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Profiling terminal *N*-acetyllactosamines of glycans on mammalian cells by an immuno-enzymatic assay

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Abstract Profiling of carbohydrate structures on cell membranes has been difficult to perform because of the complexity and the variations of such structures on cell surface glycans. This study presents a novel method for rapid profiling of cell surface glycans for terminal *N*-acetyllactosamines (Galβ1-(3)4GlcNAc-R) that are uncapped, capped with sialic acid as SA-Galβ1-(3)4GlcNAc-R, or with α1,3galactosyls as the α-gal epitope- Galα1-3Galβ1-(3)4GlcNAc-R. This method includes two enzymatic reactions: (1) Terminal sialic acid is removed by neuraminidase, and (2) α-gal epitopes are synthesized on the exposed *N*-acetyllactosamines by α1,3galactosyltransferase. Existing and *de novo* synthesized α-gal epitopes on cells are quantified by a modification of radioimmunoassay designated as "ELISA inhibition assay," which measures binding of the monoclonal anti-Gal antibody M86 to α-gal epitopes. This binding is proportional to the number of cell surface α-gal epitopes. The amount of free M86 antibody molecules remaining in the solution is determined by ELISA using synthetic α-gal epitopes linked to albumin as solid phase antigen. The number of α-gal epitopes on cells is estimated by comparing binding curves of M86 incubated with the assayed cells, at various concentrations of the cells, with the binding of M86 to rabbit red cells expressing 2×10^6 α-gal epitopes/cell. We could demonstrate large variations in the number of sialic acid capped *N*-acetyllactosamines, α-gal epitopes and uncapped *N*-acetyllactosamines on different mammalian red blood cells, and on nucleated cells originating from a given tissue in various species. This method may be useful for rapid identification of changes in glycosylation patterns in cells subjected to various treatments, or in various states of differentiation.

Keywords Glycoproteins · α-gal epitope · Carbohydrate profiling · α1,3galactosyltransferase

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Introduction

Terminal carbohydrate structures (epitopes) on mammalian cell surface glycans have been found to vary during embryogenesis, cellular activation, differentiation, oncogenesis and inflammation [1–8]. Because of the complexity of the various cell surface carbohydrate epitopes, the information on these structures is limited, in comparison to the information on carbohydrate chains of purified glycolipids and glycoproteins [1–8]. Precise structure of carbohydrate chains on purified glycans can be analyzed by hydrolysis and isolation of oligosaccharides, and subsequent analysis of each monosaccharide composition by a variety of techniques including: high performance liquid chromatography (HPLC), gas–liquid chromatography (GLC), GLC-mass spectrometry (GLC-MS), and nuclear magnetic resonance (NMR) [3, 8]. These methods require the use of special instruments, are labor intensive and can be performed only with highly purified glycans. Because of these difficulties, many studies on cell surface carbohydrate epitopes include only staining with lectins, which provide mostly qualitative information on expression or absence of carbohydrate epitopes. In the present study we describe a novel method that enables a more precise quantitative profiling of carbohydrate epitopes that are common on

mammalian cells, including: uncapped *N*-acetyllactosamines (Galβ1-(3)4GlcNAc-R), *N*-acetyllactosamines capped with sialic acid (SA-Galβ1-(3)4GlcNAc-R), or capped with terminal α1,3galactosyl units (Galα1-3Galβ1-(3)4GlcNAc-R), designated α-gal epitopes. Although the quantitative profiling of these carbohydrate epitopes is not analytical, but provides only a rough estimate of the number of epitopes per cell, this method may be useful in studies in which glycoconjugate analysis and cell biology interface with each other.

N-acetyllactosamine is a major component of *N*-linked carbohydrate chains, some *O*-linked carbohydrate chains and on glycolipids of the cell membrane. Whereas *N*-acetyllactosamines are mostly type II chains (Galβ1-4GlcNAc-R), they are also found as type I chains (Galβ1-3GlcNAc-R), which in humans are usually restricted to epithelia of the gastrointestinal and reproductive tracts [8]. A large proportion of *N*-acetyllactosamines on cell surface glycans are capped with sialic acid which is negatively charged, thus generating repulsion forces (zeta [ζ] potential) that prevent nonspecific binding of various molecules to cells. Another very common carbohydrate epitope is the α-gal epitope which is abundantly expressed on cells of non-primate mammals, prosimians and New World monkeys [9–12]. In contrast, human, apes and Old World monkeys do not express α-gal epitopes, because of evolutionary inactivation of the α1,3galactosyltransferase gene [9, 10, 12–14]. This gene encodes the glycosyltransferase that synthesizes α-gal epitopes [10, 13–21]. Humans, apes and Old World monkeys produce large amounts of the anti-Gal antibody that binds to α-gal epitopes on both type I and type II carbohydrate chains [8, 19, 22–24]. The binding of anti-Gal to α-gal epitopes expressed on carbohydrate chains of pig endothelial cells is considered to be a major cause for rapid rejection of pig organs in human [25–28].

We have developed a method for effective synthesis of α-gal epitopes on human tumor cells in order to increase their immunogenicity as autologous tumor vaccines [29–33]. For this purpose, sialic acid is removed by *Vibrio cholerae* neuraminidase, and α-gal epitopes are synthesized by recombinant α1,3galactosyltransferase on de-sialylated *N*-acetyllactosamines. The *de novo* synthesized α-gal epitopes readily bind the natural anti-Gal antibody which targets the vaccinating tumor cells to antigen presenting cells, thereby increasing the immunogenicity of these cells [29–33]. The number of *de novo* synthesized α-gal epitopes could be estimated by the ELISA inhibition assay ("Experimental procedures"), in which the binding of the monoclonal anti-Gal antibody M86 to α-gal epitopes on the processed tumor cells is measured at different concentrations of the assayed cells. Comparison of M86 binding to that observed with control cells expressing a known number of α-gal epitopes, enables determination of the number of α-gal epitopes on various cells [32, 34].

In the present study we combine the enzymatic synthesis of α-gal epitopes with the ELISA inhibition assay, for profiling some of the most common carbohydrate epitopes on various cells. ELISA inhibition assay performed on untreated cells quantifies the number of α-gal epitopes naturally expressed on cells. Since uncapped *N*-acetyllactosamine on type I and type II carbohydrate chains is the specific acceptor for α1,3galactosyltransferase [16–21, 24], ELISA inhibition assay with cells that were subjected to α-gal epitope synthesis by this enzyme, determines the sum of uncapped *N*-acetyllactosamine epitopes and that of naturally expressed α-gal epitopes on cells. By subtracting the number of naturally expressed α-gal epitopes, it is possible to determine the number of uncapped *N*-acetyllactosamines. Similarly, the number of sialic acid units on penultimate *N*-acetyllactosamines can be inferred from quantification of α-gal epitopes on neuraminidase treated cells that are further incubated with α1,3galactosyltransferase and UDP-Gal. Thus, we could quantify the number of α-gal epitopes, *N*-acetyllactosamines and sialic acid on *N*-acetyllactosamines, by performing a combination of ELISA inhibition assays on untreated cells, cells incubated with α1,3galactosyltransferase, and cells incubated with both neuraminidase and with α1,3galactosyltransferase, respectively. This analysis demonstrated a wide heterogeneity in carbohydrate profile on red blood cells and on nucleated cells originating from the same tissue but from different species.

