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Modifications of cell surface sialic acids modulate cell adhesion mediated by sialoadhesin and CD22*

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An increasing number of mammalian cell adhesion molecules, including sialoadhesion, CD22 and the family of selectins, have been found to bind cell surface glycoconjugates containing sialic acids. Here we describe how the structural diversity of this sugar influences cell adhesion mediated by the related molecules sialoadhesin and CD22 in murine macrophages and B-cells respectively. We show that the 9-*O*-acetyl group of Neu5,9Ac$_2$ and the *N*-glycoloyl residue of Neu5Gc interfere with sialoadhesin binding. In contrast, CD22 binds more strongly to Neu5Gc compared to Neu5Ac. Of two synthetic sialic acids tested, only CD22 bound the *N*-formyl derivative, whereas a *N*-trifluoroacetyl residue was accepted by sialoadhesin. The potential significance for the regulation of sialic acid dependent cell adhesion phenomena is discussed.

Keywords: sialic acid; cell adhesion; *O*-acetylation; *N*-glycoloylneuraminic acid; *N*-formylneuraminic acid; *N*-trifluoroacetylneuraminic acid.

Introduction

Cell adhesion is important in many biological processes such as embryogenesis, haematopoiesis, inflammation, immune responses and metastasis. The roles of cell surface glycoconjugates carrying sialic acids in these events have drawn an increasing interest since the discoveries of sialic acid binding molecules involved in these functions, as reviewed in [1]. Examples include sialoadhesin [2], CD22 [3] and the family of selectins [4].

Sialoadhesin is a sialic acid-dependent cell adhesion molecule of 185 kDa expressed by murine macrophage subpopulations. Highest expression is found in haematopoietic and lymphoid tissues like bone marrow, spleen and lymph nodes [5]. Sialoadhesin mediates cell adhesion by binding to cell surface glycoconjugates terminating in Neu5Acα2 → 3Galβ1 → 3GalNAc [2] and/or Neu5Acα2 → 3Galβ1 → 3(4)GlcNAc [51] and has been proposed to play a role in the interactions of macrophages with lymphocytes and other leukocytes [6, 7]. The primary amino acid sequence deduced from cDNA cloning has defined sialoadhesin as a new member of the immunoglobulin superfamily closely related to certain other members of this family which includes CD22 [52].

CD22 is a B-cell-restricted adhesion molecule [3, 8, 9] which can mediate binding to B- and T-cells as well as to neutrophils, monocytes and erythrocytes [3, 10, 11]. The interaction with T-cells has been proposed to be involved in early B-cell activation. CD22 has also been implicated to modulate signalling through the surface IgM-B-cell antigen receptor complex [12]. Recent studies on ligands for CD22 have demonstrated that this adhesion molecule recognizes α2,6-linked sialic acid on branched *N*-linked oligosaccharides [13–15].

Sialic acids occur in nature in about 30 modifications [16]. Most common sialic acids are 5-*N*-acetylneuraminic acid (Neu5Ac), 5-*N*-acetyl-9-*O*-acetylneuraminic acid (Neu5,9Ac$_2$) and 5-*N*-glycoloylneuraminic acid (Neu5Gc). However, the expression of these modifications seems to be regulated, since besides being species-specific their distribution depends also on the tissue, cell type and develop-

* Dedicated to Professor Dr Gerhard Uhlenbruck on the occasion of his 65th birthday.

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mental stage. Numerous roles for this structural diversity have been found, including higher resistance towards sialidases and other metabolizing enzymes [16] or influences on the binding capacity of pathogens such as influenza viruses [17] or *Plasmodium falciparum* [18]. For example, whereas influenza A and B strains recognize only Neu5Ac or in some cases Neu5Gc [17], influenza C viruses need Neu5,9Ac_2 as a receptor determinant [19]. In addition to the haemagglutinin, all influenza virus strains contain a receptor destroying activity. For influenza A and B this is a sialidase and for influenza C virus it is an esterase that removes the acetyl group at position 9 of cell surface sialic acid residues [20]. Whereas the binding specificities of many plant and animal lectins that bind sialic acids have been characterized with respect to sialic acid modifications, as reviewed in [21], the influence of sialic acid modifications on mammalian adhesion molecules is mainly unknown.

To investigate the influence of 9-*O*-acetyl groups on the recognition of sialic acids by sialoadhesin and CD22, these were removed by treatment with influenza C virus or alkali from murine erythrocytes which naturally carry Neu5,9Ac_2 [18]. Furthermore, sialidase-treated human erythrocytes were resialylated with sialic acids containing either *N*-acetyl, *N*-glycoloyl, *N*-formyl or N-trifluoroacetyl (TFA) residues at C-5 to probe the specificity of recombinant sialoadhesin and CD22 towards this structural part of sialic acids.

Materials and methods

Materials

Radiochemicals were obtained from Amersham, Germany. Microtitre plates were from Sarstedt, Germany (round bottom polystyrene), from Costar, Germany (flat bottom PVC) or from Greiner, Germany (flat bottom polystyrene, high binding capacity). Goat anti-human IgG antibody, specific for the Fc portion, catalogue No. I-2136, (anti-IgG) and peroxidase substrate o-phenylenediamine hydrochloride were obtained from Sigma, Germany. Sialidase from *Vibrio cholerae* was from Behringwerke, Germany. Influenza C virus (strain C/Johannesburg/1/66) was grown and purified as described before [22]. COS-1 cells were provided by the ICRF cell bank.

For resialylation experiments the following sialyltransferases were used to obtain cells containing exclusively sialic acids (Sia) in the corresponding sialylated terminal oligosaccharide structures. Galβ1 → 4GlcNAc α2 → 6-sialyltransferase (ST6N) giving Siaα2 → 6Galβ1 → 4GlcNAc (6N) was purified from rat liver [23] and Galβ1 → 3GalNAc α2 → 3-sialyltransferase (ST3O) giving Siaα2 → 3Galβ1 → 3GalNAc (3O) structures was purified from porcine liver [24]. Recombinant Galβ1 → 3(4)GlcNAc α2 → 3-sialyltransferase (ST3N) [25] giving Siaα2 → 3Galβ1 → 3(4)-GlcNAc (3N) was kindly donated by Dr J. C. Paulson at Cytel Inc., La Jolla, USA.

