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Monoclonal antibodies for the detection of desialylation of erythrocyte membranes during haemolytic disease and haemolytic uraemic syndrome caused by the *in vivo* action of microbial neuraminidase‡

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Especially in childhood, the *in vivo* action of microbial neuraminidase may cause haemolytic anaemia or life-threatening haemolytic uraemic syndrome. The exposure of the Thomsen-Friedenreich (T) crypto-antigen and T-antigen polyagglutinability of erythrocytes has been described as the first sign of toxic cleavage of *N*-acetylneuraminic acid (Neu5Ac) from sialoglycoproteins of cell membranes. This phenomenon may, however, be too unspecific to initiate treatment for toxin elimination. The present study investigated the diagnostic effectiveness of a panel of three monoclonal antibodies (mcabs) for the estimation of the clinical significance of neuraminidase action *in vivo*. Depending on the amount of Neu5Ac released, the mcabs I-C4, II-Q9 and III-Y12 recognized different epitopes on erythrocyte asialoglycophorin. In 1345 patients, the mcab II-Q9 detected cleavage of Neu5Ac in 32 children who had T-antigen polyagglutinability and mild to moderate haemolytic anaemia. However, only 10 patients, whose erythrocytes were agglutinated by the mcabs III-Y12 or I-C4, developed severe haemolysis, thrombocytopenia, and finally the life-threatening haemolytic uraemic syndrome ($p < 0.0002$). In conclusion, these mcabs provided an early marker of the *in vivo* action of neuraminidase. Two different degrees of erythrocyte desialylation, as defined by these mcabs, are suggested to reflect the severity of toxin-associated disease.

***Keywords*: microbial neuraminidase, haemolytic disease, Thomsen-Friedenreich antigen, asialoglycophorin, erythrocyte membrane**

Introduction

The *in vivo* action of the microbial toxin neuraminidase may cause destruction of erythrocytes [1] and platelets [2, 3], alterations of cardiac myocytes [4], and endothelial cells of renal capillaries [5–7], leading to haemolytic disease associated with thrombocytopenia, renal cortical necrosis, and life-threatening cardiac injury [8–10]. *Streptococcus pneumoniae*, *Clostridium perfringens*, *Klebsiella pneumoniae*, and other anaerobic bacteria are known to be the most important source of neuraminidase production [11]. Particularly in infancy and early childhood, the formation of abscesses or empyema may be the source of overwhelming amounts of toxin. The clinical course of the associated disease has been described by several authors to vary considerably from mild or moderate haemolytic anaemias [12, 13] to the life-threatening haemolytic uraemic syndrome (HUS) with mortality rates of up to 60% [5, 9, 10, 14–19]. This type of neuraminidase-associated HUS can only be treated by immediate exchange transfusion and haemodialysis for toxin elimination [7, 15].

The toxic release of neuraminic acid (Neu5Ac) from sialoglycoproteins of the erythrocyte membrane causes an exposure of the Thomsen-Friedenreich crypto-antigen (T-antigen), rendering the cells polyagglutinable in most human sera [12, 14, 20]. The detection of this type of T-antigen polyagglutinability by haemagglutination tests using the Anti-T lectin derived from peanuts [arachis hypogaea] [21] lacks specificity, since the target antigen of the lectin, the Gal-GalNAc disaccharide, is widely distributed among glycoproteins, and even glycolipids and gangliosides of cell membranes [22]. Several authors have reported the peanut agglutination phenomenon in patients without any

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evidence of haemolytic disease [23]. The destruction of neuraminidase-treated erythrocytes by macrophages depends on the amount of sialic acid release, which is not necessarily correlated with the threshold or titre of peanut reactivity [1].

To evaluate the clinical significance of T-antigen polyagglutination of erythrocytes in childhood, we generated a panel of monoclonal antibodies (mcabs) to study their diagnostic properties in 32 patients. The hypothesis tested in this study was that neuraminidase action may cause different types of crypto-antigen expression in erythrocyte membranes, which are assumed to be associated with the severity of haemolytic disease. The detection of markers for the development of HUS was as important as the early detection of toxin action *in vivo*.