Experimental procedures

Materials

Recombinant α1,3galactosyltransferase was produced in the *Pichia pastoris* expression system of the marmoset (New World monkey) α1,3galactosyltransferase gene [31]. Neuraminidase from *V. cholerae* was purchased from Sigma (St. Louis, MO). UDP-Gal was a generous gift from Neose Pharmaceuticals (Horsham, PA). Synthetic α-gal epitopes linked to BSA (Galα1-3Galβ1-4GlcNAc-BSA, α-gal BSA) were purchased from Dextra Laboratories (Reading, UK). Human anti-Gal was isolated from human AB sera by affinity chromatography on columns with synthetic α-gal epitopes (Chembiomed, Edmonton, Canada) as previously described [23, 24]. The mouse monoclonal anti-Gal antibody M86 was obtained in tissue culture medium of the hybridoma cells secreting this antibody, as

we previously described [34]. Rabbit anti-human IgG conjugated with horseradish peroxidase (HRP) was purchased from Dako (Denmark). Goat HRP-conjugated anti-mouse IgM was purchased from Accurate Chemical & Scientific (Westbury, NY). FITC-conjugated *Bandeiraea* (*Griffonia*) *simplicifolia* IB_4 (BS lectin) was purchased from Sigma.

Cells

Red blood cells were obtained from various species via the animal facility or from vendors including Hemostat (Dixon, CA) and Colorado Serum Company (Denver, CO). To avoid the lysis of red blood cells, blood samples were fixed for 2 h with 0.5% glutaraldehyde, washed and the remaining reactive aldehyde groups were blocked with 0.1 M glycine. Human myeloid leukemia cell line K562 and human B lymphoma cell line Raji were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS). All other cells listed below were cultured in Dulbecco's Modification of Eagle's medium (DMEM) supplemented with 10% FBS, including: human melanoma cell line HTB-70, human cervical adenocarcinoma cell line HeLa, human pancreatic carcinoma cell line Panc-1, human kidney cell line HEK293, pig kidney cell line PK15, monkey kidney cell line COS-1, dog kidney cell line MDCK (Madin-Darby canine cells), bovine kidney cell line MDBK (Madin-Darby bovine cells), mouse melanoma cell lines B16 and B16–BL6, mouse fibroblast cell line NIH3T3 and human fibroblasts grown from foreskin. Cells were harvested with PBS containing 1 mM EDTA, and subjected to the profiling assays.

Synthesis of α-gal epitopes on cells

Synthesis of α-gal epitopes on cells was performed as previously described [29–32]. Briefly, 250×10^6 fixed red blood cells/ml, or unfixed 10×10^6 nucleated cells were suspended in enzyme buffer comprised of saline (0.9% NaCl) containing 25 mM MES (pH 6.2) and 25 mM $MnCl_2$. α1,3Galactosyltransferase (30 μg/ml) and UDP-Gal (1 mM) were added into the cell suspension. Synthesis of α-gal epitopes occurred according to the following reaction:

$$\underset{N-\text{acetyllactosamine}}{\text{Gal}\beta1-(3)4\text{GlcNAc}-\text{R}} + \text{UDP}-\text{Gal} \xrightarrow{\alpha1,3\text{galactosyltransferase}} \underset{\alpha-\text{gal epitope}}{\text{Gal}\alpha1-3\text{Gal}\beta1-4\text{GlcNAc}-\text{R}} + \text{UDP}$$

In cells analyzed for replacement of sialic acid with *de novo* synthesized α-gal epitopes, neuraminidase was included in the enzyme solution as 1 mU/ml. Cell suspensions were incubated at 37°C for 2 h with gentle rotation. Subsequently, cells were washed three times and re-suspended in PBS containing 1% BSA (BSA/PBS), as 1×10^9 red blood cells/ml or as 40×10^6 nucleated cells/ml.

ELISA inhibition assay

Quantification of α-gal epitopes on cells was performed by ELISA inhibition assay, as we previously described [34]. The assayed cells are subjected to serial two fold dilutions in 100 μl of BSA/PBS, starting with 1×10^9 cells/ml for red blood cells and 40×10^6 cells/ml for nucleated cells. The cells in each dilution are mixed with equal volume of the monoclonal anti-Gal M86 at the final dilution of 1:100 of the antibody. This dilution of M86 is at the slope of the antibody binding curve to α-gal BSA in ELISA and it yields an absorbance of ~1.5 O.D., which represents 50% of maximum binding in ELISA. The assay is performed in two steps. First, the suspensions of cells with M86 are incubated at 4°C overnight in microfuge tubes, with constant gentle rotation. This incubation allows for maximum binding of the monoclonal anti-Gal antibody to α-gal epitopes on the assayed cells. Subsequently, the cells and bound antibody are removed by centrifugation, and 50 μl aliquots of the supernatants are placed in ELISA wells precoated with 10 μg/ml α-gal BSA. Anti-Gal M86 antibody binding to the solid phase α-gal BSA is determined by HRP-conjugated goat anti-mouse IgM and subsequent color reaction with *o*-phenylene diamine (OPD) (1 mg/ml, Sigma). Absorbance is measured at 492 nm. Since the overnight incubation results in binding of the monoclonal anti-Gal M86 antibody to cells at a level that is proportional to the number of α-gal epitopes expressed on the cells, the amount of M86 remaining in the supernatant is inversely proportional to the number of α-gal epitopes on cells, *i.e.,* the higher the number of α-gal epitopes per cell, the lower the subsequent binding of anti-Gal M86 to α-gal BSA in ELISA (*i.e.,* increased inhibition of M86 binding). By comparing the results obtained at each cell concentration with those of rabbit red blood cells (which express 2×10^6 α-gal epitopes/cells [34]), the number of α-gal epitopes per cell can be determined. Since M86 is an IgM antibody with