CMP-Neu5Ac was purchased from Boehringer Mannheim, Germany, CMP-Neu5Gc [26] was a kind gift from Dr L. Shaw, Kiel University, Germany. CMP-Neu5formyl and CMP-Neu5TFA were synthesized as described [27].

Adhesins

Sialoadhesin was purified from mouse spleen as described before [2]. cDNA clones for sialoadhesin were isolated from a cDNA library constructed from mRNA isolated from mouse peritoneal macrophages which had been induced to express sialoadhesin. Details of the cloning procedure, the characterization of the clones and the expression of recombinant sialoadhesin will be described elsewhere [52]. Fragments of cDNAs encoding for the first four extracellular domains of sialoadhesin, the first three or seven extracellular domains of the murine homologue of CD22 [10] or the five extracellular domains of MUC-18 [28] were amplified by PCR, sequenced and subcloned in frame into the pIG expression vector containing a genomic fragment encoding the H, CH2 and CH3 domains of human IgG1-Fc [29]. COS-1 cells were transfected with these constructs and secreted Fc-chimaeras were purified on protein A-agarose [29]. Purified sialoadhesin or Fc-chimaeras of the adhesion molecules were iodinated in Iodogen-coated tubes as described [2].

Erythrocytes

Human erythrocytes were obtained from heparinized blood, washed and sialidase-treated as described before [30]. Sialidase-treated erythrocytes were resialylated by incubation with one of the purified sialyltransferases in the presence of 750 µM CMP-Neu5Ac, CMP-Neu5Gc, CMP-Neu5formyl or CMP-Neu5TFA for 3 h at 37 °C [30]. To determine the incorporation of sialic acids, to each reaction CMP-^{14}C-Neu5Ac (7 µM final concentration, 9.66 Bq pmol^{-1}) was added and the amount of cell bound ^{14}C was quantified [30]. For Neu5Ac the following incorporations were obtained with the corresponding sialyltransferases: 38 nmol ml^{-1} erythrocytes for Neu5Acα2 → 6Galβ1 → 4GlcNAc, 58 nmol ml^{-1} erythrocytes for Neu5Acα2 → 3Galβ1 → 4GlcNAc and 246 nmol ml^{-1} erythrocytes for Neu5Acα2 → 3Galβ1 → 3GalNAc. Since the initial transfer rates for the modified sialic acids are different for the sialyltransferases used [Groß and Brossmer, unpublished], incorporation of ^{14}C-Neu5Ac was not a suitable method to quantify the incorporation of these sialic acids. Therefore, remaining free acceptor sites were estimated by transfer of 5-*N*-fluorescein-labelled sialic acid with the corresponding sialyltransferases to residual acceptor sites on glutardialdehyde-fixed erythrocytes and the fluorescent label was quantified by fluorescence flow cytometry [31, 32]. Untreated and sialidase-treated erythrocytes were used as controls for background and total acceptor sites available before and after sialidase treatment respectively. This analysis revealed for the ST6N and for the ST3O a similar

total incorporation of Neu5Ac, Neu5Gc and Neu5formyl, but a lower incorporation of Neu5TFA (50–60% compared to Neu5Ac). For the ST3N a similar incorporation of all sialic acid derivatives used was obtained.

For binding assays with recombinant Fc-chimaeras, erythrocytes were fixed with 0.25% glutardialdehyde in PBS for 10 min at room temperature as described before [33]. The reaction was quenched by centrifugation and three washes in PBS/1% BSA followed by at least 1 h in PBS/1% BSA at 4 °C. The modified cells were then stored in PBS/0.25% BSA/0.02% NaN_3 (PBA) at 4 °C and were stable for weeks.

Murine erythrocytes were obtained from heparinized blood of C57Bl/6 or Balb/c mice. After five washes in PBS, the cells were used directly or fixed as described above for human erythrocytes. To hydrolyse 9-*O*-acetyl groups from Neu5,9Ac_2, purified influenza C virus (0–25 μg) was incubated with erythrocytes (50 μl packed cells) in a total volume of 750 μl PBS supplemented with 150 mM glucose at 37 °C overnight with continuous gentle mixing in a rotor. In control experiments the virus esterase was inactivated by treatment of the virus with 3 mM diisopropylfluorophosphate (DFP) for 30 min at room temperature before adding the erythrocytes (final concentration 1 mM DFP). Following the incubation, the cells were washed five times with 1 ml PBS and used immediately in binding assays. In other experiments, 5–10 × 10^6 fixed cells were incubated with 10 μg purified virus in 100 μl PBA at 37 °C for 4 h with occasional shaking and then washed five times in 1 ml PBA. To release *O*-acetyl groups by alkali treatment, 10^7 fixed cells were incubated with 0.1 N NaOH (1 ml) for 60 min on ice, followed by three washes with 1 ml PBS and two washes with PBA.

Binding assays with recombinant Fc-chimaeras

^{125}I-labelled Fc-chimaeras (0.4 μg ml^{-1}) were preincubated with anti-IgG (0.5 μg ml^{-1}) in PBA for 60 min at room temperature. In round bottom polystyrene microtitre plate wells 25 μl anti-IgG-complexed ^{125}I-Fc-chimaera were added to 25 μl cell suspension and incubated for either 60 min at room temperature or overnight at 4 °C. The cells were resuspended by shaking, 125 μl PBA were added, the cells collected by centrifugation of the plates for 1 min at 750 × **g** and the supernatants were aspirated. The cells were washed once in 200 μl PBA, followed by one wash in PBS. The cells were then suspended in 200 μl PBS, transferred to flat bottom PVC microtitre plates and collected, dried and the radioactivity was determined in a gamma counter. In all experiments non-specifically bound radioactivity was less than 0.5% of the input. All assays were done in triplicate and repeated at least three times.

