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RECONSTITUTION OF THE MASKING EFFECT OF SIALIC ACID GROUPS ON SIALIDASE-TREATED ERYTHROCYTES BY THE ACTION OF SIALYLTRANSFERASES

SOERGE KELM*, ASHOK K. SHUKLA*, JAMES C. PAULSON† AND ROLAND SCHAUER*,‡

**Biochemisches Institut, Christian-Albrechts-Universität Kiel, Olshausenstr. 40, D-2300 Kiel (Federal Republic of Germany) and †Department of Biological Chemistry, University of California School of Medicine, Los Angeles, California 90024 (U.S.A.)*

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ABSTRACT

Glutardialdehyde-fixed or native rat erythrocytes were partially desialylated by the action of *Vibrio chlolerae* sialidase, resulting in the binding of these cells to homologous peritoneal macrophages. Resialylation of these erythrocytes by purified α-(2→3)- or α-(2→6)-sialyltransferases with CMP-*N*-acetylneuraminic acid led to the incorporation of 60–80% of the enzymically released sialic acid. Binding of the resialylated erythrocytes to peritoneal macrophages was reduced when compared with corresponding, partially desialylated erythrocytes. Thus, the amount of transferred sialic acid was sufficient to demonstrate reconstitution of the masking effect of sialic acids.

INTRODUCTION

Sialic acids are known to mask ligands on the surface of erythrocytes[1]. Galactose has been found to be responsible for the binding of sialidase-treated erythrocytes to macrophages *in vivo* and *in vitro*[1–5]. To get more insight whether the recognition of galactose by the receptor is due only to the removal of sialic acids or to other effects of sialidase-treatment, *e.g.*, adsorbed sialidase molecules[6], sialidase-treated erythrocytes were partially resialylated and studied for their interaction with macrophages. In these experiments, glutardialdehyde-fixed erythrocytes were used. They behave similarly to native cells in the interaction with macrophages[7] and are best suited for the sialyltransferase (ST) reaction.

EXPERIMENTAL

Preparation of cells. — Peritoneal macrophages and erythrocytes from Wistar rats were prepared according to ref. 5. The glutardialdehyde fixation of erythro-

‡To whom correspondence should be addressed.

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cytes was carried out according to ref. 8. Washed erythrocytes (10^8/mL) were incubated for 5 min at 20° with 0.125% glutardialdehyde in phosphate-buffered saline (PBS) (pH 7.2) and excess reagent was removed by reaction with 1% bovine serum albumin, centrifugation, and washing of the erythrocytes in PBS 4–5 times.

Sialidase treatment. — Erythrocytes (5×10^9) were treated with various amounts (0–250 mU) of sialidase from *Vibrio cholerae* (VCS) (Behringwerke, Marburg, FRG) in PBS (pH 7.2, 2 mL) for 60 min at 37° before or after fixation. VCS was removed by centrifugation and repeated washing of the erythrocytes. Aliquots of the supernatants were analyzed for sialic acid content by a micro-adaption of the periodic acid–thiobarbituric acid assay according to Warren[9,10].

Resialylation of erythrocytes. — Resialylation of erythrocytes was performed as described earlier[11]. Briefly, erythrocytes (2×10^8) treated with VCS (2 or 5 mU/10^9 cells) were incubated with α-(2→3)-sialyltransferase[12] (10 mU) or α-(2→6)-sialyltransferase[13] (100 mU) in 25mM sodium cacodylate buffer [pH 6.5, 200 μL; 75mM NaCl, 100mM glucose, bovine serum albumin (10 mg/mL), penicillin (100 U/mL), and streptomycin (100 U/mL)] in the presence of mM CMP-*N*-acetylneuraminic acid (CMP-[^{14}C]Neu5Ac) (185 KBq/mmol) for 16 h at 37°. CMP-*N*-acetyl-[4,5,6,7,8,9-^{14}C]neuraminic acid (5.55 GBq/mmol, New England Nuclear) was diluted with unlabeled CMP-Neu5Ac (Sigma Chemical Co.) accordingly. Cells were washed with PBS 4–5 times and transfer of sialic acid was determined by estimating the amount of [^{14}C]Neu5Ac incorporated into the erythrocytes in a Beckman LS 9000 D beta-counter correcting each estimation for quenching automatically. Fixed cells do not release hemoglobin and, therefore, oxidation before counting was not necessary.

Assay for erythrocyte–macrophage interaction. — For the binding assay, erythrocytes were incubated with homologous, adherent peritoneal macrophages as described earlier[4]. Briefly, erythrocytes (2×10^7) were incubated with peritoneal macrophages (adherent to 100 mm^2) in medium 199E (300 μL) for 60 min at 37°, unbound erythrocytes were removed by washing, and the number of erythrocytes bound to 100 macrophages was estimated by light microscopy after staining[4]. All assays were performed in triplicate at least.