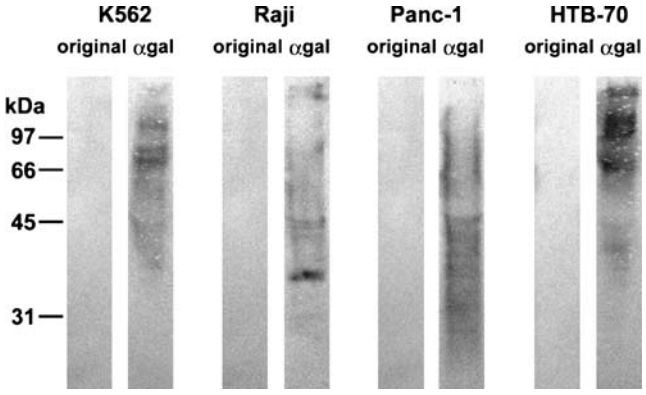

Fig. 1 Synthesis of α-gal epitopes on human tumor cell glycoproteins as assayed by Western blots stained with anti-Gal. Western blots of membrane glycoproteins of various human tumor cells, prior (*original*) or post synthesis of α-gal epitopes by neuraminidase and α1,3galactosyltransferase (*α-gal*). The α-gal epitopes were identified by binding of human anti-Gal antibody isolated from normal blood group AB serum

only part of its ten combining sites bound to α-gal epitopes on the assayed cells, the remaining free combining sites may bind to α-gal epitopes in ELISA wells, if the two steps of this assay were performed simultaneously in the ELISA wells. Thus, if the analysis would have been performed simultaneously in one step, the sensitivity of the assay would have been diminished.

Flow cytometry analysis for expression of α-gal epitopes on cells

Cells (1×10^6) in BSA/PBS are incubated with 10 μg/ml FITC-BS lectin, which specifically binds to α-gal epitopes [35], at 4°C for 30 min. Subsequently, cells are washed, fixed with 1% paraformaldehyde, and analyzed for fluorescence in a flow cytometer (Becton Dickinson, San Jose, CA).

Western blotting

The lysate of 1×10^6 cells was subjected to SDS-PAGE in a 12% polyacrylamide gel and then analyzed by Western blotting using human anti-Gal antibody for immunostaining. The protein was transferred to polyvinylidene fluoride membrane (S&S, Keene, NH) by semi-dry blotting system, and the membrane was blocked overnight with PBS containing 3% defatted milk. Membranes were incubated with 10 μg/ml human anti-Gal, and binding detected by HRP-anti-human IgG antibodies.

Results

Synthesis of α-gal epitopes on cells by α1,3galactosyltransferase

In studies preceding this work, recombinant α1,3galactosyltransferase was found capable of synthesizing α-gal epitopes on both Galβ1-3GlcNAc-BSA and Galβ1-4GlcNAc-BSA and the monoclonal anti-Gal M86 antibody displayed binding to α-gal epitopes as Galα1-3Galβ1-3GlcNAc-BSA and as Galα1-3Galβ1-4GlcNAc-BSA (not shown). These observations and previous studies on the specificity of α1,3galactosyltransferase and of the monoclonal anti-Gal antibody M86 [16–21, 24, 34] imply that the detection of *N*-acetyllactosamines and of α-gal epitopes is feasible on both type I and type II carbohydrate chains expressed on the cell surface membrane. The synthesis of α-gal epitopes by α1,3galactosyltransferase on cell surface carbohydrate chains requires the availability of uncapped *N*-acetyllactosamines as acceptors. These *N*-acetyllactosamines can be either naturally present in an uncapped form or exposed after removal of sialic acid by neuraminidase. Expression of α-gal epitopes on the enzymatically processed cells was confirmed by two independent methods:

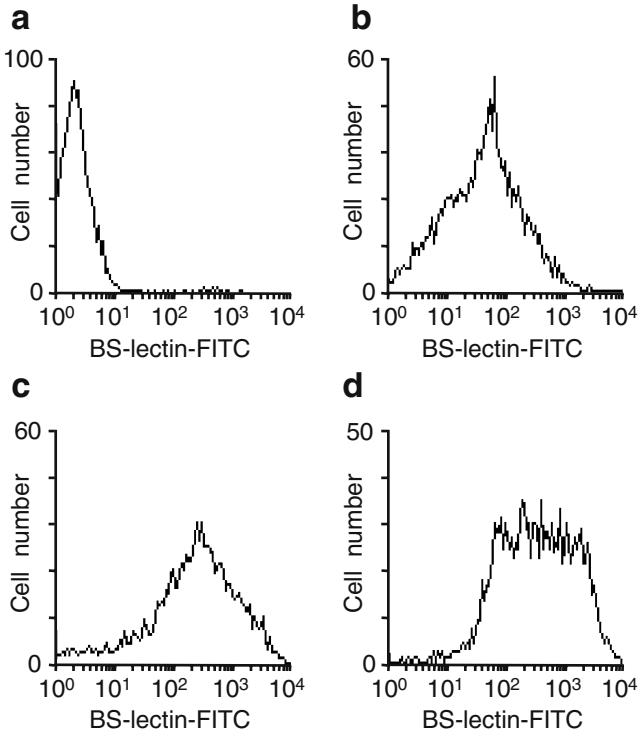

Fig. 2 Flow cytometry analysis of α-gal epitope expression on cells by staining with *Bandeiraea simplicifolia* IB_4 (*BS lectin*). (a) Raji cells prior to enzymatic treatment, (b) untreated mouse lymphocytes, (c) Raji cells treated with neuraminidase and α1,3galactosyltransferase, (d) mouse lymphocytes treated with neuraminidase and α1,3galactosyltransferase

(1) Western blots stained with human anti-Gal (Fig. 1) and (2) flow cytometry analysis with *B. simplicifolia* IB_4 (BS lectin) which binds specifically to α-gal epitopes [35] (Fig. 2). As expected, human anti-Gal did not bind to any of the glycoproteins of the human tumor cells separated by SDS PAGE gel and immunostained in Western blot (Fig. 1), confirming that these human tumor cells, like normal human cells, do not express α-gal epitopes because of the absence of endogenous α1,3galactosyltransferase activity [9, 10]. Following the incubation with both neuraminidase and α1,3galactosyltransferase, *de novo* synthesized α-gal epitopes were readily detectable in all cells as multiple bands representing a wide range of glycoproteins with this epitope (Fig. 1). This synthesis of α-gal epitopes was demonstrated on the human myeloid leukemia K562 cells, lymphoma cells Raji, pancreatic adenocarcinoma cells Panc-1 and melanoma cells HTB70. The multiple glycoproteins stained by anti-Gal in Western blots (Fig. 1) suggest that *N*-acetyllactosamines are present on a large number of different cell surface glycoproteins. Following the removal of sialic acid by neuraminidase and the exposure of the penultimate *N*-acetyllactosamines, the carbohydrate chains on these glycoproteins are subjected to synthesis of α-gal epitopes by the recombinant α1,3galactosyltransferase. Because of the very large number of glycoproteins that express α-gal epitopes following the enzymatic treatment, some of the blot sections give the impression of a "smear" where many glycoproteins binding anti-Gal are close to each other.