Adhesion assay with immobilized Fc-chimaeras

Flat bottom microtitre plates with high binding capacity were coated with 50 μl anti-IgG (15 μg ml^{-1} in 50 mM sodium carbonate buffer, pH 9.5) per well overnight at 4 °C, the plates were washed three times with PBA and blocked with 100 μl 5% skimmed milk in PBS for 1 h at 37 °C. After a further three washes in PBA, 50 μl Fc-chimaera were added at 2.0 μg ml^{-1} PBA and left at 37 °C for at least 3 h. After washing the plates three times with PBA, 100 μl unfixed erythrocyte suspension (0.1% in PBA) were added and the plates left at room temperature for 60 min. Unbound cells were resuspended by shaking and contents of the wells were flicked out. One hundred μl 0.25% glutardialdehyde in PBS were added and bound cells were fixed for 10 min at room temperature with gentle shaking. The plates were washed thoroughly with PBS, allowed to dry and the cells permeabilized with 100 μl methanol for 10 min. After removing the methanol, the plates were dried again before addition of 200 μl peroxidase substrate for quantification of haemoglobin of erythrocytes bound. The reaction was stopped after 1–2 h by addition of 50 μl 5N HCl and the reaction product was quantified by measuring E_{450}. All assays were done in triplicate and repeated at least three times.

Cell binding assays with peritoneal macrophages

Murine thioglycollate-elicited peritoneal macrophages were cultivated in the presence of 0–20% homologous serum for 2 days and erythrocyte binding assays were performed as described [2]. The percentage of macrophages binding more than four erythrocytes was estimated by light microscopy by counting at least 200 macrophages.

SDS-PAGE and Western blotting

SDS-PAGE of erythrocyte membranes, Western blotting and probing with ^{125}I-labelled sialoadhesin was performed as described before [2]. To de-*O*-acetylate sialic acids on membrane glycoproteins, Western blots were treated with 0.1 N NaOH or saline at 0 °C for 30 min and washed three times with PBS before probing with ^{125}I-sialoadhesin. To prove the specificity of binding, ^{125}I-sialoadhesin was pretreated with the inhibitory monoclonal antibody 3D6 [2] (20 μg ml^{-1}) for 1 h at room temperature.

Results

Influence of 9-O-acetylation

Whereas purified sialoadhesin bound well to human erythrocytes [2], which contain only Neu5Ac, binding to murine erythrocytes, which contain high amounts of Neu5,9Ac_2 [18], was very low. Therefore, we investigated the influence of 9-*O*-acetylation of sialic acids on sialoadhesin binding. Since *O*-acetyl groups are alkali-labile, fixed erythrocytes from human or mouse were subjected to alkali treatment and then compared for binding of ^{125}I-sialoadhesin. Whereas the alkali treatment of human erythrocytes did not affect binding, removal of *O*-acetyl groups from

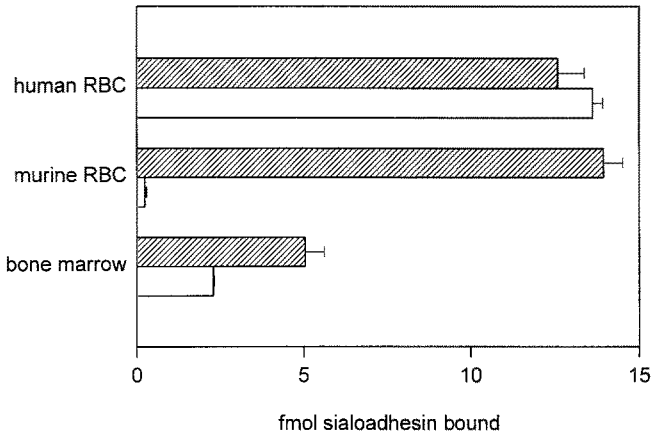

Figure 1. Alkali treatment unmasks binding sites for sialoadhesin on murine cells. Erythrocytes (RBC) or total bone marrow cells were fixed with glutardialdehyde and treated with PBS (white bars) or 0.1 N NaOH (hatched bars) for 60 min on ice. Binding of ^{125}I-sialoadhesin to 2.5×10^5 cells was estimated as described [2].

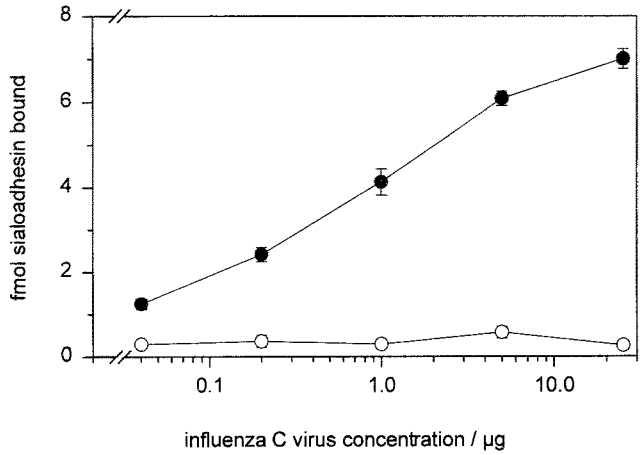

Figure 2. Influenza C virus treatment unmasks binding sites for sialoadhesin on murine erythrocytes. Cells were treated with 0–25 µg purified virus overnight at 37 °C. Binding of ^{125}I-sialoadhesin to 2.5×10^5 cells (closed circles) was estimated as described [2]. In control experiments ^{125}I-sialoadhesin was preincubated for 60 min at room temperature with the inhibitory monoclonal antibody 3D6 (20 µg ml^{-1}) (open circles).

murine erythrocytes enhanced binding to the same level as that observed with human erythrocytes (Fig. 1). This is evidence that *O*-acetyl groups can completely mask ligand determinants for sialoadhesin.