Materials and methods

Monoclonal antibodies

Mcabs were generated by immunization of Balb/c mice with *Vibrio cholerae* neuraminidase-(VCN) treated human erythrocytes according to the method of Stähli *et al.* [24]. Immunoblasts of the spleen were fused with the Balb/c nonproducing myeloma cell line P3-x63-Ag8.653 [25] according to the method described by Köhler and Milstein [26] using polyethyleneglycol [27]. For further characterization, mcabs were selected according to the following criteria: (1) specific strong agglutination of neuraminidase-treated human erythrocytes; (2) binding of ^{125}I-labelled asialoglycophorin in the liquid phase radioimmunoassay (RIA) as described below; (3) proven monoclonality; and (4) growth stability of hybridoma cell line even after the cloning by limited dilution. In all tests, culture supernatant or ascitic fluid served as the source of antibodies, which were purified by gel-chromatography using Sephacryl S 300 (Pharmacia Fine Chemicals, Uppsala, Sweden). Determination of immunoglobulin type was performed by immunoprecipitation of monospecific anti-mouse-Ig sera (Nordic, Tilburg, The Netherlands) in agarose gel and identified the mcab I-C4 as IgM, the mcab III-Y12 and the mcab II-Q9 as IgG.

Erythrocytes

Human erythrocytes used for the immunization of mice as well as the characterization of mcabs were obtained with informed consent from a healthy female donor typed as O Rh pos CCD.ee Kell neg MMss. Neuraminidase derived from *Vibrio cholerae* (Behring, Marburg, Germany), *Clostridium perfringens* (Sigma, Deisenhofen, Germany) or *Streptococcus pneumoniae* (culture supernatant) was usually added in a concentration of 0.2 U ml^{-1} and incubated for 30 min at 37 °C as described elsewhere in detail [7]. Papain (Biotest, Frankfurt, Germany), trypsin (Sigma, Deisenhofen, Germany), and pronase (Serva, Heidelberg, Germany) digestion were performed in addition to prior neuraminidase treatment of erythrocytes according to widely used methods [28].

T-antigen substances

Asialoglycophorin A was prepared from erythrocytes of healthy donors by phenolextraction, and purified by use of gel-chromatography on Sephacryl S-300 [30]. For removal of Neu5Ac, 20 mg of purified glycophorin A was incubated for 6 h at 37 °C in 0.1% $CaCl_2$ and 100 mU ml^{-1} VCN (Behring, Marburg, Germany). Heating to 100 °C for 5 min terminated enzyme action. Pure mono- and di-saccharides (galactose, glucose, galactosamine, glucosamine, Neu5Ac, mannose, fucose, lactose) were purchased from Serva (Heidelberg, Germany) and Merck (Hohenbrunn, Germany). An immunoadsorbent of synthetically prepared T-hapten (8-methoxycarbonyloctyl 2-acetamido 2-deoxy-3-O(β-D-galactopyranosyl)-α-D-galactopyranoside) was coupled to silylaminated calcined diatomaceous earth [31], was purchased from Chembiomed (Alberta, Canada) and used as recommended by the supplier.

Assay procedures

Radioimmunoassay (RIA)

For the RIA ^{125}I-asialoglycophorin A was labelled according to the lactoperoxidase-glucose-oxidase method [29] by use of the Enzymo-bead solid phase radioiodination reagent (BioRad laboratories, München, Germany). Binding curves were determined by serial two-fold dilution of the mcab. Immune complexes were precipitated by use of an anti-mouse-Ig derived from the goat (Nordic, Tilburg, The Netherlands). Non-specific binding [NSB] was found to be at least 1.5–2% of total activity. For binding inhibition studies prior to the addition of the tracer, saccharides and synthetic derivatives of T-hapten and T-antigen were mixed in serial dilutions with an amount of mcab, which precipitated 35–40% of total tracer activity (BO value = 100%). The concentration of inhibitor causing a 50% inhibition of the antibody binding (C 50%) was calculated by linear regression analysis of curves using the empirical logit B/Bo-log c function (MAG-312 gamma counter, Berthold, Hannover, Germany).

