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Brief report: a new profile of terminal *N*-acetyllactosamines glycans on pig red blood cells and different expression of α-galactose on Sika deer red blood cells and nucleated cells

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Abstract It has been reported that: (1) large variations were found in the number of sialic acid (SA) capped with *N*-acetyllactosamines (SA-Galβ1-4GlcNAc-R) and α-Gal epitopes (Galα1-3Galβ1-4GlcNAc-R) or uncapped *N*-acetyllactosamines (Galβ1-4GlcNAc-R) on different mammalian red blood cells, and on nucleated cells originating from a given tissue in various species; (2) goat, sheep, horse and mouse red blood cells lack α-Gal epitopes, despite the expression of this epitope on a variety of nucleated cells in these species, including lymphocytes differentiated from the same hematopoietic origin. In this study, flow cytometry and Western blot analyses of pig red blood cells showed that α-Gal epitopes on pig red cells developed concomitantly after treatment with neuraminidase, suggesting that the terminal *N*-acetyllactosaminide glycans were capped with SA-α-Gal epitopes. Whereas, the expression of the α-Gal epitopes on red blood cells from Sika deer (*Cevus nippon hortulorum*) were found to be absent even though the epitopes were present on their white blood cells. Thus, these results add new data not only for the terminal carbohydrate structures on cell surface glycans of various mammalian cells, but also for wide variety of epitope expression on the cells from different tissues, which might be useful for understanding their unique states resulting from differentiation and evolution.

Keywords Pig red blood cell · α-Gal epitope · Sialic acid · *N*-acetyllactosamine · α-Galactosidase · Neuraminidase · Sika deer blood cell

Abbreviations

α-Gal	Galα1-3Galβ1-4GlcNAc
FACS	Fluorescent activated cell sorting
FCM	Flow cytometry
FITC	Fluorescein isothiocyanate
GlcNAc	*N*-acetylglucosamine
GS-IB4	*Griffonia simplicifolia* isolectin B4
hRBC-A and hRBC-O	Group A and O of human red blood cell
M86	Mouse monoclonal antibody to α-gal epitopes
NeuGc	*N*-glycolylneuraminic acid
N-acetyllactosamine	Galβ1-4GlcNAc-R
PBS	Phosphate buffered saline
PK15	Pig kidney cell line
PVDF	Polyvinyidene fluoride
SA	Sialic acid
TBST	Tris-buffered saline, 0.1% Tween 20

Introduction

Investigation of species-specific terminal carbohydrate structures (epitopes) is important in understanding how

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glycan diversity was created during the course of evolution and diversification of animals [1–3], development and oncogenesis [4–6], as well as in xenotransplantation [7]. Owing to the complexity and the variations of carbohydrate structures on cell surface glycans, profiling of such structures on cell membranes has been difficult to perform. Consequnently, only limited information is available in this field. Glycosidase is a powerful tool for selectively removing carbohydrate epitopes from the cell surface. Further, the carbohydrate structures then exposed are used as acceptors by the glycosyltransferase to form new carbohydrate epitopes. Along this way, researchers have used glycosidases and glycosyltransferases as analytical tools for rapid identification of carbohydrate structures on cell membranes [8, 9] or as a method to overcome rejection occurring in transplantation [10, 11]. Pig organs, tissues and blood [12–14] were used for xenotransplantation by applying these enzymes. For instance, universal human red blood cells were prepared using two bacterial glycosidases [15].

There are two well-known carbohydrate epitopes on cell surfaces: one is the α-Gal epitope (Galα1-3Galβ1-4GlcNAc-R), which is abundantly expressed on cells of non-primate mammals, prosimians and New World monkeys [16–19]; the other is *N*-glycolylneuraminic acid (NeuGc) epitope, one of the sialic acid (SA) analogues, which are widely expressed on endothelial cells of all mammals except humans [20, 21]. *N*-acetyllactosamine (Galβ1-4GlcNAc-R) is the major component of the α-Gal epitope and the sialic acid epitope. Recently, Ogawa and Galili [9] reported that there is a significant variation in the number of uncapped *N*-acetyllactosamines, sialic acid capped, and α-Gal capped *N*-acetyllactosamines, on different mammalian red blood cells, and nucleated cells originating from a given tissue in various species. They also found that goat, sheep, horse and mouse red blood cells lack α-Gal epitopes, despite the presence of this epitope on a variety of nucleated cells in these species, including lymphocytes differentiated from the same hematopoietic origin [9].

