Determination of Glycoconjugate Residues of Erythrocytes at Different Age Groups of Rats

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ABSTRACT

Aging is a time-dependent process that contains cell injury caused by molecular damages and eventually functional impairment of tissues and organs. The possible roles of cell surface carbohydrates, which are very important molecules in cell to cell and/or cell to extracellular matrix recognition, on aging process are not yet clear. In this study, glycoconjugate alterations of membrane glycoproteins of erythrocytes in aging organism were evaluated with lectin histochemistry and lectin blotting studies in 1, 4 and 7 months old rats. Lectin histochemistry results indicated that α(2→3) and α(2→6)-linked sialic acids are intensively found in erythrocyte membranes, but this intensity of sialic acids decreases with age. Similar evidences were obtained from lectin blotting studies which performed with same lectins. These results suggest that sialic acid reactivity alters with the age of organism. We thought that (a) sialic acid containing glycoconjugates altered not only with erythrocyte senescence, but also aging process of organism or (b) the number of sialic acid containing erythrocytes decreased by age.

Keywords: Erythrocyte, Glycoconjugates, Lectin, SDS-PAGE, Sialic acid, Aging

ÖZET

Farklı Yaş gruplarına Ait Rat Eritrositlerinin Glukokonjugat İçeriklerinin Belirlenmesi

Yaşlanma, moleküler bozulmaların neden olduğu hücre hasarını ve bunu takiben doku ve organların fonksiyonel olarak zayıflamasını içeren, zamana bağlı bir süreçtir. Hücre-hücre ve/veya hücre-ekstrasellüler matriks arasındaki tanıma olaylarında önemli moleküller olan hücre yüzey karbohidratlarının yaşlanma sürecindeki olası rolleri hakkında yeterli bilgi bulunmamaktadır. Bu çalışmada, eritrositlerde membran glikoproteinlerine ait glukokonjugatların organizma yaşlanması sürecindeki değişiklikleri 1, 4 ve 7 aylık ratlarda lektin histokimyası ve lektin blotting yöntemleri kullanılarak belirlenmeye çalışıldı. Lektin histokimyası sonuçları, α(2→3) ve α(2→6) bağlı sialik asitlerin eritrosit membranlarında yoğun olduğunu, ancak bu yoğunluğun yaş ile birlikte belirgin bir azalmaya gittiğini göstermiştir. Aynı lektinler kullanarak yapılan blotting çalışmalarında benzer bulgular elde edilmiştir. Bu sonuçlar, sialik asit reaktivitesinin organizmanın yaşlanmasına bağımlı olarak azaldığını göstermektedir. Bu durum, (a) sialik içeren glukokonjugatların sadece eritrosit yaşlanması ile değil aynı zamanda organizmanın yaşlanması sürecinde de değişim gösterdiğini ya da (b) sialik asit içeren eritrosit sayısının yaşlanma ile azaldığını düşündürmüştür.

Anahtar Kelimeler: Eritrosit, Glukokonjugatlar, Lektin, SDS-PAGE, Sialik asit, Yaşlanma

INTRODUCTION

Glycan moieties of glycoconjugates, known as fundamental energy sources and building blocks of complex biological systems, are compose integral parts of many plasma and secretory proteins.[1,2] They are found in cell surfaces[3-7], cytoplasm or organelles as well as intercellular areas or fluids[8] and play crucial roles in recognition phenomena.

Sialic acids are the most important terminal sugars that play a crucial role in the achieving functional properties of carbohydrates. They belong to a diverse family of closely related nine-carbon sugars that contain 40 members and derivated from N-acetylneuraminic acids, N-glycolylneuraminic acids and deaminoneuraminic acids.[9,10] Sialic acids can be found in many glycan types such as glycoproteins, glycolipids, glycosaminoglycans and proteoglycans[11] and play a critical roles in the regulation of cell-to-cell and/or cell-extracellular matrix interactions due to their negatively charges at physiological pH.[8,9,12-14] They have dual function in recognition phenomena: they can either serve as antigenic determinants in protein-protein interactions or mask the recognition sites and therefore inhibit receptor-ligand interactions.[15] Sialic acids in erythrocyte membranes play important roles in maintaining the cell integrity and survival as well as keeping the erythrocytes in circulation.[16-19] It is suggested that amount of sialic acids in erythrocyte membranes diminished by erythrocyte senescence[20], probably in consequence of alteration of cell surface glycoproteins during the cell aging.[21,22] Nevertheless, the possible glycoconjugate alterations of erythrocytes obtained from aging organisms remain be unsolved.