Haemagglutination tests

For agglutination tests, patient erythrocytes were suspended at a concentration of 5% (v/v) in 0.15 M NaCl and incubated with an equal volume of mcab for 30 min at room temperature. Agglutination was assessed directly on a slide. Titres were determined in saline as the highest antibody dilution (two-fold serial dilution) giving macroscopically visible agglutination. For diagnostic purposes, the binding activity of mcab II-Q9, III-Y12 and I-C4 was standardized by titrating six two-fold dilutions against human erythrocytes, which had previously been desialylated with 0.2 U ml^{-1} VCN. Red cells of healthy age-matched children

and adult blood donors served as negative controls to demonstrate the specificity of each test.

Patients

Out of 1345 blood specimens from patients that were transferred to our laboratory for immunohaematological investigation of infection-associated anaemia, 32 children were included in this study. They were selected according to the following criteria: (a) demonstration of erythrocyte T-antigen polyagglutinability with peanut lectin (PNA) (Miles, Yeda); and (b) a clinical state of severe infection with presumed *in vivo* action of microbial neuraminidase and/or acquired haemolytic anaemia and/or haemolytic uraemic syndrome. None of the patients had a history of congenital haemolytic anaemia, autoimmune disease, or a malignant disorder of the bone marrow. Underlying infectious diseases were bacterial septicaemia in seven cases, meningitis in two cases, otitis/sinusitis in two cases, pneumonia in four cases, perinatally-acquired infections in five cases, necrotizing enterocolitis of the newborn in six cases, gastroenteritis/colitis in three cases and endocarditis, urinary tract infection, and central shunt infection in one case each. Microbiological investigations of blood, ascitic or cerebrospinal fluid, pleural effusions, abscesses, stool, urine, sputum, or smears of the nose and the pharyngeal space revealed neuraminidase-producing bacteriae as the cause of disease in 15 children. Erythrocytes were investigated for binding of mcabs immediately after blood was drawn at the time of admission, or in the very beginning of infectious and/or haemolytic disease prior to blood and/or platelet transfusions. Analyses were repeated, initially, up to four times per day, and continued daily until recovery from the acute disease state. Evaluating the clinical course, the haemolytic disease was defined as (I) mild in the case of anaemia; (II) moderate in the case of anaemia, thrombocytopenia, and septicaemia; or (III) severe in the case of haemolytic uraemic syndrome and septic shock.

Statistics

Statistical analysis was performed using Pearsons and Fisher exact χ^2 tests as appropriate (*Statistix* 4.1 software 1994). p-Value < 0.005 was considered indicative of statistical significance.

Results

Characterization of mcabs against human erythrocytes

Specific binding to desialylated erythrocytes was documented for mcab I-C4, II-Q9 and III-Y12. There was no difference in reactivity of antibodies using neuraminidases of different microbial origin for desialylation. Additional haemagglutination tests were performed using red cells modified with trypsin, papain and pronase in addition to prior neuraminidase treatment. In these tests, reactivity of mcab I-C4 was totally abolished by any proteolytic membrane alteration. Papain and pronase treatment destroyed the binding of both mcab III-Y12 and mcab II-Q9. Trypsin digestion of membrane proteins, however, reduced binding activity of these two antibodies only mildly or moderately, respectively.