Sialoadhesin is expressed by resident bone marrow macrophages that are in close contact with developing haematopoietic cells. Unlike murine erythrocytes, murine bone marrow cells bound sialoadhesin without prior alkali treatment. However, alkali treatment enhanced binding significantly (Fig. 1). To determine whether this could be related to the heterogeneity of this cell population, total bone marrow cells were fractionated on Percoll gradients. Fractions enriched in erythroblasts showed lower binding of sialoadhesin than fractions enriched in myeloid cells. If fixed fractionated bone marrow cells were alkali treated, those fractions enriched for erythroblasts revealed the strongest increase in sialoadhesin binding (data not shown). Since erythroblasts are likely to contain Neu5,9Ac$_2$ at a similar level to erythrocytes, unmasking of ligands on this cell population could explain the increased binding observed for the total bone marrow cells after alkali treatment.

To remove 9-*O*-acetyl groups from cell surface sialic acids more specifically, murine erythrocytes were treated with influenza C virus, which contains a 9-*O*-acetyl esterase activity in its HEF surface protein [20]. After this treatment, murine erythrocytes bound ^{125}I-sialoadhesin. The degree of binding correlated with the amount of virus used for the treatment (Fig. 2), reaching levels similar to human erythrocytes (Table 1), and thus confirming further that 9-*O*-acetyl groups are masking the ligands on murine erythrocytes. The HEF protein of influenza C virus also contains a haemagglutinating activity which binds to Neu5,9Ac$_2$ [19]. Therefore, it was necessary to prove that the effect of virus treatment was due to the action of the esterase activity and not caused by haemagglutination. The

Table 1. Ligands for sialoadhesin on murine erythrocytes are unmasked by influenza C treatment. Human or murine erythrocytes were treated as indicated. ^{125}I-sialoadhesin was incubated with 2.5×10^5 erythrocytes for 1 h at room temperature and bound radioactivity was estimated [2].

Species	*Treatment*	*Total binding*		*Non-specific binding (pretreatment with 3D6)*		*Net binding*	
		fmol	SD	*fmol*	SD	*fmol*	SD
Human	None	2.98	±0.15	0.13	±0.07	2.85	±0.14
Mouse	None	0.40	±0.04	0.15	±0.04	0.25	±0.03
	25 µg influenza C virus at 37 °C	2.70	±0.09	0.10	±0.01	2.60	±0.09
	25 µg influenza C virus at 4 °C	1.34	±0.12	0.07	±0.00	1.27	±0.11
	25 µg influenza C + DFP	0.35	±0.09	0.07	±0.00	0.28	±0.07
	DFP alone	0.12	±0.03	0.05	±0.02	0.07	±0.02

specificity was assessed in two control experiments. (1) The esterase activity is temperature-dependent leading to strongly reduced activity at 4 °C, whereas the haemagglutinin is temperature-independent [34]. If influenza C virus treatment was performed at 4 °C, the increase in binding was reduced by about 60% (Table 1). This reduction was equivalent to that found for erythrocytes treated at 37 °C with 50-fold less virus (Fig. 2), correlating to a reduction in esterase activity of 98%. (2) DFP is a strong irreversible inhibitor of the esterase but does not interfere with the haemagglutinin activity of the virus. If influenza C virus treatment was performed with DFP-inactivated virus, no increased binding of sialoadhesin to murine erythrocytes was found (Table 1).

Whereas the experiments described above demonstrate that 9-*O*-acetyl groups mask cell surface ligands for solubilized sialoadhesin, it was also necessary to investigate whether 9-*O*-acetyl groups would prevent cell–cell interactions mediated by sialoadhesin. Cultivation of murine peritoneal macrophages in the presence of increasing concentrations of homologous serum induces a dose-dependent expression of sialoadhesin on these cells [35]. We used this system to investigate whether the removal of 9-*O*-acetyl groups also leads to increased cell adhesion mediated by sialoadhesin expressed on these macrophages. As shown in Fig. 3A, only murine erythrocytes treated with influenza C virus containing an active esterase formed sialoadhesin-dependent rosettes with the macrophages.

Further evidence for masking by 9-*O*-acetyl groups was obtained with chicken erythrocytes. These cells contain mainly Neu5Ac after hatching, but high amounts of $Neu5,9Ac_2$ as adult birds [36]. Serum-induced peritoneal macrophages formed a high number of sialoadhesin-dependent rosettes with erythrocytes from newborn chickens, whereas fewer rosettes were observed with erythrocytes from adult animals (Fig. 3B), confirming the masking effect of increasing levels of 9-*O*-acetylation of sialic acids. Taken together, these findings prove that 9-*O*-acetyl groups on murine erythrocytes prevent cell adhesion mediated by sialoadhesin.

We then analysed the binding of ^{125}I-sialoadhesin to erythrocyte membrane glycoproteins (Fig. 4A) from human and murine erythrocytes on Western blots. As shown in Fig. 4B, sialoadhesin bound to glycoprotein bands from human membranes, whereas binding to certain murine glycoproteins could only be detected with influenza C virus-treated membranes. Even stronger binding was observed if 9-*O*-acetyl groups had been hydrolysed after blotting by alkali treatment of the blot (Fig. 4C). Binding was sialic acid-dependent in all cases, since it was abolished if sialic acid residues had been cleaved with sialidase (data not shown). In addition, preincubation of ^{125}I-sialoadhesin with the inhibitory monoclonal antibody 3D6 prevented any detectable binding (Fig. 4B and C). This finding provided evidence that 9-*O*-acetylation of sialic acid residues on murine erythrocyte glycoproteins masks the ligand determinants for sialoadhesin on these glycoconjugates.