The synthesis of α-gal epitopes was further confirmed by flow cytometry analysis following the staining with BS lectin (Fig. 2). One example is the human Raji B lymphoma cell line. As other human cells, Raji cells lack α-gal epitopes and therefore, do not bind BS lectin (Fig. 2a). These cells displayed extensive staining by BS lectin following incubation with neuraminidase and α1,3galactosyltransferase, (Fig. 2c). Non-primate mammalian cells such as mouse cells, naturally express α-gal epitopes [10, 36]. These cells display increased numbers of these epitopes following incubation with neuraminidase and α1,3galactosyltransferase. This is exemplified with mouse lymphocytes (Fig. 2b and 2d). These lymphocytes express endogenous α-gal epitopes [34] and thus bind BS lectin (Fig. 2b). The mean fluorescence intensity, indicating the binding level of this lectin, increased by eight fold after these cells were incubated with neuraminidase and α1,3galactosyltransferase (Fig. 2d). This increase in α-gal epitope expression was the result of α-gal epitope synthesis on naturally uncapped *N*-acetyllactosamines and on additional *N*-acetyllactosamines that are capped with sialic acid, which become acceptors to α1,3galactosyltransferase following desialylation by neuraminidase.

The optimum concentration of α1,3galactosyltransferase (*i.e.*, concentration allowing for maximum synthesis of α-gal epitopes) was determined by performing α-gal epitopes synthesis on human red blood cells at various enzyme

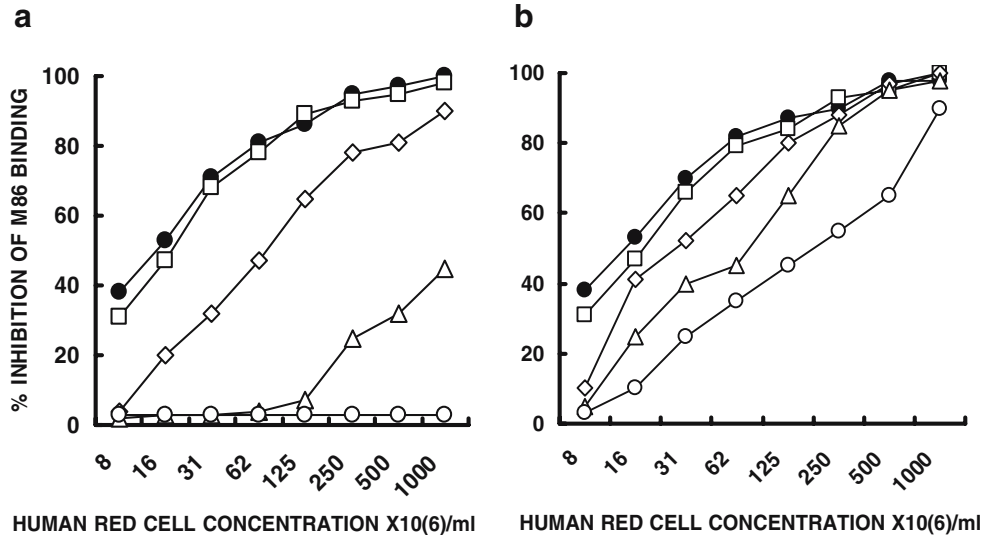

Fig. 3 Kinetic studies on synthesis of α-gal epitopes on human red blood cells as measured by the ELISA Inhibition Assay. (a) Analysis of α-gal epitope synthesis with α1,3galactosyltransferase at the following concentrations of the enzyme: *open circle* 0.3 μg/ml; *open triangle* 1 μg/ml; *open diamond* 3 μg/ml; *open square* 10 μg/ml; *filled circle* 30 μg/ml. Neuraminidase concentration was 1 mU/ml. Note that similar numbers of α-gal epitopes are synthesized with 10 and 30 μg/ml of α1,3galactosyltransferase. (b) Analysis of α-gal epitope synthesis on human red blood cells after different incubation periods with 30 μg/ml of α1,3galactosyltransferase and 1 mU/ml of neuraminidase: *open circle* 2 min; *open triangle* 10 min; *open diamond* 30 min; *open square* 60 min; *filled circle* 120 min. Note that the maximum number of α-gal epitopes is synthesized within the first hour of incubation

concentrations for 2 h and subjecting these cells to the ELISA inhibition assay (Fig. 3a). As described in "Experimental procedures," the extent of inhibition of anti-Gal M86 binding in the ELISA wells (presented as percent inhibition) is proportional to the number of α-gal epitopes expressed on the cells that were incubated overnight with this antibody. Neuraminidase concentration in these assays was 1 mU/ml. Whereas incubation of the red blood cells with 0.3 μg/ml of α1,3galactosyltransferase resulted in no measurable synthesis of α-gal epitopes during the 2 h incubation period, incubation with a 30 fold higher concentration (10 μg/ml) reached the maximum α-gal epitope synthesis. This can be inferred from the finding that incubation with 30 μg/ml enzyme did not increase the amount of synthesized α-gal epitopes above the level observed with 10 μg/ml of the enzyme (Fig. 3a). Based on these findings we chose the concentration of 30 μg/ml as the enzyme concentration used in the assays below, in order to provide a level of α1,3galactosyltransferase activity, sufficient to run the reaction to completion. The incubation for 2 h with 30 μg/ml of α1,3galactosyltransferase indeed resulted in maximum synthesis of α-gal epitopes, as no difference was observed in the amount of α-gal epitopes synthesized within 2 h incubation period at that enzyme concentration, in comparison to red blood cells subjected to the enzyme activity only for 1 h (Fig. 3b). Additional incubation for a total of 3 h yielded the same amount of α-gal epitopes as observed in 2 h incubation (not shown). Similarly, increasing neuraminidase concentration from 1 mU/ml to 5 or 10 mU/ml, or increasing UDP-Gal concentration to >1 mM did not increase the extent of α-gal epitope synthesis (not shown).