Binding of mcab to T-hapten

Only mcab I-C4 was found to bind well with the pure synthetic preparation of the T-hapten. For adsorption tests the mcabs were used in standard dilution giving an agglutination titre of six steps of serial two-fold dilution against neuraminidase-treated erythrocytes. The incubation of mcab I-C4 with a synthetic T-hapten immunoadsorbent before testing completely abolished the binding to desialylated erythrocytes. Adsorption of mcabs II-Q9 with this immunoadsorbent only slightly reduced the binding, decreasing the titre of haemagglutination for only one step of serial two-fold dilution. The binding of mcab III-Y12 was not at all influenced by preincubation with T-hapten immunoadsorbent.

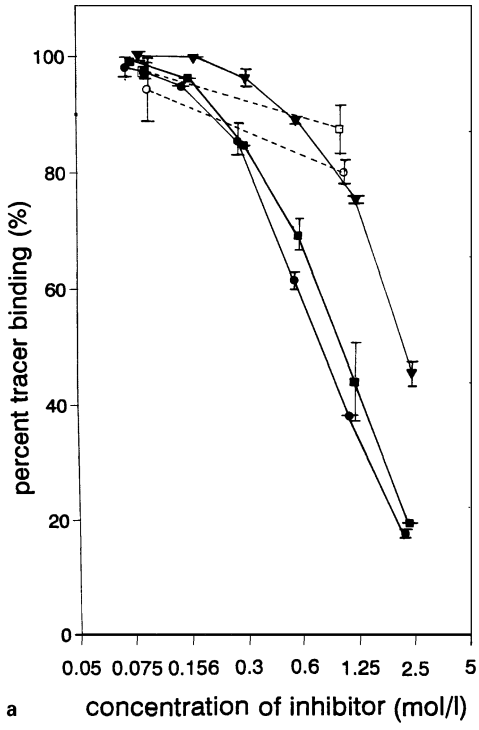
Glycopeptide and carbohydrate specificity of mcabs

In the RIA all antibodies showed specific binding to the asialoglycophorin tracer. Inhibition studies were performed for further characterization of the binding specificities. The inhibitory activity of different glycoproteins and carbohydrates was calculated from the concentration of inhibitor that causes a 50% inhibition of antibody binding to the tracer. As expected, all antibody binding was inhibited by even low concentrations of asialoglycophorin (Table 1). This reflected the specificity for desialylated erythrocytes. The binding of mcab I-C4 and mcab III-Y12, however, was not influenced by even high concentrations of pure native glycophorin. Mcab II-Q9 was inhibited by a 40-fold higher concentration of glycophorin compared to that of asialoglycophorin. Using pure carbohydrates, the binding of

Table 1. Glycoprotein–specificity of monoclonal antibodies. Concentration of inhibitor [$\mu g\ ml^{-1}$] causing 50% inhibition of ^{125}I-asialoglycophorin binding.

Inhibitor [$\mu g\ ml^{-1}$]	*mcab I-C4*	*mcab II-Q9*	*mcab III-Y12*
Asialoglycophorin	0.25	1.29	0.52
Glycophorin	> 500	39.1	> 500

Mcabs derived from culture media and used in the RIA at lowest concentrations still giving saturated tracer binding; glycoproteins prepared from human red cell membranes used as inhibitors in five two-fold serial dilutions (0.05–500 $\mu g\ ml^{-1}$). The concentration of inhibitor which caused 50% inhibition of tracer binding was calculated from the inhibitor dilution curves by linear regression analysis (MAG-312 gamma counter, Berthold, Hannover, Germany).