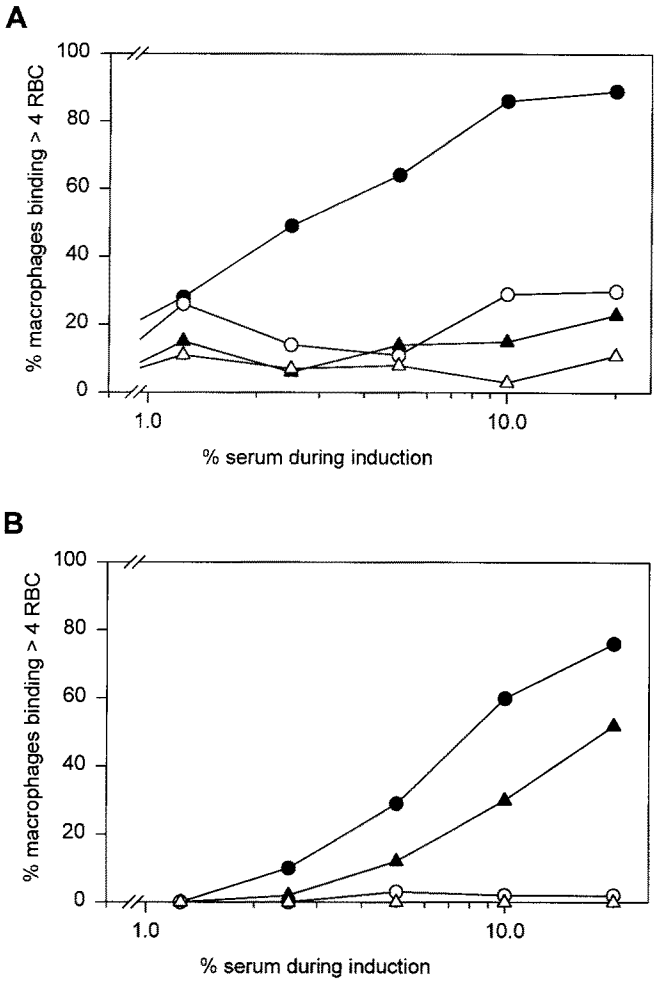

Figure 3. Binding of erythrocytes to serum-induced peritoneal macrophages. Macrophages were induced to express sialoadhesin by cultivation in the presence of homologous serum at the indicated concentrations. The percentage of macrophages binding more than four erythrocytes (RBC) was estimated by light microscopy (closed symbols). In control experiments, macrophages were pretreated with the inhibitory monoclonal antibody 3D6 (open symbols). (A) Binding of murine erythrocytes treated with influenza C virus (circles) or mock-treated in PBS (triangles). (B) Binding of chicken erythrocytes from newborn (circles) or adult birds (triangles).

Sialoadhesin and CD22 are members of a newly defined family of sialic acid-dependent cell adhesion molecules of the Ig-superfamily containing 17 and seven Ig-like domains respectively [51]. For quantitative binding assays recombinant protein chimaeras were constructed containing the Fc portion of human IgG1, together with four (sialoadhesin) and three or seven (CD22) extracellular domains of the adhesion molecules. Since, in previous studies, oligomeric preparations of purified sialoadhesin showed better binding than preparations containing mainly monomers

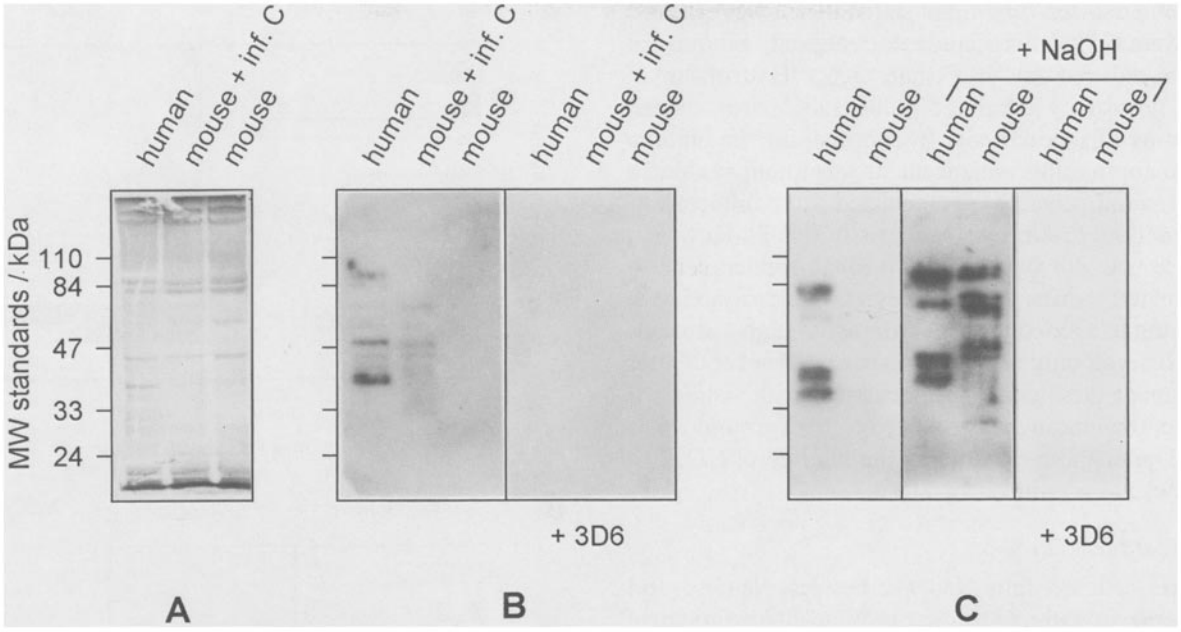

Figure 4. Binding of sialoadhesin is only observed after removal of 9-*O*-acetyl groups. Erythrocyte membrane proteins were run on SDS-PAGE, blotted on nitrocellulose and probed with ^{125}I-sialoadhesin as described [2]. Preincubation of ^{125}I-sialoadhesin with the inhibitory monoclonal antibody 3D6 at 20 μg ml^{-1} for 1 h at room temperature, where indicated at the bottom of blots B and C, completely inhibited binding of the labelled protein to the blots. (A) Western blots of membrane proteins from human or murine erythrocytes were stained for total protein with Ponceau red solution. (B) In the lanes indicated, untreated murine erythrocytes (mouse) or treated with influenza C virus (mouse + inf. C) before SDS-PAGE to remove 9-*O*-acetyl groups and blots were probed with ^{125}I-sialoadhesin [2]. (C) Blots were treated with 0.1 N NaOH or saline for 30 min at 0 °C before probing with ^{125}I-sialoadhesin. The variability in band patterns as detected by ^{125}I-sialoadhesin on blot B and C possibly represents different heterodimers of glycophorin molecules [2].