Profiling carbohydrate epitopes on red blood cells

The profile of naturally uncapped *N*-acetyllactosamine and those capped with sialic acid, or capped with α1,3galactosyls (*i.e.*, α-gal epitopes) on red blood cells was determined by performing the synthesis of α-gal epitopes in presence or absence of neuraminidase and subsequently measuring α-gal epitope synthesis in the ELISA inhibition assay. The curves of percent inhibition of M86 binding to α-gal BSA in ELISA wells were compared with that of percent inhibition curve measured with rabbit red blood cells. These red blood cells express ~2×10^6 α-gal epitopes per cell [34]. Three ELISA inhibition assays were performed with each cell type: (1) An assay performed with untreated cells to measure the natural expression of α-gal epitopes on the cells; (2) An assay performed with cells incubated with α1,3galactosyltransferase, enabling the identification of uncapped *N*-acetyllactosamines by subtracting the number of α-gal epitopes on untreated cells from that measured

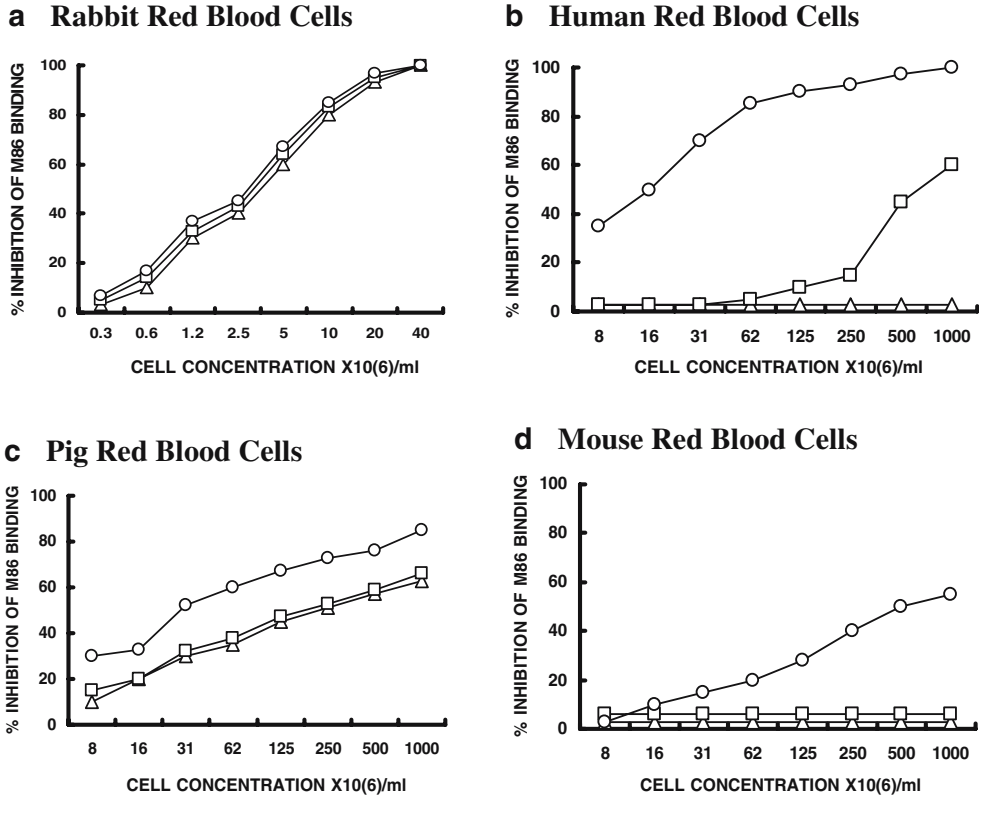

Fig. 4 Profiling carbohydrate chains on red blood cells from various species. Red blood cells prior to enzymatic treatment (*open triangle*); red blood cells incubated with α1,3galactosyltransferase (*open square*); red blood cells incubated with neuraminidase and α1,3galactosyltransferase (*open circle*)

with cells incubated with α1,3galactosyltransferase; (3) An assay performed with cells treated with both neuraminidase and α1,3galactosyltransferase in which the number of *N*-acetyllactosamine capped with sialic was calculated by subtracting the results obtained with cells treated only with α1,3galactosyltransferase (*i.e.,* naturally expressed α-gal epitopes and α-gal epitopes synthesized on uncapped *N*-acetyllactosamines) from the results with cells incubated with the two enzymes. The percent inhibition binding curves measured with rabbit, human, pig and mouse red blood cells are presented in Fig. 4 and demonstrate the variations in carbohydrate profile in various species. The calculated numbers of epitopes on the various cells are summarized in Table 1.

Rabbit red blood cells

Rabbit red blood cells (Fig. 4a) are unique among mammalian red blood cells in that they express relatively large amounts of α-gal epitopes (2×10^6 epitopes/cell [34]) which are present both on glycolipids [37–39] and glycoproteins. This number of α-gal epitopes per cell is based on early studies which measured binding of radiolabeled (iodinated) *B. simplicifolia* IB4 lectin (specific for α-gal epitopes [35]) and calculation of epitope number per cell by Scatchard plot analysis [10]. Rabbit red blood cells subjected to α-gal epitope synthesis by incubation only with α1,3galactosyltransferase, or with neuraminidase and α1,3galactosyltransferase displayed no changes in the percent inhibition curve of M86 binding. This implies that no uncapped *N*-acetyllactosamine epitopes, or *N*-acetyllactosamine epitopes capped with sialic acid, were detected by this analysis. These data imply that most, and possibly all *N*-acetyllactosamines on the rabbit red cell membranes are present as α-gal epitopes.

Human red blood cells

As expected, human red blood cells (Fig. 4b) express no α-gal epitopes. This is indicated by the complete lack of M86 binding to these cells (*i.e.,* 0% inhibition) even when the antibody was incubated overnight with 1×10^9 human red blood cells/ml. However, these red blood cells have ~1×10^4 naturally uncapped *N*-acetyllactosamines. This is inferred from the synthesis of α-gal epitope following incubation with α1,3galactosyltransferase, as measured in the ELISA inhibition assay. The subsequent concentration of red blood cells yielding 50% inhibition of M86 binding

Table 1 Estimated numbers of terminal epitopes on *N*-linked carbohydrate chains on cell surface

Cells	Number of epitopes ($\times10^6$/cell)		
	α-gal epitopes	*N*-acetyllactosamine	SA-*N*-acetyllactosamine
Human RBC	0[a]	0.008	0.4
Rabbit RBC	2	0	0
Pig RBC	0.016	0	0.12
Dog RBC	0.008	0.008	0.8
Bovine RBC	0.008	0	0.4
Goat RBC	0	0	2
Sheep RBC	0	0	0.16
Horse RBC	0	0	<0.008
Mouse RBC	0	0	0.016
Chicken RBC	0	0	2
Rabbit Lymphocytes	0.8	0.8	1.0
Mouse Lymphocytes	<0.2	0	0.2
K562 (human leukemia)	0	<0.2	5
Raji (human lymphoma)	0	0.8	12
HeLa (human cervical carcinoma)	0	0.3	3.7
Panc-1 (human pancreatic carcinoma)	0	1.6	7
HTB-70 (human melanoma)	0	0	27
BL6 (mouse melanoma)	0	0	4
B16 (mouse melanoma)	0	0	10
PK15 (pig kidney)	13	0	40
MDBK (bovine kidney)	13	0	27
MDCK (dog kidney)	0.2	0.2	2.8
COS-1 (monkey kidney)	0	3.2	10
HEK293 (human kidney)	0	<0.2	1.6
Mouse NIH/3T3 fibroblasts	1	1	1.5
Human fibroblast	0	1.0	20

RBC Red blood cells, *SA-N-acetyllactosamine* *N*-acetyllactosamine capped with sialic acid

[a] The value 0 indicates that the number of epitopes is lower than the limit of detection which is 8,000 per cell.

was 200 fold higher than that of rabbit red cells inhibiting 50% of M86 binding (~3×10^6 cells/ml, displayed in Fig. 4a). The extent of inhibition by the human red cells increased by ~50 fold when the assayed red blood cells were incubated with both neuraminidase and α1,3galactosyltransferase, in comparison with those incubated only with α1,3galactosyltransferase. This implies that the number of *N*-acetyllactosamine chains capped with sialic acid is 50 fold higher than that of uncapped *N*-acetyllactosamines. This inhibition was five fold lower than that measured with rabbit red blood cells (50% inhibition at 15×10^6 human red cells/ml *vs.* 50% inhibition at 3×10^6 rabbit red cells/ml), implying that ~4×10^5*N*-acetyllactosamines were exposed by neuraminidase removing sialic acid and served as acceptors for α1,3galactosyltransferase.