It is well known that α-Gal is the major xenoantigen in pigs recognized by natural antibodies in human sera [22]. While, NeuGc epitopes are important non-αGal xenoantigens, though some doubt remains regarding its importance in xenotransplantation [23–25]. Miwa *et al.* [26] reported that neuraminidase treatment could markedly reduce the expression of NeuGc epitopes and the binding of human anti-nonGal antibodies to pig red blood cells. In this study, we used two glyosidases, neuraminidase and α-galactosidase, to investigate the effect of neuraminidase on the change of xenoantigens on pig red blood cells. Neuraminidase catalyzes the hydrolysis of sialic acids including NeuGc epitopes from glycoproteins and oligosaccharides. α-Galactosidase removes the α-galactosyl terminal residues in glycoconjungates and has been used frequently to reduce the antigenicity of pig organs for xenotransplantation. To reduce the antigenicity of pig red blood cells, effects of these two enzymes on α-Gal epitope were studied by flow cytometry (FCM) and Western blotting using *Griffonia simplicifolia* Isolectin B4 (GS-IB4, a probe for terminal α-galactose). Along with this, we also investigated α-Gal expression on Sika deer (*Cervus nippon hortulorum*) blood cells by FCM and hemagglutination test using GS-IB4 and monoclonal antibody M86.

Materials and methods

Reagents

α-Galactosidase from green coffee beans, neuraminidase from *Vibrio cholerae*, fluorescein isothiocyanate (FITC) and biotin labeled *Griffonia simplicifolia* Isolectin B4 (GS-IB4) were purchased from Sigma (St. Louis, Mo, USA). Horseradish peroxidase (HRP)-linked streptavidin was from Zhongshan Reagent Company (Beijing, China). PageRuler™ Prestained Protein Ladder in the range 10–170 kDa was from Fermentas (Vilnius, Lithuania). Enhanced chemiluminescence (ECL) reagents were from Applygen Technologies Inc (Beijing, China). BCA protein assay kit was from Pierce (Rockford, IL, USA). The M86 monoclonal antibody (mouse IgM) to α-Gal epitope was from Alexis Biochemicals (Axxora, Germany). BD FACS™ Lysing Solution was from BD Biosciences (BD Biosciences, Japan).

Preparation of cells

Pig (Large White pig) blood was obtained from Specific-Pathogen-Free Pig Breeding Center of Beijing in China. Sodium-heparinized anticoagulant blood samples were centrifuged at 1,000 g for 5 min to remove plasma and the pelleted red cells were washed by phosphate buffered saline (PBS, pH7.3). Group O and A of human red blood cells were from Beijing Red Cross Blood Center (Beijing, China).

Sika deer EDTA anticoagulant blood samples were from Beijing Zoo (Beijing, China). The red blood cells were collected by centrifugation of the blood followed by washing with PBS; the white blood cells were obtained after removing red blood cells by lysing in 1×FACS™ Lysing Solution (incubation 15–30 min at room temperature). Pig kidney cell line PK15 was cultured in RPMI 1,640 medium supplemented with 10% fetal bovine serum (FBS). Cells were harvested with PBS containing 0.25% trypsin.

Enzyme treatment on pig red blood cells

The pig red blood cells were subjected to treatment with α-galactosidase and neuraminidase, respectively. PBS washed pig red blood cells were treated by α-galactosidase (10 units of α-galactosidase were added to 100 μl pig red blood cells) in 200 μl phosphate-citric acid-sodium chloride buffer (56.8 mmol Na_2HPO_4, 21.6 mmol $C_6H_8O_7.H_2O$ (monohydrate citric acid) and 77.0 mmol NaCl, pH5.5) [13]. After incubating at 26°C for 2 h, the cells were extensively washed with PBS. To get asialo-forms, the terminal sialic acids were removed with reference to the method of Miwa *et al.* [26]. Briefly, pig red blood cells were incubated with neuraminidase (0.05 units/50 μl/5×10^6 cells) in calcium- and magnesium-free phosphate-buffered saline for 40 min at 37°C.

Flow cytometry analysis

The effect of enzyme treatment on α-Gal epitopes of pig red cells was confirmed by flow cytometry analysis. The red blood cells (1×10^6) were fixed with 2.0% paraformaldehyde, washed with PBS, and then incubated with FITC-labeled GS-IB4 (10 μg/ml). The percentage of fluorescently labeled red blood cells was analyzed by a flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA). Cells without incubation with the probe were used as background control.