The aim of this study was to evaluate the alterations in glycoconjugate patterns of erythrocytes from 1, 4 and 7 month old rats by lectin histochemistry and lectin blotting studies.

MATERIAL AND METHODS

Preparation of Erythrocytes and Membranes

One (n=16), four (n= 12) and seven (n= 12) months old rats were used respectively with permission of Celal Bayar University Medical Faculty Animal Research Ethic Council (Protocol no: 2007/0025). Blood samples were obtained by intracardiac puncture and collected to EDTA containing tubes. After the centrifugation at 3000 rpm for 5 min at +4^{0}C, supernatant and buffy coat were removed by aspiration and erythrocyte washing solution was added to pellet. These steps were repeated three times at same conditions. The washed and separated erythrocyte mass divided in two fragments. One portion diluted with isotonic NaCl at 1:3 ratio, smeared to slides and fixed with methanol for the lectin histochemistry. Other fraction of erythrocyte mass was lysed by addition of ice-cold membrane preparation reagent in a ratio 1:20, (vol/vol) (5 mM Tris-HCl, 0.1 mM EDTA, pH 7.4). In order to isolation of erythrocyte membranes, lysates were centrifuged at 15000 rpm for 20 min at +4^{0}C, supernatant was removed, and instead membrane preparation reagent was added. These steps were repeated a few times until the erythrocyte membranes extracted.[23] Thereafter, extracted membranes scaled and stored at –86^{0}C until the employ for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Lectin Histochemistry

In order to identify the glycoconjugates within the erythrocytes, a panel of digoxigenin (DIG) labelled lectins, a series of plant derived non-immune proteins possessing binding sites for different types of mostly specific terminal carbohydrate residues of mammalian cells, (DIG Glycan Differentiation Kit, Cat# 11210238001) was used (Table 1). Briefly, slides were blocked with 10% (w/v) blocking buffer (Roche Applied Science) in Tris-buffered saline

Table 1. Data concerning the lectins used in this study

Lectins	Binding specificity	Reference
Maackia amurensis agglutinin Lectin (MAA)	NeuNAcα(2→3Gal	24
Sambucus nigra agglutinin Lectin (SNA)	NeuNAcα(2→6)Gal	25
Peanut agglutinin Lectin (PNA)	Galβ(1→3)GalNAc	26

NeuNAc= N-acetylneuraminic acid; Gal= galactose; GalNAc= N-acetylgalactosamine

(TBS)/ 0.5% Tween 20, pH 7.5 for 30 min and washed 2 times with TBS (pH 7.5) and 1 times buffer 1 [TBS, 1mM $MgCl_2$ (Fluka), 1mM $MnCl_2$ (Sigma), 1mM $CaCl_2$ (Sigma), pH 7.5] for 10 min each step. Afterwards, slides were washed with TBS and incubated with buffer-1 diluted DIG labelled Maackia amurensis agglutinin (MAA, 10 μg/ml), Sambucus nigra agglutinin (SNA, 20 μg/ml) and peanut agglutinin (PNA, 10 μg/ml) lectins (DIG Glycan Differentiation Kit, Cat# 11210238001, Roche Applied Science) in a humidified chamber for 1 h, washed two times with TBS and incubated with 1 μl/ml anti-DIG-alkaline phosphatase (AP) (Roche Applied Science) for 1 h. After washing with TBS, sections were incubated with 20 μl/ml nitro-blue-tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) staining reagent (Roche Applied Science) in buffer 2 (0.1 M Tris-HCl, 0.05 M $MgCl_2$, 0.1 M NaCl, pH 9.5) for 3-5 min. Reaction was stopped with ultra pure distilled water when dark-brown staining appeared. All the steps above were performed at room temperature.