Pig red blood cells

These red blood cells (Fig. 4c) express α-gal epitopes at a level that is 250 fold lower than that on rabbit red blood cells (*i.e.,* ~1.6×10^4 α-gal epitopes/cell). The number of these epitopes did not change after the cells were incubated with α1,3galactosyltransferase, implying the absence of uncapped *N*-acetyllactosamine. However, the number of α-gal epitopes increased by ~9 fold when these red blood cells were incubated with both neuraminidase and α1,3galactosyltransferase, implying expression of ~1.2×10^5 sialic acid residues/cell capping *N*-acetyllactosamines (Table 1).

Mouse red blood cells

Whereas various mouse nucleated cells express α-gal epitopes [10, 36], no such epitopes were detected by the immuno-enzymatic assay on mouse red blood cells (Fig. 4d). Similarly, these red blood cells lack free *N*-acetyllactosamine residues. Upon removal of sialic acid by neuraminidase, ~1.6×10^4 α-gal epitopes were synthesized by α1,3galactosyltransferase on these red blood cells (Fig. 4d; Table 1).

Red blood cells from various species

Profiling of α-gal epitope expression on other species revealed low expression of these epitopes on bovine red blood cells (Table 1), although many α-gal epitopes were reported to be present on bovine red blood cell glycolipids [9, 40–42]. The low number of α-gal epitopes detected by M86 on the intact red blood cells suggests that most of the α-gal epitopes on glycolipids may not be accessible to the monoclonal anti-Gal binding as they may be "buried" among the longer chains of cell surface glycoproteins. Interestingly, red blood cells from goat, sheep and horse express no α-gal epitopes, although these epitopes are expressed in significant amounts on nucleated cells in these species [10, 43]. Horse red blood cells displayed even lower numbers of sialic acid capped *N*-acetyllactosamines than those measured in mice (Table 1). It may be possible that red blood cells displaying low numbers of the assayed epitopes (*e.g.,* horse, sheep and goat) also have many *N*-acetyllactosamines, however, these carbohydrate chains are capped with various carbohydrate units that cannot be removed by *V. cholerae* neuraminidase.

The only non-mammalian red blood cells assayed were those of chicken. In accord with previous findings on the lack of α-gal epitopes in non-mammalian vertebrates [9, 10], chicken red blood cells were found to be devoid of this epitope (Table 1). These red blood cells also lack uncapped *N*-acetyllactosamine residues, as no α-gal epitopes were synthesized on chicken red blood cells incubated with α1,3galactosyltransferase and UDP-Gal. However, as many as 2×10^6 α-gal epitopes were synthesized on chicken red blood cells that were de-sialylated by neuraminidase, implying that this is likely to be the number of *N*-acetyllactosamines capped with sialic acid.

Profiling carbohydrate chains on nucleated cells

The profiles of capped or uncapped *N*-acetyllactosamines on nucleated cells greatly differ from those on red blood cells of the same species. One example is that of rabbit red blood cells and rabbit lymphocytes. The expression of α-gal epitopes on rabbit lymphocytes is ~60% lower than that on rabbit red blood cells (0.8×10^6 epitopes/lymphocyte *vs.* 2.0×10^6 epitopes/red blood cells), although both cells are from the same hematopoietic origin (Table 1). Moreover, rabbit red blood cells have no detectable uncapped *N*-acetyllactosamines, or sialic acid capped *N*-acetyllactosamines, whereas rabbit lymphocytes were found to express ~0.8×10^6 uncapped acetyllactosamine residues per cell and a similar number of *N*-acetyllactosamines capped with sialic acid. In contrast to the rabbit lymphocytes, mouse lymphocytes do not express uncapped *N*-acetyllactosamine epitopes. Differences in the profile were observed also with mouse red blood cells and lymphocytes. Whereas mouse red blood cells lack α-gal epitopes, such epitopes are readily detectable on mouse lymphocytes at approximately 1.5×10^5 epitopes/cell (Table 1).

As expected, human fibroblasts and human tumor cells do not express α-gal epitopes, since humans lack active α1,3galactosyltransferase. However, the number of uncapped *N*-acetyllactosamines varied in the human cells assayed. Human fibroblasts (grown from foreskin) had 1.0×10^6 such epitopes per cell (Fig. 5a) and the pancreatic tumor cell line Panc-1 had as many as 1.6×10^6 uncapped *N*-acetyllactosamine epitopes per cell (Fig. 5b; Table 1). The K562 human leukemia cells, Raji human lymphoma