To investigate α-Gal epitope expression on Sika deer red blood cells and white blood cells, the cells were subjected to the enzymatic treatment as described above. Pig red blood cells were used as a positive control. Group O and A of human red blood cells were used as negative controls (human blood groups of B and AB have a similar structure to the α-Gal epitope). Pig kidney cell line PK15 was a positive control of deer white blood cells.

Western blot analysis

To analyze the change of α-Gal epitope expression on pig red blood cells after treatment by α-galactosidase and neuraminidase, membrane proteins were prepared by the following procedures: (1) pig red blood cells (treated and untreated) were resuspended in 40 volumes of cold hypotonic buffer (10 mM Tris-HCl, pH7.4) and incubated at 4°C for 30 min; (2) after hemolysis, cells were centrifuged at 12,000×g for 15 min, and subsequently washed with the hypotonic buffer for 3–4 times. All the operations were carried out at 4°C. Protein concentration was determined by the BCA protein assay kit.

According to protein concentration, equal quantity of control and treated porcine red blood cell membrane was loaded on a 12% SDS-PAGE gel. After separation by electrophoresis, the proteins were transferred to polyvinyidene fluoride (PVDF) membrane. The PVDF membrane was blocked with 5% nonfat milk in TBST (Tris-buffered saline containing 0.1% Tween 20) at room temperature for 4 h. The membrane was probed at 26°C for 2 h with biotin-labeled BS-IB4 (1:200 diluted in 5% nonfat milk TBST), followed by four washings of 10 min in TBST. The membrane was then incubated for 30 min with HRP-linked streptavidin (1:500 diluted in 5% nonfat milk TBST), then washed with TBST three times followed by incubation with ECL reagents and exposure of film for 1 to 2 min.

Analysis of the expression of α-Gal epitopes on red blood cells by hemagglutination test with anti-Gal and GS-IB4 lectin

To further identify α-Gal expression on deer red blood cell, 25 μl anti-αGal epitope monoclonal antibody of (M86; IgM) or 10 μl GS-IB4 was incubated with 25 μl 2.5% pig, deer, and human red blood cells (type A and O) suspension in saline. After incubation at room temperature for 10 min, the strength of hemagglutination was determined under through a microscope.

Results

Analysis of α-Gal epitope expression on neuraminidase treated pig red blood cells

The changes of α-Gal epitope expression on α-galactosidase and neuraminidase treated pig red blood cells, respectively, were detected by two independent methods: flow cytometry

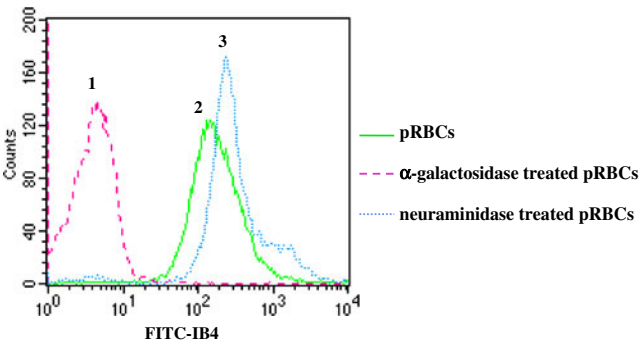

Fig. 1 Fluorescence overlay histogram showing flow cytometry analysis of α-Gal epitope expression on pig red blood cells (RBC) by staining with FITC labeled *Griffonia simplicifolia* isolectin B4 (GS-IB4). The result reveals that neuraminidase treatment resulted in increase of α-Gal positive signal in comparison with untreated and α-galactosidase treated samples. *Peaks* in the histogram represent 1) pig RBC treated with α-galactosidase; 2) pig RBC prior to enzymatic treatment; 3) pig RBC treated with neuraminidase