Table 2. Ligands for sialoadhesin are masked by 9-*O*-acetyl groups on murine erythrocytes. Glutardialdehyde-fixed erythrocytes were treated as indicated. After complexing with anti-IgG ^{125}I-Fc-sialoadhesin (four extracellular domains) or ^{125}I-Fc-CD22 (three extracellular domains) were incubated with 2.5 × 10^5 erythrocytes overnight at 4 °C and cell bound radioactivity was estimated as described under Materials and methods. In control experiments, anti-IgG complexed ^{125}I-Fc-CD22 bound well to murine lymphocytes (data not shown) and derivatized human erythrocytes containing Neu5Gcα2 → 6Galα1 → 4GlcNAc (see Fig. 5).

Species	*Treatment*	*^{125}I-Fc-sialoadhesin bound*		*^{125}I-Fc-CD22 bound*	
		fmol	SD	*fmol*	SD
Human	None	14.99	±0.6	0.07	±0.01
	Sialidase	0.98	±0.34	0.04	±0.01
Mouse	None	0.43	±0.02	0.40	±0.01
	Sialidase	0.12	±0.08	0.29	±0.01
	20 μg influenza C virus	6.82	±0.08	0.56	±0.03
	0.1 N NaOH	20.82	±0.2	0.38	±0.06
	0.1 N NaOH + sialidase	0.13	±0.02	0.08	±0.01

and dimers [2], the valency of Fc-adhesin chimaeras was increased by complexing with anti-IgG prior to binding assays. Titration of anti-IgG concentrations revealed optimal binding at about 1:1 molar ratios [51]. Therefore, complexed Fc-adhesins were used in all binding assays with Fc-chimaeras in solution.

Murine erythrocytes treated with sialidase, alkali or influenza C virus were assayed for binding of Fc-sialoadhesin (four domains), and Fc-CD22 (three domains) (Table 2). Binding of both proteins to untreated cells was

very low. Whereas the binding of sialoadhesin was reduced by more than 75% after sialidase treatment, binding of CD22 was reduced by less than 30%. Hydrolysis of 9-*O*-acetyl groups by alkali or influenza C virus caused strong binding of sialoadhesin. In contrast, for the binding of CD22 no comparable enhancement was found (Table 2), although a small increase was detected after influenza C virus treatment of murine erythrocytes (Table 2). However, this increase was not observed with alkali-treated cells. If sialidase-treated murine erythrocytes were hydrolysed with alkali, binding to sialoadhesin was almost as high as to cells treated with alkali only (data not shown). However, if after alkali treatment these cells were incubated with sialidase a second time, the binding was reduced to background levels (Table 2). Under these conditions, the binding of CD22 to these cells was also reduced by 80%.

Effect of substituents at C-5

Since mouse cells contain Neu5Gc besides Neu5Ac and Neu5,9Ac$_2$, we investigated how the *N*-acyl substituent of sialic acids might influence their recognition by the adhesion molecules. To study this aspect, sialic acids with *N*-acetyl, *N*-glycoloyl, *N*-formyl or *N*-TFA substituents at C-5 were transferred to sialidase-treated human erythrocytes. These derivatized cells were then used in binding assays with ^{125}I-labelled Fc-adhesins (Fig. 5A) and in adhesion assays with immobilized Fc-adhesins (Fig. 5B). Assays with CD22 were done with Fc-chimaeras containing either all seven or just the first three extracellular domains. Similar results were obtained with both constructs. In either assay, specific binding patterns were obtained for sialoadhesin and CD22. As a negative control, assays were also performed with a Fc-chimaera containing the extracellular domains of MUC-18, a member of the Ig family with unknown binding properties [28]. No significant binding was detected with MUC-18-Fc in either binding assay system (Fig. 5B and data not shown).

In both assays, sialoadhesin bound to Neu5Ac in Neu5Acα2 → 3Galβ1 → 3GalNAc (3O) and in Neu5Acα2 → 3Galβ1 → 3(4)GlcNAc (3N) to a similar extent. Whereas a *N*-TFA substituent instead of the *N*-acetyl group in Neu5Acα2 → 3Galβ1 → 3(4)GlcNAc (3N) did not significantly influence sialoadhesin binding, the same change on Siaα2 → 3Galβ1 → 3GalNAc (3O) reduced binding significantly in the assay with soluble Fc-sialoadhesin complexes (Fig. 5A). In contrast, in the adhesion assay with immobilized Fc-sialoadhesin no such reduction was observed for the same substitution (Fig. 5B). Binding of sialoadhesin to resialylated erythrocytes carrying Neu5Gc or Neu5formyl was very low. These results demonstrate that sialoadhesin binds poorly to glycoconjugates carrying Neu5Gc or Neu5formyl. Furthermore, no binding occurred to erythrocytes carrying Neu5Acα2 → 6Galβ1 → 4GlcNAc (6N) or any other sialic acid in this linkage. This is in agreement with the specificity of sialoadhesin for

