protein remains at the top of the gel). Absorption of human sera on pig platelets abolished the binding of Ig to all these components (not shown), indicating that proteins were labeled on Western blots by XNA that specifically bind native pig platelets. Of note, human antibodies also bound to antigens of lower molecular weight, but this binding was assumed to be nonspecific, since these components were also present on immunoblots prepared with human platelets and were still detectable with absorbed serum (not shown). Affinity-purified anti-Gal IgG or IgM (synsorb− fractions) strongly stained components of 135, 150, 180, and 210 kD. In contrast, the large–molecular-weight bands (>240 kD) were only faintly stained, whereas the 115- and 125-kD antigens were barely detectable. Nonretained IgM (synsorb− fractions) showed the >240- and the 125-kD bands as the predominant components but failed to label gpts of 150, 180, and 210 kD, showing that these gpts are specific targets of anti-Gal IgM. Similar patterns were obtained with total and nonretained IgG.

Human platelet gpts carrying N-glycans, particularly gp IIb and IIIa, can be isolated by Con A–Sepharose chromatography.24 Porcine platelet gpts purified by this method were analyzed by immunoblotting (Fig 3). NHS revealed two ma-
ajor components of molecular weight 90 and 135 kD under nonreducing conditions, or 110 and 125 kD under reducing conditions. Under nonreducing conditions, the 115- and 180-kD N-glycans not recognized by Con A. Under reducing conditions, the bands at 150, 210, and 240 kD were no longer detectable, and new bands appeared at 170, 180, and 220 kD. The 90- and 135-kD bands were also strongly stained by IgG and IgM anti-Gal (synsorb™ fractions). According to the data obtained with platelet lysates, the >240-kD molecule was not the target of the anti-Gal IgM. Also, only one band of the doublet at 210 kD was observed with total or anti-Gal IgG and IgM.

Effect of enzymatic treatments on gps recognized by XNA. The same gps were isolated by LCH- or Con A-Sepharose affinity chromatography. However, the 90-kD gp was better isolated by Con A chromatography, whereas the 210-kD component was preferentially enriched by LCH. Porcine platelet gps were purified on the LCH-Sepharose column and treated with glycosidases to characterize the epitopes recognized by human natural antibodies. Three major LCH-reactive gps of 135, 210, and more than 240 kD were detected with NHS (Fig 4A, lane B). α-Galactosidase treatment abrogated all reactivity with 135- and 210-kD components, whereas only a weak staining of a gp of more than 240 kD was still detectable (lane A). After β-galactosidase digestion, new strong bands appeared, complicating the interpretation of the resulting pattern (lane C). This pattern strongly suggests that β-galactosidase unmasked new epitopes to which natural antibodies bind. Both treatments with N-glycanase and O-glycosidase of LCH-enriched gps caused a diminution of the apparent molecular weight of the 135-kD component without eliminating its XNA reactivity (lanes E or J, respectively). However, O-glycosidase treatment of the purified 135-kD component completely abolished its reactivity with XNA, whereas N-glycanase treatment did not significantly modify its intensity (Fig 4B). These data strongly suggest that α-galactosyl epitopes are carried by only O-glycans that could not be totally cleaved during treatment of the crude gp extract. A mass loss of about 10 to 20 kD after N-glycanase treatment suggests that the 135-kD gp carries a few N-glycans. After neuraminidase treatment (Fig 4A, lanes H and J), decreased reactivity was detected on the 210-kD doublet, suggesting that sialic acid residues are involved in the epitope recognized by the corresponding antibodies. On the other hand, the electrophoretic mobility of both components of the doublet was slightly enhanced after N-glycanase treatment (Fig 4A, lane E).