Control of Lectin Histochemistry

MAA lectin and a control glycoprotein, fetuin (Roche Applied Science), were mixed in a ratio of 1:2.5 (10 μg/ml MAA: 25 μg/ml fetuin). In order to confirm of accuracy of the method, some slides were incubated with MAA-fetuin mix prior to lectin histochemistry.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed on a 4% stacking gel and a 7.5% separating gel according to the discontinuous system of Laemmli.[27] Gels were stained with 25% Coomassie Brillant Blue (CBB, Sigma).

Lectin Blotting

After the SDS-PAGE, gels were sandwiched with wetted 0.2 μm pore size polyvinylidene difluoride (PVDF) membranes (ImmunBlot®, Bio-Rad) supported by filter papers and pads on each side. Blotting was carried out at 100 V, 350 mA for 2 h with a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) according the standard procedure.[28] Blotted membranes were stained with 0.1% Ponceau S (Sigma). In order to determine cell surface glycoconjugates in erythrocyte of rats at different age groups, PVDF membranes were incubated with three different DIG labelled plant lectins, MAA, SNA and PNA, (DIG Glycan Differentiation Kit, Cat#11210238001, Roche Applied Science), according to the manufacturer's instructions, as in lectin histochemistry (Table 1). Briefly, PVDF membranes were blocked with 10% (w/v) blocking buffer (Roche Applied Science) in TBS/0.5% Tween 20, pH 7.5 for 30 min at room temperature and washed with TBS (pH 7.5) and buffer 1 for 10 min each step. MAA, SNA and PNA lectins were diluted with buffer 1 in a ratio of 1:200, 1:1000 and 1:100, respectively. Thereafter, PVDF membranes were incubated with pre-diluted lectins for 1 h at room temperature, washed three times with TBS for 10 min each step, and incubated with 1 μl/ml anti-digoxigenin-AP, in TBS for 1 h. After washing with TBS, in order to determine bound lectins, PVDF membranes were stained with 20 μl/ml NBT/BCIP reagent (Roche Applied Science) in buffer 2 until the bands become visible, and reaction was halted with ultra pure distilled water.

Controls of Lectin Blotting

Two different negative controls were accomplished. First, erythrocyte membrane proteins obtained from 1 month old rats were incubated with 1U/ml of Type X *Clostridium perfiringens* neuraminidase (Sigma), which hydrolyses α(2→3), α(2→6) and α(2→8) glycosidic linkages of terminal sialic acids, at 38°C for 45 min. Enzyme treated protein and a control glycoprotein, fetuin, were subjected SDS-PAGE and transferred to PVDF membranes. Thereafter, samples were incubated with MAA as described above. Second, blotted rat erythrocyte membrane proteins incubated with MAA, SNA and PNA lectins which are pre-diluted with control glycoproteins (for the dilutions, 25 μg/ml fetuin, 50 μg/ml asialofetuin and 5 μg/ml transferring were used respectively).

RESULTS

Lectin Histochemistry

Lectin histochemistry is a morphological technique that takes advantage of the carbohydrate binding characteristics of proteins. Figure 1 summarizes the staining patterns observed in the erythrocytes at all age groups analyzed incubated with DIG labelled lectins.