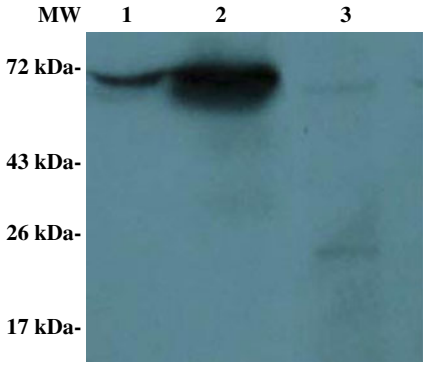

Fig. 2 Western blot analysis of the expression of α-Gal epitope on pig RBC treated with α-galactosidase and neuraminidase, respectively. The α-Gal epitopes are probed with biotin-labeled GS-IB4 and HRP-labeled streptavidin. Untreated RBC showed one α-Gal positive band with a molecular mass about 70 kDa (lane 1), while this band became much stronger in the neuraminidase treated pig RBC (lane 2). The α-galactosidase treated RBC showed two weak bands (lane 3)

analysis (Fig. 1) and Western blot analysis (Fig. 2) using GS-IB4 as a probe, which binds specifically to α-Gal epitopes.

The FCM profiles of the enzymatically treated pig red blood cells were different from those of untreated cells (Fig. 1). Untreated pig red blood cells had strong GS-IB4 signal. In comparison, the mean fluorescence intensity (MFI) level decreased by almost 100% (99.27%) in α-galactosidase treated group (Table 1), which indicates that α-Gal was almost completely removed by the treatment with α-galactosidase. In contrast, neuraminidase treatment resulted in an increase at the level of MFI by 38.83%, indicating exposing of α-Gal epitope after removal of sialic acid by neuraminidase (Table 1). The results suggest that *N*-acetyllactosamine epitopes are capped with SA-αGal- carbohydrate structure on pig red blood cell membranes.

Western blot analysis is shown in Fig. 2. A considerably stronger α-Gal positive band with a molecular mass about 70 kDa was detected in neuraminidase treated pig red cell membrane proteins (lane 2), in comparison with untreated and α-galactosidase treated samples (lane 1 and lane 3, respectively). It should be noted that two weak bands were observed in the α-galactosidase treated sample, which might be trace amounts of α-Gal epitope resistant to α-galactosidase. Therefore, the results supported the conclusion of flow cytometry that a very large number of α-Gal epitopes were exposed after the removal of sialic acid by neuraminidase.

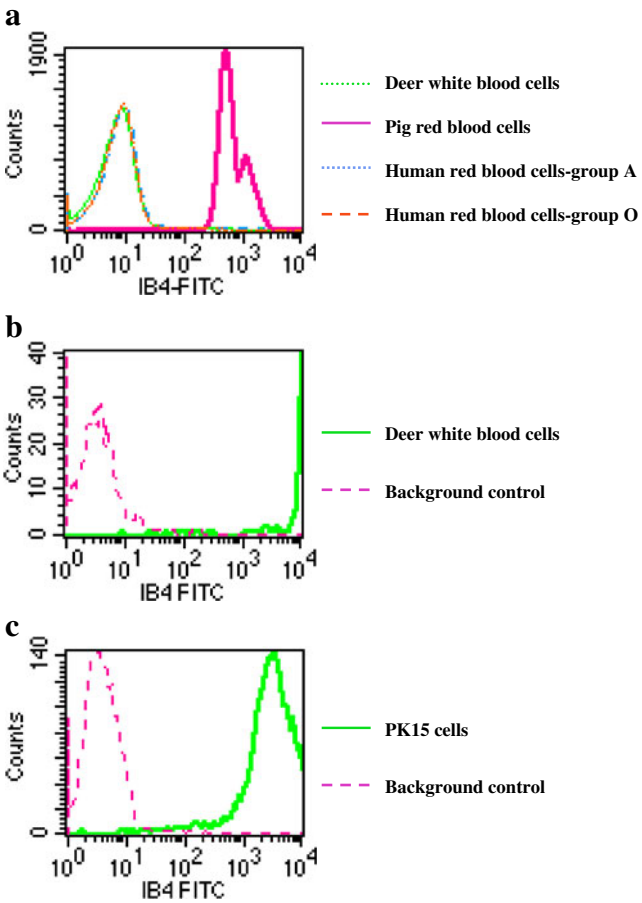

Fig. 3 α-Gal expression on Sika deer blood cells detected by flow cytometric analysis with FITC labeled GS-IB4. **a** Fluorescence overlay histogram of α-Gal expression on Sika deer RBC (*green line*), pig RBC (*red line*, positive control), and group A and O of human RBC (*blue and orange lines*, respectively, negative control). The results show that no GS-IB4 binding is detectable on deer red cells; **b** α-Gal expression on Sika deer white blood cells pre- (*red line*) and incubation with FITC labeled GS-IB4 (*green line)*; **c** Expression of α-Gal on pig kidney cell (PK15) as positive control (*green line*) and blank (*red line*)