RESULTS AND DISCUSSION

The use of glutardialdehyde-fixed erythrocytes in the present studies, as compared to native cells, had the following advantages. Fixed cells do not hemolyze if subjected to detergents like Triton X-100 or to slightly acidic, alkaline, hypo-, or hyper-tonic conditions. Furthermore, fixed cells do not loose sialic acid or protein during storage for weeks at 4°. Therefore, the fixed cells from one batch can be used for experiments with macrophages on different days to give similar results, which is not the case with native cells[7]. The radioactivity of fixed erythrocytes can also be counted after being incorporated in liquid scintillation fluid without prior oxidation.

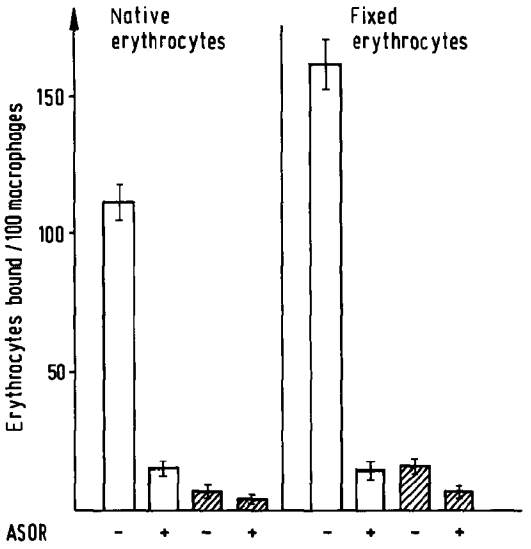

Fig. 1. Binding of rat erythrocytes to homologous peritoneal macrophages. Erythrocytes (5×10^9 native or glutardialdehyde-fixed) were treated with VCS (250 mU) (white bars) or without sialidase (cross-hatched bars), washed, and incubated with macrophages in the presence or absence of 0.6μM ASOR as described in the Experimental section (n = 8).

Sialidase liberates sialic acid from fixed erythrocytes at rates similar to those observed with native cells[7]. Sialidase treatment of fixed erythrocytes leads to binding with receptors on macrophages in a similar way as found with native, sialidase-treated erythrocytes (Fig. 1). This is demonstrated by the inhibition of interaction with compounds containing terminal galactose residues like asialoorosomucoid (ASOR) (Fig. 1).

It was shown with native cells that only 15–20% of the total sialic acid content must be removed from erythrocytes to initiate binding to macrophages[4]. With further sialidase treatment the binding increased to a maximum which was reached when 50–70% of the sialic acid residues representing the total amount hydrolyzable from rat erythrocytes by *Vibrio cholerae* sialidase (VCS)[4] had been removed. The binding of fixed cells was shown to depend on the degree of sialidase treatment in a similar manner (Fig. 2).

On the basis of these experiments, it was expected that reincorporation of sialic acid into erythrocytes would reconstitute the masking effect of this sugar, if binding to macrophages is due to a loss of sialic acid residues only. Fixed erythrocytes are ideal for the study of resialylation with purified ST-containing detergent and glycerol in the buffer for storage, as removal of these substances causes loss of sialyltransferase activity.

As ST does not incorporate as much sialic acid as was removed by the action of sialidase[11], we treated the erythrocytes with amounts of VCS such that the binding to macrophages was significantly dependent on the content of sialic acid (see

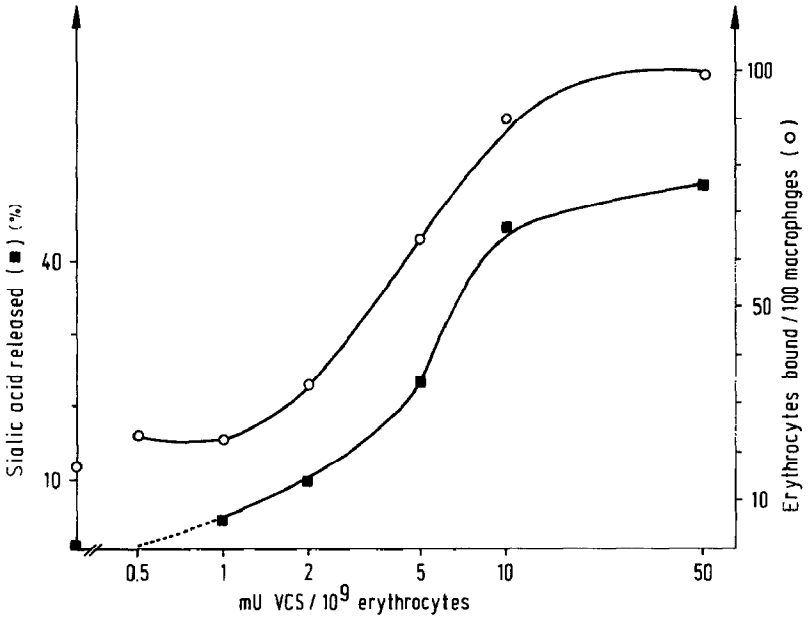

Fig. 2. Removal of sialic acid from fixed erythrocytes and binding of these cells to peritoneal macrophages depending on the amount of VCS during preceding sialidase treatment. The curves represent one typical experiment out of a series of 6. The total sialic acid content is 22.5 nmol/10^9 erythrocytes. Treatment with sialidase, estimation of sialic acid, and binding assay are described in the Experimental section.