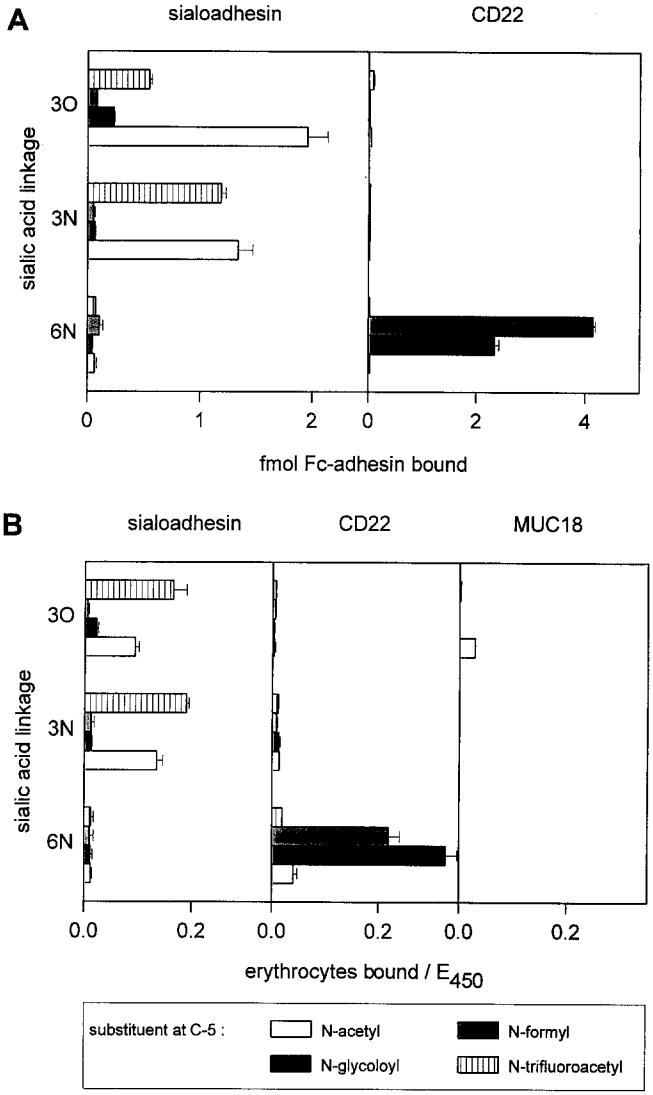

Figure 5. The substituent at C-5 of sialic acids influences the binding of sialoadhesin and CD22 to erythrocytes. Sialidase-treated erythrocytes were resialylated with CMP-sialic acids (Sia) with the corresponding substituent at C-5 in the resialylation reaction as indicated. The sialyltransferases used synthesized the following terminal oligosaccharide sequences on the cell surface: Siaα2 → 3Galβ1 → 3GalNAc (3O), Siaα2 → 3Galα1 → 4GlcNAc (3N), or Siaα2 → 6Galα1 → 4GlcNAc (6N). (A) Anti-IgG-complexed ^{125}I-Fc-sialoadhesin (four extracellular domains) or ^{125}I-Fc-CD22 (three extracellular domains) were incubated with 2.5×10^5 derivatized human erythrocytes overnight at 4 °C and the bound adhesins were estimated as described in the Materials and methods section. (B) Binding of derivatized human erythrocytes to immobilized Fc-sialoadhesin (four extracellular domains), Fc-CD22 (seven extracellular domains) or Fc-MUC-18 was estimated by measuring E_{450} as described under Materials and methods.

Neu5Acα2 → 3Galβ1 → 3GalNAc and Neu5Acα2 → 3Galβ1 → 3(4)GlcNAc.

Human CD22 requires Neu5Acα2 → 6Galβ1 → 4GlcNAc (6N) structures for binding [13–15]. Our results presented

in Fig. 5 confirm the same sialic acid linkage specificity for the murine homologue. However, in contrast to sialoadhesin, binding of CD22 to Neu5Ac was very low and could only be detected in adhesion assays with immobilized Fc-CD22 (Fig. 5B). Moreover, strong binding was found with Neu5Gc or Neu5formyl on Siaα2 → 6Galβ1 → 4GlcNAc in both assays (Fig. 5), suggesting that the murine homologue of CD22 prefers glycoconjugates with Neu5Gc or Neu5formyl over those with Neu5Ac. The low binding to Neu5TFA could be explained by the lower density of this sialic acid obtained in the resialylation reaction.

Discussion

The expression of sialic acid modifications is known to be tissue- and cell-specific [16]. Therefore, it was of considerable interest to study the influence of such modifications on cellular interactions mediated by sialic acid-dependent adhesion molecules. To date, only limited information has been available on this issue. For example, shortening of the glycerol side chain of sialic acids by periodate/borohydride treatment revealed that this moiety is not important for the binding of E-selectin [37], of L-selectin [38] and of P-selectin [39]. In contrast, for recognition by human CD22 the glycerol side chain seems to be an essential structural element [13–15]. However, in none of these studies were modifications of naturally occurring sialic acids studied.

Here we describe the potential role of sialic acid modifications as modulators of cell adhesion mediated by sialoadhesin and CD22. Using different approaches, we were able to show that in the mouse these two related sialic acid-dependent adhesion molecules of the immunoglobulin superfamily have distinct preferences for sialic acid species. Our experiments demonstrate that 9-*O*-acetylation of sialic acids masks binding sites for sialoadhesin. This finding is of particular interest, since macrophages expressing sialoadhesin are in close contact with erythroblasts in haematopoietic clusters of the murine bone marrow [6, 40]. In these clusters sialoadhesin is highly concentrated at contact sites between macrophages and myeloid cells but not at contact sites with erythroblasts. An intriguing possibility is that masking of ligands by 9-*O*-acetyl groups prevents interactions with erythroblasts mediated by sialoadhesin in these clusters. Furthermore, this masking may also protect circulating erythrocytes from being trapped in the spleen by binding to macrophages expressing sialoadhesin in this organ.