Identification of gp 135 and gp 90. As described earlier, pig platelet gps of 135 and 90 kD (125 and 110 kD under reducing conditions) were strongly recognized by human XNA. In addition, our data suggest that gp 135 carries N- and O-glycans. Altogether, these results show that gp 135 and gp 90 have some homology (apparent molecular weight and glycosylation) with human platelet gps IIb and IIIa. This hypothesis was confirmed by microsequencing the two gps purified by electrophoresis. Amino-terminal sequences obtained by Edman degradation (Table 1) show that gp 135 is effectively homologous to human IIbα (apparent molecular weight 125 kD under reducing conditions) chain and gp 90 is similar to human IIIa (apparent molecular weight 108 kD under reducing conditions). The percent identity of the peptide sequences between human and pig is 75% and 86% for aIIb and β3, respectively.

Characterization of >240-kD component. Among the murine MoAbs produced against LCH affinity-purified pig platelets, monoclonal antibody NaM147-7B6 (IgG1) strongly bound to a porcine platelet component of >240 kD on immunoblots prepared with platelet lysates (not shown).
The antibody recognized purified porcine fibrinogen, particularly its β-chain, which migrates at 56 kD after reduction (Fig 5). For comparison, immunoblots of purified porcine and human fibrinogen (fraction I from porcine and human plasma; Sigma) with NHS show that XNA react with porcine fibrinogen (with and without reduction) but faintly with human fibrinogen, especially under reducing conditions (Fig 5). Natural antibodies bind the three chains (α, β, and γ of 67, 56, and 47 kD, respectively), and more particularly the α-chain of pig fibrinogen, whereas MoAb NaM147-7B6 recognizes the β-chain. Because fibrinogen is present in the α-granule of platelets, these results show that the >240-kD component recognized by human XNA is fibrinogen.

**DISCUSSION**

In our previous study using anti-Gal antibodies purified on a melibiose (Galα1-6Glc) affinity gel, we found that the bulk of human XNA directed to porcine platelets was not retained on the column. However, in agreement with Galili et al., the present study shows clearly that the immunoadsorbent, Synsorb-14, bearing the glycosidic epitope, Galα1-3Galβ-R, is more effective than melibiose-Sepharose in binding anti-Gal and indicates that the non-anti-Gal antibodies are not as quantitatively important as previously estimated. Moreover, IgG and IgM antibodies against several carbohydrates have been characterized in NHS by passive hemagglutination and quantitative tests of inhibition of hemagglutination. However, antibodies against cell-bound sugars detected by this method were variably inhibited by the addition of free saccharides, indicating that such antibodies cross-react with other glycosidic epitopes with lower affinity.

Galili et al had initially reported that up to 1% of IgGs are anti-Gal antibodies; subsequent studies have shown that IgMs also react strongly with the Galα1-3Gal epitope. Consistent with these data, we showed that pig platelet gps are recognized by both IgG and IgM anti-Gal. This supports our recent observation that both IgG and IgM bind to RRBC and porcine endothelial cells, although anti-Gal IgMs are only able to kill porcine endothelial cells in the presence of human complement. Non-anti-Gal IgMs do not agglutinate RRBC and are not cytolytic for pig cells, but still react with whole-pig platelet lysates, suggesting that they recognize intracellular platelet components. The present study clearly confirms our previous observations that IgMs are more reactive than IgGs when immunoblots are used, although the magnitude of the signal mostly depends on the secondary antibodies used.

Having shown that XNA preferentially bound the Galα1-3Gal epitope carried by several gps, it was important to identify these components. The use of lectins provides an efficient method for analyzing platelet gps. Con A and LCH both recognize many asparagine-linked oligosaccharides that contain α-mannosyl residues, although the LCH receptor contains an additional fusocysyl residue. Using biotinylated lectins, human platelet gps Ia, IIb, and IIIa, fibrinogen, and thrombospondin were stained by Con A and LCH. Human gp IIb, gp IIIa, fibrinogen, and thrombospondin were isolated on Con A-Sepharose, whereas gps Ia and IIb were isolated with LCH.