α-Gal epitope expression on deer red blood cells and white blood cells

Expression of α-Gal epitopes on Sika deer red blood cells and white blood cells was assessed by flow cytometry analysis,

Table 1 The mean fluorescence intensity (MFI) level of pig red blood cells (pRBCs). α-Gal epitope is increased by 38.83% after neuraminidase treatment and decreased by 99.27% after α-galactosidase treatment, in comparison with the non-treated pRBC

pRBC	α-galactosidase treated pRBC		neuraminidase treated pRBC	
MFI	MFI	MFI decrease (%)	MFI	MFI increase (%)
308.29	3.12	99.27	428.00	38.83

respectively, after incubation with FITC-conjugated GS-IB4. The FCM profile (Fig. 3a) showed that almost no GS-IB4 binding was detectable on deer red blood cells, which was consistent with the negative control (human red blood cells, type O and A), indicating lacking of α-Gal epitopes on the deer red blood cells. In contrast, the deer white blood cells express substantial amount of α-Gal epitopes (Fig. 3b), which is higher than PK15 cells (α-Gal positive) (Fig. 3c). Thus the flow cytometry results indicated that the α-Gal epitope is strongly expressed on deer white blood cells, but not on deer red blood cells, although both cell types were from the same hematopoietic origin.

Similar to the FCM result, the hemagglutination test showed strong agglutination reaction and bulky agglutinated clots in pig red blood cells. In contrast, deer red blood cells and human red blood cells (type A and O) showed negative reaction, suggesting there was no M86 binding epitope on deer red blood cells (Fig. 4). Due to the strict specificity for α-Gal residues, GS-IB4 can induce hemagglutination of α-Gal positive red blood cells [1]. In this experiment, hemagglutination mediated by GS-IB4 paralleled that observed with anti-αGal. Pig red blood cells were agglutinated by the GS-IB4. But no agglutination was detected with the deer red blood cells and human red blood cells (type A and O) (data not shown).

Discussion

At present, three types of terminal *N*-acetyllactosamines carbohydrate structures are known: α-Gal epitopes (Galα1-3Galβ1-4GlcNAc-R), uncapped *N*-acetyllactosamines (Galβ1-4GlcNAc-R), and *N*-acetyllactosamines capped with sialic acid (SA-Galβ1-4GlcNAc-R). As presented in this study, the α-Gal epitope was increased by 38.83% on pig red blood cells after neuraminidase treatment as analyzed by FCM (Table 1), indicating that some of the *N*-acetyllactosamine on the carbohydrate moiety of the pig red blood cells are capped with SA-αGal. This result is confirmed by Western blot analysis (Fig. 2). Therefore, it is concluded that at least about 38% of the *N*-acetyllactosamines on pig red blood cell membrane have a SA-αGal- terminal structure (SA-Galα1-3Galβ1-4GlcNAc-R). It should be pointed out that the Western blot ananlysis only detected the protein contents in the membrane of pig red blood cell, while the membrane-