It is more difficult to explain the results obtained with CD22 binding to murine erythrocytes showing that the weak binding to untreated cells was not significantly enhanced after the hydrolysis of 9-*O*-acetyl groups. Unmasking Neu5Ac would not be expected to result in binding of murine CD22, since this adhesin prefers Neu5Gc. Although murine erythrocytes express Neu5Gc [18], the overall low levels of CD22 binding to murine erythrocytes could be a consequence of an unfavourable ratio of α2,6 linked over α2,3 linked Neu5Gc (see also next paragraph). Taken together, the influence of 9-*O*-acetyl groups on murine CD22 binding remains unclear. To prove conclusively whether murine CD22 can bind to 9-*O*-acetylated sialic acids, it would be necessary to investigate Neu5Gc,9Ac on appropriate glycoconjugates, i.e. α2,6 linked to *N*-glycans. Interestingly, a recent study on human CD22 demonstrated that recognition of Neu5Ac is masked by 9-*O*-acetyl groups [50].

Neu5Gc is an abundant sialic acid in mice which shows tissue and cell specific expression patterns. Therefore, it was important to investigate whether the adhesion molecules could recognize this sugar. Whereas sialoadhesin bound only to Neu5Ac in the α2,3-linkage, murine CD22 preferred α2,6-linked Neu5Gc over Neu5Ac. An interesting aspect is the possible evolutionary shift in specificity between murine and human CD22. In contrast to murine CD22, the human homologue binds well to Neu5Ac-containing glycoproteins [13–15], which is not surprising since normal human tissues do not contain Neu5Gc [16]. Furthermore, recent experiments showed that human CD22 can recognize Neu5Gc besides Neu5Ac [50]. CD22 mediates binding of B-cells to lymphocytes and monocytes [10, 11, 51]. In this context, our results may be of biological significance, since evidence has been presented that, at least in gangliosides, murine T-cells [42], B-cells [43] and peritoneal macrophages [44] express developmentally regulated levels of Neu5Gc.

Only small differences in the affinity of a single binding site are necessary to result in pronounced differences in the multimeric interactions of receptors with glucoconjugate ligands, which are likely to mediate cell adhesion *in vivo*. Whereas cells carrying similar levels of sialic acids were compared in this study, an improved presentation of receptors and ligand determinants might also allow the adhesion of cells expressing only sialic acids with lower affinities. For example, murine CD22 binds also to Neu5Ac if the density of adhesion molecules and ligand determinants on a cell surface is high enough [51] and cells transfected with murine CD22 were shown to bind human B-cell Daudi lymphoma cells [10], which are not expected to express Neu5Gc.

Two synthetic sialic acids were also investigated in this study. Sialoadhesin, which preferred Neu5Ac, bound to Neu5TFA-containing ligands but not to those with Neu5-formyl. The loss of binding activity in derivatives with the more hydrophilic *N*-glycoloyl or the shorter *N*-formyl substituent supports the idea that a hydrophobic interaction of the methyl group of the *N*-acetyl residue with the protein stabilizes the binding, similar to the situation in the binding pocket of influenza A strain X31 [45]. The strong binding of CD22 to Neu5Gc suggested an additional stabilizing interaction of the hydroxyl group for this adhesion molecule not possible with Neu5Ac or Neu5TFA, which were only poorly bound. However, similar strong

binding was found if the methoxy group of the *N*-glycoloyl substituent was removed as in the *N*-formyl derivative. This finding would argue against such a stabilizing effect in the *N*-glycoloyl substituent. Further studies using a series of derivatives with different substituents at C-5 will be helpful in the elucidation of the binding specificity of these adhesion molecules. Such studies could lead to the development of synthetic sialylated inhibitors tailored for the binding specificity of one of these sialic acid-dependent cell adhesion molecules.

The metabolic pathways leading to sialic acid modifications and their intracellular locations are well documented [16]. With respect to cellular interactions, some of their characteristics are of particular interest. Neu5Ac bound to glycoconjugates is *O*-acetylated in the trans Golgi network [46]. This allows the modification of sialic acids on specific glycoconjugates depending on the enzyme(s) involved in this modification. In addition, the transport rate of glycoconjugates through this compartment could have an impact on the relative amount of $Neu5,9Ac_2$ on the cell surface. In contrast, CMP-Neu5Ac is converted to CMP-Neu5Gc by a cytoplasmic monooxygenase system [47, 48]. Since transport to the Golgi apparatus and transfer to the glycoconjugates does not seem to be specific for either donor substrate [26], the amount of Neu5Gc in glycoconjugates on the cell surface would be regulated by the ratio of CMP-Neu5Ac to CMP-Neu5Gc generated by the monooxygenase [49]. If cells containing mainly Neu5Gc produce the appropriate terminal oligosaccharide structures, Neu5Gcα2,6-linked to *N*-glycans, they carry ligands for CD22 but not for sialoadhesin, which requires Neu5Acα2,3-linked to *N*-glycans or *O*-glycans.

Another characteristic difference between $Neu5,9Ac_2$ and Neu5Gc on glycoconjugates is their catabolism. Whereas intra- or extracellular esterases can remove the 9-*O*-acetyl group from $Neu5,9Ac_2$ leading to Neu5Ac, no comparable enzymatic system is known to convert Neu5Gc to Neu5Ac. Cells expressing Neu5Ac can mask their ligands for sialoadhesin by 9-*O*-acetylation, but this mask could be removed by appropriate extracellular esterases, allowing interactions of Neu5Ac on the ligands with sialoadhesin. In contrast, Neu5Gc can only be removed by sialidases. However, this would lead to the asialooligosaccharides, which are not ligand determinants for sialoadhesin or CD22. Thus, regulation of the complex metabolic pathways that lead to the biosynthesis and successive degradation of glycoconjugates carrying different sialic acid modifications can specifically and dramatically influence cell–cell interactions mediated by these adhesins.

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