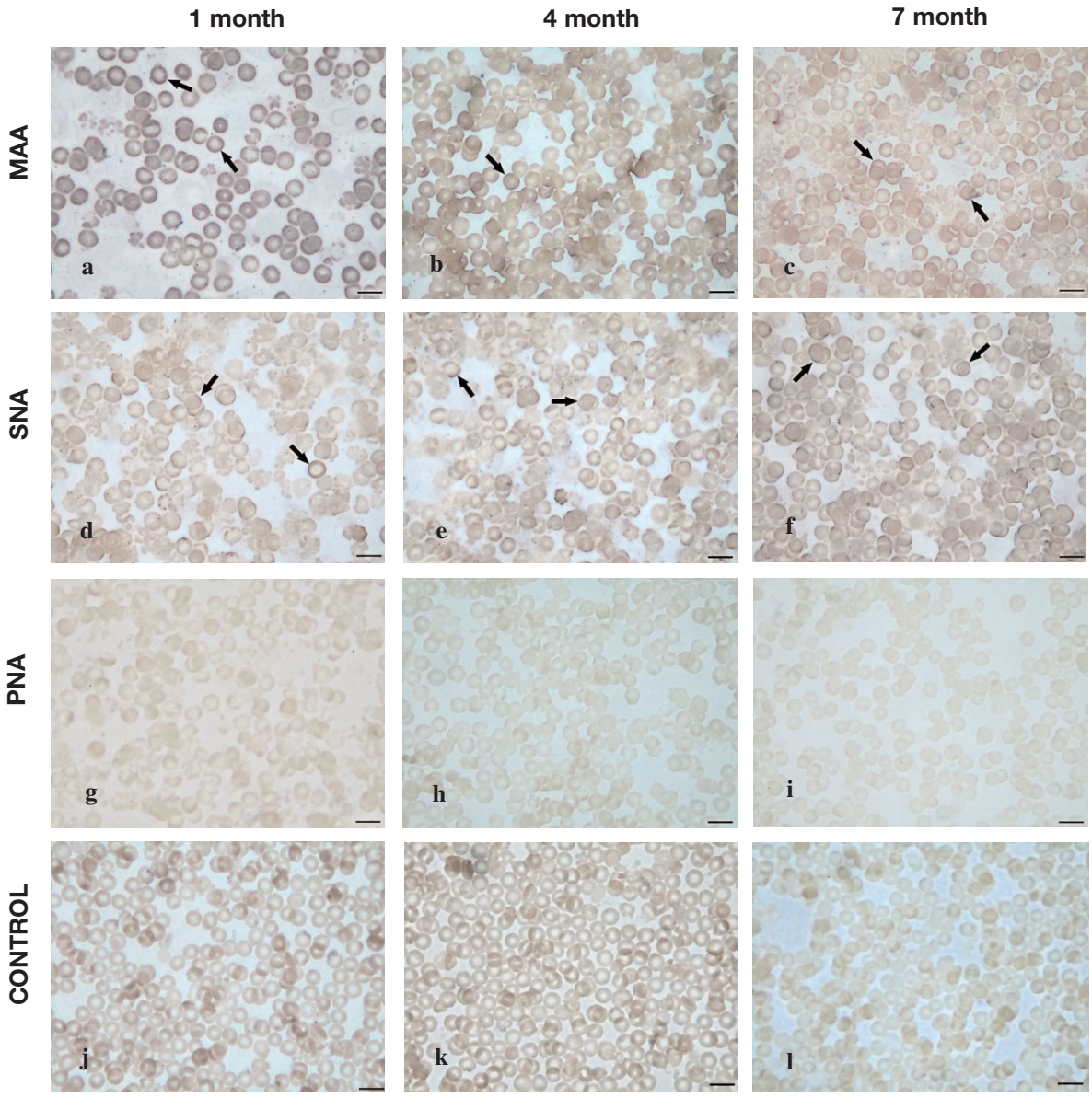

Figure 1. Lectin activities and MAA controls of erythrocyte smears at different age groups
Arrows indicate cells recognized by lectins. Bars: 20 µm

Lectin histochemistry results of MAA, which specific for sialic acid α(2→3) galactose structures, indicated that erythrocytes from the all evaluated age groups were stained. This positive reaction was more intense in 1-month-old rats (Figure 1a) when compared to 4 and 7-month-old ones (Figure 1 b, c). SNA lectin is specific for sialic acid α(2→6) galactose linkages. Similar to MAA, positive SNA stainings were observed nearly all the erythrocytes at all age groups and binding intensity of this lectin was stronger in 1-month-old group than others (Figure 1 d-f). However, reactivity of PNA lectin, specific for the Galβ(1→3)GalNAc disaccharide, was decreased in all groups (Figure 1 g-i).