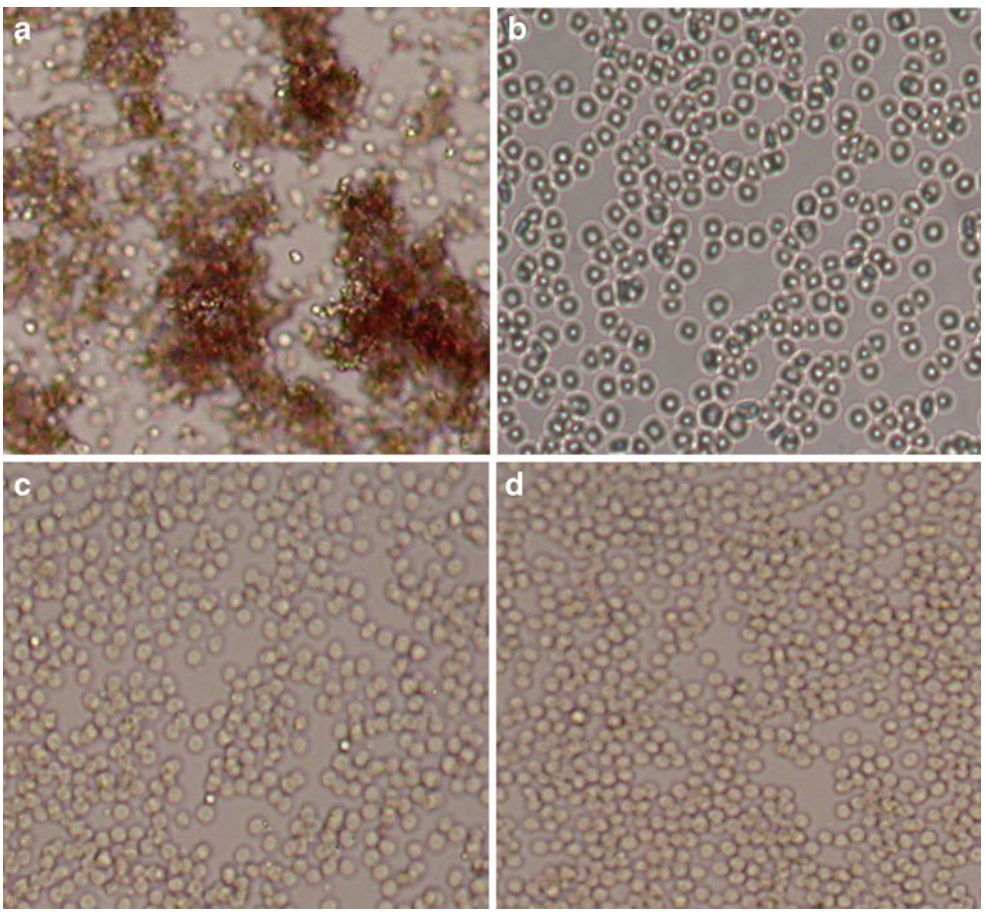

Fig. 4 Hemagglutination test of red blood cells with M86 monoclonal antibody to α-Gal epitope. **a** Pig red blood cells; **b** Deer red blood cells; **c** Human red blood cells group A and **d** Human red blood cells group O

associated glycolipids were not detected. Hence, it cannot excluded that the glycans linked to the lipid molecules may also have the SA-Galα1-3Galβ1-4GlcNAc-R epitopes.

Interestingly, the FCM results revealed that deer red blood cells lack α-Gal expression (Fig. 3a), while white blood cells expressed substantial amounts α-Gal epitopes, which are higher than PK15 cells (α-Gal positive) (Fig. 3b, c). To exclude the possible masking effect of paraformaldehyde fixation that prevented the interaction of red blood cells with GS-IB4 lectin [1], we performed hemagglutination test using M86 anti-αGal antibody and GS-IB4. The results confirmed a distinct pattern of hemagglutination reactivity in pig red blood cells. In comparison, deer red blood cells showed negative in this reaction at the same condition, which was similar to the result of human type A and O red blood cells (α-Gal negative) (Fig. 4). Based on the high specificity of GS-IB4 and M86 monoclonal antibody to the α-Gal epitope, we concluded that there were no α-Gal epitopes on the Sika deer red blood cells. This is an unexpected finding since white blood cells have high expression of the epitope. In fact, the same expression pattern was also found in Fallow deer (*Dama dama L.*) blood cells (data were not shown).

Absence of the α-Gal epitope in red blood cells of other mammals, including goat, sheep, horse and mouse, has been previously described by Ogawa and Galili [9]. The underlying mechanism for the differential expression of α-Gal epitopes on the membrane of different mammalian red blood cells, and on nucleated cells originating from a given tissue in various species remain to be elucidated. It was reported that lack α-Gal epitopes from human cells and chicken red blood cells results from diminished activity of the α1,3-galactosyltransferase [1, 16]. Ogawa and Galili [9] indicated that the lack of this epitope on red blood cells may be the result of either diminished α1,3-galactosyltransferase activity in erythropoietic cells in these species, or a much higher activity of a competing glycosyltransferase, such as sialyltransferase [9]. Why do the white blood cells of these species express α-Gal epitopes is a problem of interest to find out in future research.

In summary, this study reported two novel findings. By examination of pig red blood cells treated with α-galactosidase and neuraminidase, we demonstrated a new profile of cell surface glycans for terminal *N*-acetyllactosamines that are capped with the SA-αGal epitope. On the other side, we detected expression of α-Gal epitopes on the nucleated cells from Sika deer blood, but not on the red blood cells from Sika deer. Our results point to a differential expression of the α-Gal epitope in mammalian blood cells, which may have important biological relevance yet to be found out.

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