middle part of the curves of Fig. 2). Therefore, 10^9 erythrocytes were treated with 2 or 5 mU of VCS for 60 min at 37°. This amount of enzyme removed sufficient quantities of sialic acid (2–6 nmol/10^9 cells) to show a pronounced increase in binding to macrophages when compared with control cells treated without sialidase (Fig. 2). Incubation with either the α-(2→3)-ST or α-(2→6)-ST transferred back to the cell 60–80% of the amount of enzymically released sialic acids, as shown in Fig. 3 for the α-(2→3)-enzyme. This degree of resialylation reduced the binding of fixed erythrocytes to macrophages by 50–80%. However, it did not decrease the binding rate to the level of control cells. The residual macrophage binding of resialylated cells is probably due to the remaining exposed galactose residues and not to an increase of nonspecific binding, as was shown by inhibition with ASOR (Fig. 3). The degree of resialylation and the biological effect were similar for both α-(2→3)-ST and α-(2→6)-ST.

This points to the possibility that about equal proportions of D-galactose groups linked β-(1→3) to 2-acetamido-2-deoxy-β-D-galactopyranosyl or β-(1→4) to 2-acetamido-2-deoxy-β-D-glucopyranosyl residues, the substrate-sugar sequences of the two sialyltransferases used[12,13], may have been exposed by sialidase treatment. The occurrence of α-Sia-(2→6)-β-D-Gal *p*-(1→4)-D-GlcNAc sequence has been described for asparagine-linked sugar chains of rat-erythrocyte-membrane glycoproteins[14]. In contrast to the action of the sialyltransferases, the galactose

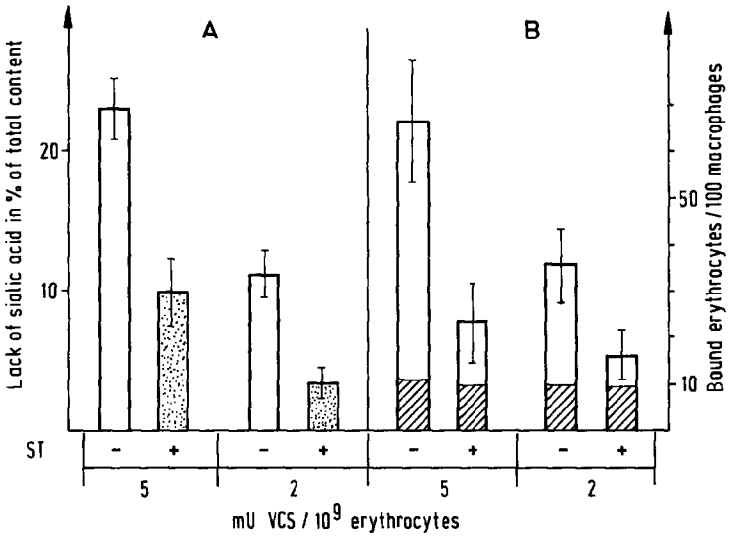

Fig. 3. (A) Partial resialylation of fixed erythrocytes by the action of α-(2→3)-ST: Fixed erythrocytes were treated with VCS (2 or 5 mU/10^9 cells; liberated amount of sialic acid, see white bars) and incubated with purified α-(2→3)-ST as described in the Experimental section. The remaining difference in sialic acid content to control cells is indicated by dotted bars. Removal of sialic acid by VCS and incorporation of sialic acid by ST were estimated as described (n = 3).

(B) Effect of partial resialylation on the binding of fixed erythrocytes to macrophages: Binding of erythrocytes from Fig. 3A to macrophages was assayed as described. Unspecific, not-galactose-mediated binding was estimated by incubation in the presence of 0.6μM ASOR (cross-hatched bars) (n = 3).

residues of both disaccharide sequences seem to be recognized by the macrophage lectin to a similar extent, as the effects of resialylation by both enzymes are similar.

Since Jancik and Schauer[15] observed the rapid disappearance of sialidase-treated rabbit erythrocytes from blood stream, the discussion on the cause of this phenomenon has persisted. Although the involvement of a galactose-specific receptor on macrophages was well established[1–5], factors other than exposed galactose residues could not be ruled out completely. Relevant to this observation was the suggestion that sialidase molecules adhere to the erythrocyte surface and could mediate binding to the macrophages[6]. This explanation, however, was largely ruled out by experiments with an immobilized enzyme[16] or the sialidase inhibitor "*N*-acetyl-2-deoxy-2,3-didehydroneuraminic acid" (5-acetamido-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-non-2-enonic acid)[17]. The experiments described herein are a further proof that the sialic acid residues themselves prevent binding of erythrocytes to macrophages by masking the ligand on erythrocytes for the receptor on macrophages. This antirecognition effect is dependent on the density of negatively charged sialic acid residues on the cell surface. This adds to the observation made for a variety of biological systems, summarized in ref. 1, that sialic acid residues efficiently mask recognition sites.

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