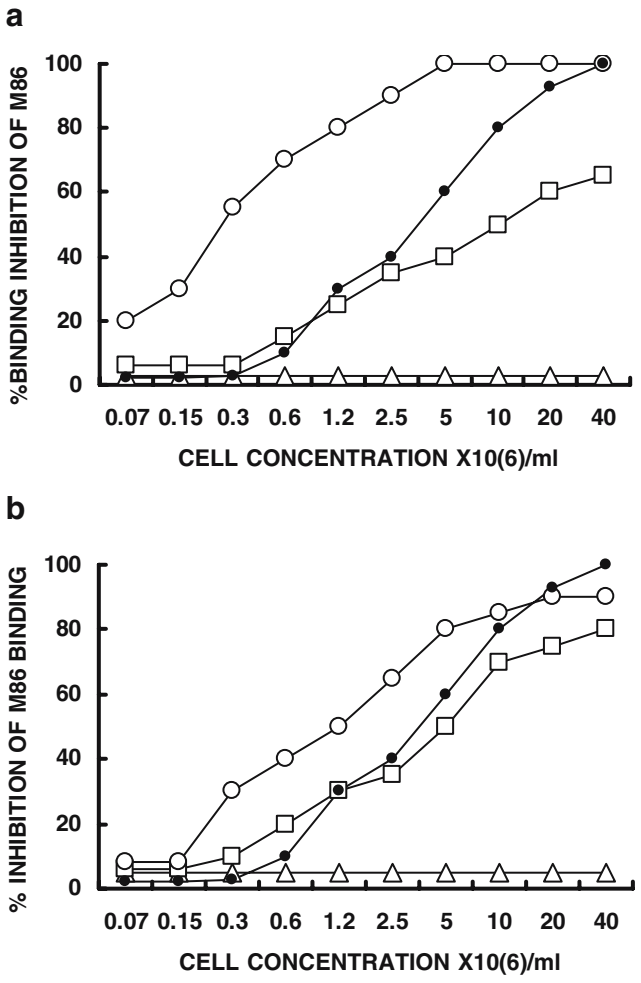

Fig. 5 Examples of carbohydrate chain profiling on human nucleated cells. (a) Human fibroblasts, (b) human pancreatic cell line Panc-1. Cells prior to enzymatic treatment (*open triangle*); cells incubated with α1,3galactosyltransferase (*open square*); cells incubated with neuraminidase and α1,3galactosyltransferase (*open circle*); rabbit red blood cells as standard (*filled circle*)

cells and HeLa human cervical carcinoma cells displayed $<1.0\times10^6$ uncapped *N*-acetyllactosamine epitopes per cell (Table 1). The number of *N*-acetyllactosamines capped with sialic acid on human tumor cells was consistently high. Based on measuring of *de novo* synthesis of α-gal epitopes following desialylation by neuraminidase, human fibroblasts displayed the expression of as many as 20×10^6 sialic acid capped *N*-acetyllactosamines per cell (Fig. 5a; Table 1) and Panc-1 human pancreatic adenocarcinoma cells expressed 7×10^6 epitopes/cell (Fig. 5b; Table 1). Most other tumor cells displayed sialic acid capped *N*-acetyllactosamines ranging from 2.7×10^6 to 27×10^6 epitopes/cell, with the human melanoma cell line HTB-70 displaying the highest number of such epitopes. Mouse melanoma B16 also expresses a high level of *N*-acetyllactosamine capped with sialic acid (10×10^6 epitopes per cell). Interestingly, B16–BL6, which is a highly tumorigenic subclone of mouse B16 melanoma [44] displayed 60% less sialic acid residues on *N*-acetyllactosamine residues than the parental less tumorigenic clone (Table 1).

The great variations in carbohydrate epitope expression on cells from the same organ, but originating in different species, are demonstrated in Table 1 and in Fig. 6b, with a collection of non-malignant kidney cell lines from various mammals. Pig kidney PK15 cells and bovine kidney MDBK cells express α-gal epitopes as 13×10^6 epitopes per cell, and *N*-acetyllactosamine capped with sialic acid at even higher level (40×10^6 epitopes per cell on PK15 and 27×10^6 epitopes per cell on MDBK). Both cells did not display uncapped *N*-acetyllactosamines (Table 1). In contrast, the dog kidney MDCK cells express α-gal epitopes and *N*-acetyllactosamine capped with sialic acid at a much lower level, as 0.2×10^6 and 2.8×10^6 epitopes/cell, respectively. However, these cells also express 0.2×10^6 uncapped *N*-acetyllactosamines per cell. African green monkey kidney COS-1 cells express as many as 3.2×10^6 uncapped *N*-acetyllactosamine/cell, and 1×10^7*N*-acetyllactosamines capped with sialic acid. Analysis of these epitopes on human kidney HEK293 cells revealed a much lower

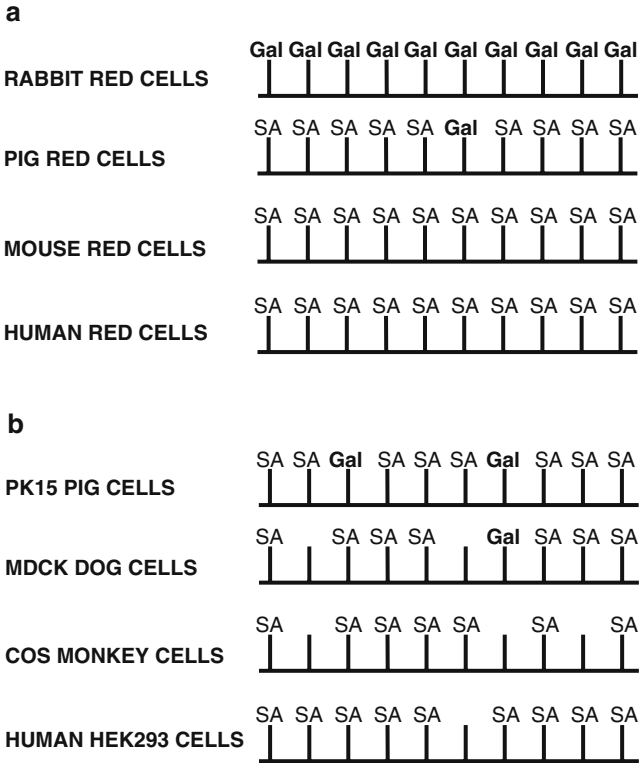

Fig. 6 Schematic presentation of carbohydrate profile on red blood cells and nucleated cells from various species. Ten *N*-acetyllactosamine units linked via carbohydrate chains to the membrane (*vertical line*), are either uncapped, capped with sialic acid (*SA*), or capped by terminal α1-3galactosyls to form α-gal epitopes (*Gal*). The profile is based on the relative proportion of each of these epitopes measured on: (a) Red blood cells, or (b) Kidney cells, from various species

expression of both capped and uncapped *N*-acetyllactosamines (Table 1). As expected, monkey and human kidney cells do not express α-gal epitopes.

Discussion

The present study demonstrates a novel immuno-enzymatic assay for profiling *N*-acetyllactosamines on cell surface glycans. This method does not require the isolation of each oligosaccharide, but exploits the enzymatic specificity of recombinant α1,3galactosyltransferase for synthesis of α-gal epitopes on *N*-acetyllactosamines [21, 24, 45, 46]. This method measures the number of existing, or *de novo* synthesized α-gal epitopes on *N*-acetyllactosamines by an assay that is based on the high specificity of the monoclonal anti-Gal antibody M86 to the α-gal epitope [34]. This method enables the profiling of three types of terminal carbohydrate structure: α-gal epitopes, uncapped *N*-acetyllactosamines, and *N*-acetyllactosamines capped with sialic acid. This analysis requires two reagents produced in our lab, the recombinant α1,3galactosyltransferase and the monoclonal anti-Gal antibody. It should be stressed that this non-radioactive profiling can be performed also with other types of epitopes, by the use of a glycosyltransferase specific for the assayed acceptor and an antibody or lectin that bind specifically to the epitope synthesized *de novo* on the cells assayed.