Human fibrinogen is a globular protein (340 kD) consisting of three polypeptide chains: Aα (67 kD), Bβ (56 kD), and γ (47 kD). The overall structure contained one pair of each polypeptide chain type (A2, B2, and γ2). It has been shown that human fibrinogen molecules have four N-linked carbohydrate chains with the terminal NeuAc-Galβ1-4GlcNAc residues. On the other hand, Thall and Galili have shown that the Galα1-3Galβ1-4GlcNAc residues are present on porcine and bovine fibrinogen and are concentrated on approximately 1% of the fibrinogen molecules. Our results are consistent with all these data. We show that anti-Gal IgM and IgG bound a gp of more than 240 kD isolated on a Con A or LCH affinity column, a component also recognized by a murine MoAb (NaM147-7B6) directed against the β-chain of porcine fibrinogen. Treatment with α-galactosidase eliminates only part of the reactivity of human antibodies against porcine fibrinogen. Together with the fact that the non-anti-Gal fraction still recognized the fibrinogen, it indicates that it carries a structure(s) other than the α-galactosyl epitope that is a target of XNA.

Platelet gp Ia was efficiently isolated by Con A or LCH affinity chromatography, suggesting that this component carries N-linked carbohydrate chains. Human gp Ia (integrin α2) is described as a 150-kD gp (170 kD under reducing conditions). Platt and Holzknecht demonstrated a sequence homology between a porcine platelet gp (the 135-kD component) recognized by human XNA, and human integrin α2. Taken together, these data suggest that the 150-kD component that binds anti-Gal antibodies on Western blot is the porcine platelet gp Ia.

The gps IIb and IIIa are the major cell-surfacegps in the platelet plasma membrane. The Ca2+-dependent gp IIb-IIIa complex (integrins αIIb/β3) serves as the inducible receptor for fibrinogen and soluble von Willebrand factor at the surface of activated platelets, and plays a major role in platelet aggregation. The gp IIb (136 kD, nonreduced) is formed.
from two subunits, gp IIbα (114 kDa) and gp IIbβ (22.5 kDa) that are split under reducing conditions, inducing a reduction of the molecular weight. Gp IIbα has only one polypeptide chain (92 kDa, nonreduced) with a large number of disulfide bridges. The apparent molecular weight increased after reduction (Fig 3). Distinct distributions of amino sugars and glycosylation sites are mostly known. Five N-linked glycosylation sites and three O-glycosylation sites were located on gp IIb, whereas gp IIbα carries only six potential N-glycosylation sites.35 Our results show that these two gps are well conserved between pig and man: (1) N-terminal sequences of pig and human gps IIb and IIa show a high degree of homology (Table 1), (2) gp IIb from both species carries N- and O-glycans and (3) in man and pig, gp IIb and gp IIa undergo a similar shift of apparent molecular weight between native and reducing conditions.

Although platelets are likely not involved in the hyperacute rejection process of pig tissues triggered by XNA and complement, they constitute a good material for the study of XNA targets because they are abundant and easily obtained. Moreover, (1) similar but not identical immunoblot patterns were obtained with platelets and endothelial cells from pigs,33 (2) purified gp 115/1135 inhibited binding of xenoreactive human natural antibodies to cultured porcine endothelial cells.2 These findings indicate that a similar epitope recognized on both cell types is probably present on different proteins. It was also clearly established that endothelial cells synthesize and express plasma membrane gpⅠb glycoecochemically and immunologically similar to some of the major platelet membrane components.34 The endothelial gp IIb/IIIa-like complex has been extensively studied, and it was shown that platelet and endothelial gp IIb/IIIa have identical amino acid sequence,5 although platelet gp IIb is structurally and antigenically different from its endothelial counterpart, the vitronectin receptor α-chain.56 The gp complex la-Ila is present on the membrane of both platelets and endothelial cells.7 Recent studies have shown that endothelial cells express platelet gp IV38 and the platelet α-granule membrane P-selectin (GMP-140).39 Owing to the easy availability of pig platelets, these findings indicate that a similar epitope recognized on both pig and man: (1) N-terminal sequences of pig and human gps IIb and IIa show a high degree of homology (Table 1), (2) gp IIb from both species carries N- and O-glycans and (3) in man and pig, gp IIb and gp IIa undergo a similar shift of apparent molecular weight between native and reducing conditions.

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