Control of Lectin Histochemistry

MAA reactivity was disappeared when the lectin incubated with fetuin prior to lectin histochemistry

Lectin Blotting

Glycoconjugate changes of erythrocyte membranes belonging to the different age groups were evaluated by lectin blotting technique. The majority of

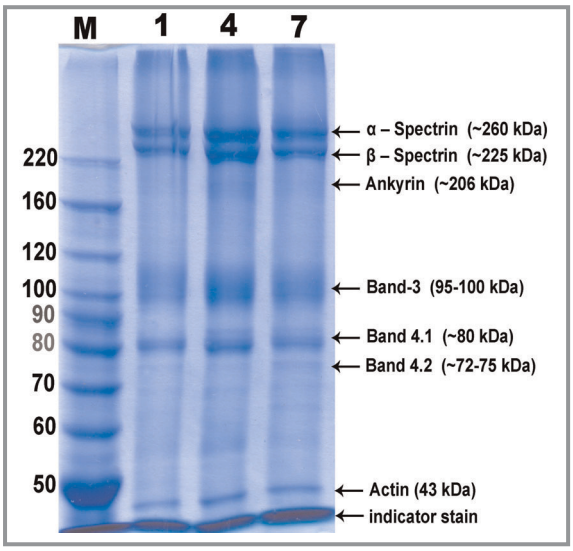

Figure 2. SDS-PAGE analysis of total protein extracts of rat erythrocyte membranes belonging to different age groups. M, marker; 1, 4 and 7 indicate 1 month; 4 month; 7 month old rat erythrocyte membranes, respectively (stained with CBB). A set of broad range molecular mass protein standard (Benchmark Broad Range Protein Marker, Invitrogen Cat#10747012) indicated on the left (M).

proteins detected by coomassie blue staining were not labelled by the lectins (Figure 2).

MAA: Blots of erythrocyte membranes revealed approximately 5 different MAA-reactive bands. While the 90 kDa band (band-3) strongly stained with MAA lectin in 1-month-old rats, staining intensity was decreased in 4- and 7-month-old groups. Other protein bands were weak-positive for MAA in 1 month old erythrocytes and their affinity were also reduced in 4- and 7-month old rats.

SNA: Lectin blotting study with SNA was shown that at least 5 different protein bands with molecular mass ranging from 50 to 220 kDa and one protein of *Mr* upon 220 kDa were reactive in all age groups. However, binding capacities of band-3 and other proteins to SNA were decreased throughout the age (Figure 3a). These results and the lectin histochemistry investigations indicated that α(2→3) and α(2→6) linked sialic acids were decreased during the aging.

PNA: With the PNA lectin blotting, three protein bands with molecular weight upon 100 kDa were weakly stained in all age groups (Figure 3c), so that these proteins might contain galactose residues which are come in slight following by the sialic acid degradation.

Control of Lectin Blotting

For the check of lectin blotting technique, pure fetuin and Type X *C. Perfiringens* neuraminidase treated erythrocyte membrane proteins obtained from 1-month-old rats were separately blotted with MAA. Strong affinity of MAA to fetuin (Figure 4a) was disappeared when erythrocyte membranes were treated with enzyme (Figure 4b). Secondly, lectins, which are pre-incubated with control glycoproteins (fetuin for MAA, transferrin for SNA and asialofetuin for PNA) were incubated with erythrocyte membrane proteins and a decreased lectin activity was observed (Figure 5 a, b, c).

DISCUSSION

Due to their pivotal roles in cell-cell and/or cell-extracellular matrix interactions, cell surface glycan molecules were became an important target in cell biology.[1,2,8,9] Previous studies indicated that erythrocyte membrane proteins (and lipids) are glycosylated forms and glycosylation status of these molecules is closely related with functions of erythrocytes.[16-19] A considerable amount of work has been elucidate the diminution of erythrocyte membrane sialic acids during the erythrocyte senescence.[21,29] However, data on the alterations of glycosylation status in erythrocyte membranes during the organism's ageing is absent. Our lectin histochemistry results shown that MAA and SNA lectins, which are specific for α(2→3) and α(2→6) linked sialic acids respectively, recognize some erythrocytes, but not all. This situation can be interpreted as meaning that MAA- and SNA-reactive erythrocytes are younger than non-reactive ones.