The immuno-enzymatic assay measuring α-gal epitopes may not be accurate enough to serve as an analytical tool. However, the assay provides information on the relative expression of α-gal epitopes, free *N*-acetyllactosamine, and *N*-acetyllactosamine capped with sialic acid (Fig. 6). The assay further allows for rough estimation of the number of these epitopes on the assayed cells (Table 1). By performing this assay with many types of cells, we could confirm a well established fact that the majority of *N*-acetyllactosamines on most cells are capped with sialic acid (Table 1; Fig. 6). This is implied from the limited synthesis of α-gal epitopes on cells that were not subjected to desialylation by neuraminidase *vs.* the much higher synthesis of this epitope following incubation with both neuraminidase and α1,3galactosyltransferase. In addition to their detection by binding of mouse monoclonal anti-Gal M86, the *de novo* synthesized α-gal epitopes on cells incubated with these two enzymes were readily detectable by the binding of human anti-Gal antibody in Western blot analysis (Fig. 1). These Western blots demonstrated the expression of the carbohydrate chains on multiple glycoproteins of different sizes.

Whereas this profiling method was found to be effective in all nucleated cells assayed, some red blood cells yielded unexplainable results, such as the very low numbers of uncapped *N*-acetyllactosamines, or of *N*-acetyllactosamines capped with sialic acid on mouse, goat or horse red blood cells. It is possible that this is the result of expression of other structures that cannot be detected by this method, *e.g.*, abundance of Forssman antigen, or sialic acid units that cannot be cleaved by *V. cholerae* neuraminidase. Alternatively, it may be possible that cross linking by the glutaraldehyde fixation in these particular red blood cells prevents subsequent access of the enzymes and/or of the antibody to the carbohydrate chains. Additional studies are required for evaluating this issue. However, in the absence of fixation, red blood cells are lysed during the washes subsequent to the enzyme reaction, possibly due to osmotic shocks, whereas nucleated cells can "adapt" much more effectively to the various buffers.

Identification of cell surface *N*-acetyllactosamines, by using them as acceptors for a glycosyltransferase has been previously described also by Whiteheart *et al.* [47, 48] who used a sialyltransferase-mediated radiolabeling system. According to that method, radiolabeled CMP-sialic acid is used as a sugar donor. The present method allows for profiling which does not require the use of a radioactive sugar donor and expands the analysis also to α-gal epitopes. The profile of cell surface carbohydrate epitopes may be further expanded by the use of additional enzymes cleaving terminal carbohydrate units other than sialic acid and exposing the penultimate *N*-acetyllactosamines (*e.g.,* fucosidase to remove terminal fucosyl in blood group O carbohydrate chains) which are then subjected to synthesis of α-gal epitopes.

The analysis of carbohydrate profiles on the many types of cells demonstrated the wide diversity of carbohydrate chain expression on cells from various species (Table 1; Fig. 6). Two extreme examples for such differences are rabbit and human red blood cells (Fig. 6a). Whereas rabbit red blood cells were found to express only α-gal epitopes, >90% of the *N*-acetyllactosamines on human red blood cells are capped with sialic acid. With the exception of rabbit red blood cells, red blood cells from the various species display no, or very little expression of α-gal epitopes, and relatively few uncapped *N*-acetyllactosamines (Table 1). It may be possible that the erythropoietic cells in the rabbit bone marrow are devoid of sialyltransferases capping *N*-acetyllactosamines. In contrast, these erythropoietic cells have a high α1,3galactosyltransferase activity [16], ultimately resulting in capping of the cell surface *N*-acetyllactosamines with terminal α-galactosyls to form α-gal epitopes (Fig. 6a).

It was expected that human and chicken red blood cells lack α-gal epitopes, as these species are devoid of α1,3galactosyltransferase activity [9, 10]. However, goat, sheep, horse and mouse red blood cells were also found to lack α-gal epitopes, despite the expression of this epitope

on a variety of nucleated cells in these species, including lymphocytes produced from the same hematopoietic origin [10, 34]. The lack of this epitope on red blood cells may be the result of either diminished α1,3galactosyltransferase activity in erythropoietic cells in these species, or because of a much higher effective activity of a competing glycosyltransferase, such as sialyltransferase.

The very low expression of α-gal epitopes on untreated bovine red blood cells (Table 1) was an unexpected finding since these red blood cells have α-gal epitopes on neutral glycolipids and on bi-antennary gangliosides [9, 40–42]. The relatively low binding of the monoclonal anti-Gal M86 antibody to intact bovine red cells raises the possibility that this antibody binds preferentially to α-gal epitopes on cell surface glycoproteins and much less, or not at all, to this epitope on glycolipids. In ELISA assays, this monoclonal antibody was found to bind equally well to α-gal epitopes on glycolipids such as ceramide pentahexoside (Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer), and on glycoproteins [34]. However, since anti-Gal M86 is a large size IgM immunoglobulin (m.w. 950,000) and since carbohydrate chains on glycolipids are usually "buried" among the much larger glycoprotein molecules, it is possible that many of the α-gal epitopes on glycolipids are not accessible to this antibody when expressed on cell surface membranes. Based on these considerations, it is possible that the proposed method is preferentially profiling cell surface *N*-acetyllactosamine on glycoproteins, as they may be much more accessible to enzymatic manipulation and detection by anti-Gal M86 binding.

The ability of this method to detect differences in carbohydrate epitope profiles on cells of same tissue origin, but from different species, was demonstrated with non-malignant kidney cell lines (Table 1; Fig. 6b). In accord with the lack of endogenous α1,3galactosyltransferase, human HEK293 kidney cells and African green monkey COS-1 kidney cells lack α-gal epitopes. Although both cells express uncapped *N*-acetyllactosamines and *N*-acetyllactosamine capped with sialic acid, the number of these epitopes was found to be approximately ten times higher in monkey COS-1 cells than in the human HEK293 cells, despite similarity in the size of these cells. The α-gal epitope is abundantly expressed on pig PK15 kidney cells and on bovine MDBK kidney cells (13×10^6 per cell in both cell types). These two types of kidney cells also express a higher number of *N*-acetyllactosamine capped with sialic acid (40×10^6 and 27×10^6 per cells, on pig and bovine cells, respectively). In contrast, the dog kidney cells MDCK express much lower numbers of both α-gal epitopes and of *N*-acetyllactosamines capped by sialic acid. It may be possible that either there are fewer glycans on dog kidney cells, or that many of the carbohydrate chains are capped with other epitopes which are not detectable by this immuno-enzymatic assay.

Overall, this study demonstrates many variations in *N*-acetyllactosamine and in carbohydrates capping it on mammalian cell glycans. Correlation between functional changes in cells at different stages of differentiation and the profile of carbohydrate epitopes, as described in the present study, may ultimately help to identify specific associations between pattern of carbohydrate expression and certain functions in the assayed cells.

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