In agreement with previous studies[30-33], our data provide direct evidence that MAA- and SNA-reactive erythrocytes bear O-linked and hybrid or complex *N*-linked glycosylated molecules in their membranes. Meanwhile, decreased MAA and SNA reactivity in erythrocytes from 4- and 7-month-old rats thought that sialylation pattern of erythrocytes belonging to older rats is diminished. Lectin blotting studies were confirmed the lectin histoche-

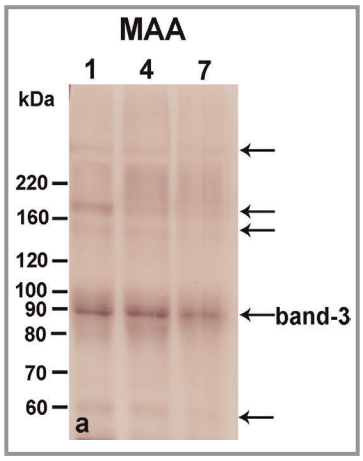

Figure 3a. MAA lectin blotting analysis of total protein extracts of rat erythrocyte membranes from different age groups.

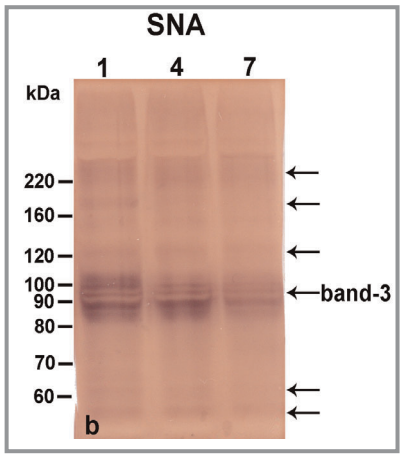

Figure 3b. SNA lectin blotting analysis of total protein extracts of rat erythrocyte membranes from different age groups.

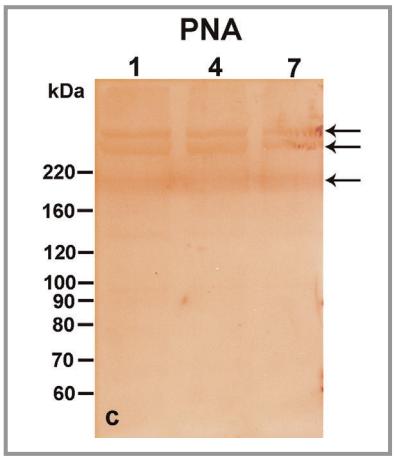

Figure 3c. PNA lectin blotting analysis of total protein extracts of rat erythrocyte membranes from different age groups. M, marker; 1, 4 and 7 indicate 1 month; 4 month; 7-month-old rat erythrocyte membranes, respectively.

mistry. Similar to histochemical analyses, affinity of protein bands to lectins was diminished at 4 and 7 months. Bratosin and her colleagues[34] have suggested that binding affinity of SNA, Wheat germ agglutinin and Limulus polyhemus agglutinin in young erythrocytes is more than senescent ones. Similarly, Seaman and co-workers[35] have proposed that reduction of sialic acids is result from erythrocyte membrane loss. While these works suggest the sialylation of erythrocyte membranes decreased by the erythrocyte aging, reason(s) of this diminution is poorly understood. Three possible situations can be supposed: First, diminution of glycoprotein content in erythrocyte membranes, secondly, loss of erythrocyte membranes and finally, loss of oligosaccharide moieties in erythrocyte membrane proteins. These situations can be valid for our results. In conclusion, it can be said that, amount of sialic acid is decreased in aging erythrocytes and also in erythrocytes from aging organisms. On the other hand, alteration in the glycophorin A, an integral membrane glycoprotein which consists of large numbers of sialic acid molecules, is responsible for the diminished levels of sialic acids in ageing erythrocytes.[36] Our lectin blotting studies with MAA and SNA showed that band-3 is also an important factor for sialic acid diminution. Contrary to previous studies[34,37], our lectin histochemistry studies indicated that PNA lectin slightly recognize erythrocytes at all the stages. In addition to this, PNA-positive protein bands were also recognized by MAA and SNA in lectin blotting studies. In this instance, it can be conceive that $\alpha(2\rightarrow3)$ and $\alpha(2\rightarrow6)$ linked sialic acids mask the PNA binding regions of membrane glycoproteins in young rats and during the aging, sialic acid amount is decreased, therefore PNA-reactive structures appeared in erythrocyte membrane glycoproteins of old animals.

Band-3 is an erythrocyte membrane glycoprotein of molecular weight approximately 100 kDa.[38] Its carbohydrate content separates two fractions: high molecular weight and low molecular weight oligosaccharides.[39] Peripheral sides of high molecular weight oligosaccharides carry polylactosaminyl side chains consisting of various numbers of a repeating disaccharide $\alpha(1\rightarrow4)$GlcNAc $\alpha(1\rightarrow3)$. Low molecular weight fraction contains complex type oligosaccharides such as galactose, mannose, fucose and N-acetylglycosamine (GlcNAc).[40] Sialic acid groups of band-3 are located in terminal region of polylactosaminyl side chains.[41] In agreement with the previous suggestions[42], our MAA and SNA lectin blotting studies indicated that 100 kDa band (band-3) proteins contain both $\alpha(2\rightarrow3)$ and $\alpha(2\rightarrow6)$ linked sialic acids.

In conclusion, while the erythrocyte membrane glycoconjugates alterations during the erythrocyte senescence well known[20], the identify of lectin bin-

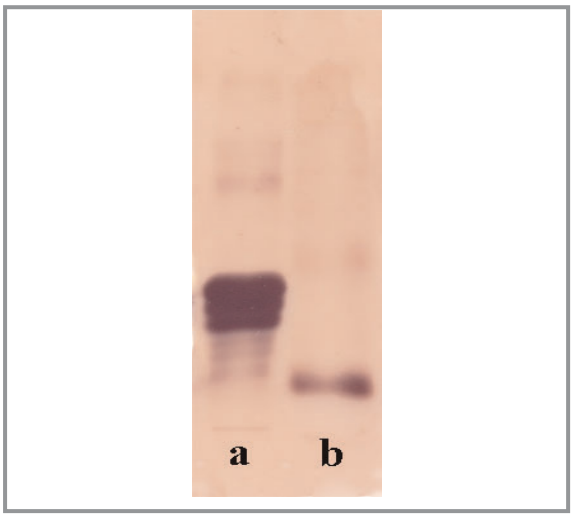

Figure 4. Control of lectin blotting. MAA strongly reacted with fetuin (lane **a**), whereas Type X neuraminidase treated erythrocyte membrane extracts from 1 month old rats did not react (lane **b**).

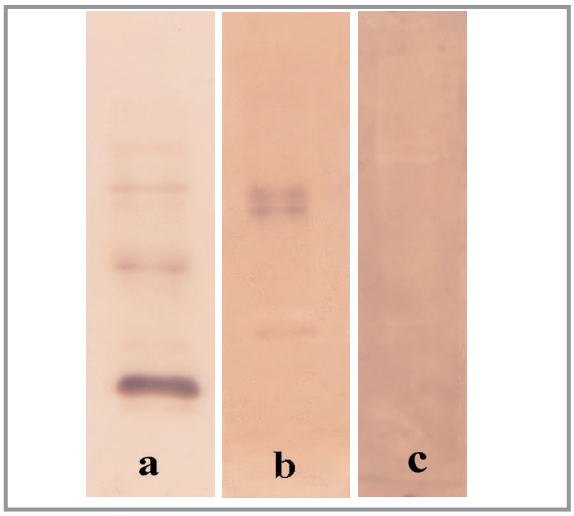

Figure 5. Incubation of erythrocyte membrane extracts from 1 month old rats with lectins pre-incubated with control glycoproteins. (**a**) MAA-fetuin, (**b**) SNA-transferrin, (c) PNA-asialofetuin

ding pattern of erythrocytes in aging organism remains to be established. We examined this question in the present study in order to whether the relationship between erythrocyte membrane glycoconjugate alterations and organism's aging. Our results indicated that some alterations occur in erythrocyte membrane glycoconjugates during oganism's aging process, probably according to well orchestrated alterations of aging. Therefore, detailed investigation of alterations in cell surface glycoconjugates during the aging process will facilitate to understand this complex course.

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