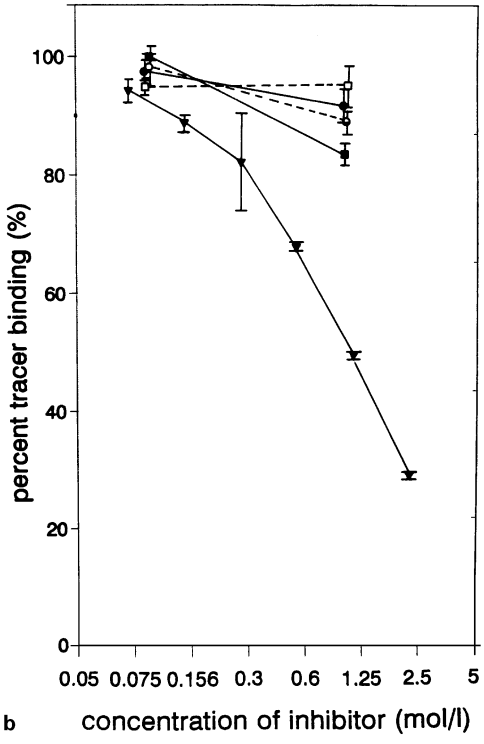


Figure 1. Carbohydrate specificity of mcab I-C4 [a], mcab III-Y12 [b] and mcab II-Q9 [c] measured by the inhibition of ^{125}I-T-antigen binding by different saccharides; galactose ●, lactose ■, mannose ▼, in a RIA. Mcabs were used at dilutions giving best tracer binding [B0]. The data are given as the percentage binding inhibition [B/B0] (mean ± SD). Glucose □ and fucose ○ were used as controls.

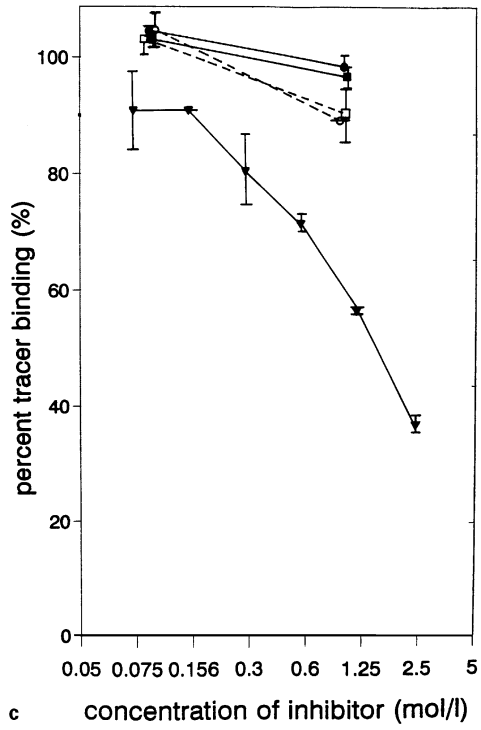


Figure 1. Continued.

all antibodies was found to be specifically inhibited by mannose (Figure 1). Mcab I-C4, however, was even more inhibited by the carbohydrates galactose and lactose. Surprisingly, mcab II-Q9 was also inhibited by low concentrations of Neu5Ac. We found no significant inhibition by glucose, sucrose, fucose, galactosamine and glucosamine.

Binding of mcabs to erythrocytes after different amounts of desialylation

Haemagglutination assays revealed striking differences in: (1) the affinity and strength of binding to desialylated erythrocytes and (2) the threshold of haemagglutination for mcabs I-C4, II-Q9 and III-Y12. Reactivity appeared to depend on the amount of Neu5Ac released. This was further examined using erythrocytes treated with increasing amounts of neuraminidase and the measurement of binding activity by haemagglutination (Figure 2). Mcab II-Q9 agglutinated erythrocytes that were treated with only 0.37 $U l^{-1}$ of neuraminidase, but the maximum effect was observed after treatment of the cells with more than 100 $U l^{-1}$. Mcab III-Y12 agglutinated erythrocytes after treatment with 1.5 $U l^{-1}$ of enzyme, and using 12 $U l^{-1}$ of neuraminidase caused the maximum effect. On the other hand, mcab I-C4 reacted with erythrocytes only after treatment with more than 25 $U l^{-1}$ neuraminidase, and showed the highest effect after treatment with more than 100 $U l^{-1}$. After incubation with 200 $U l^{-1}$ of enzyme, human erythrocytes were strongly agglutinated by all antibodies.

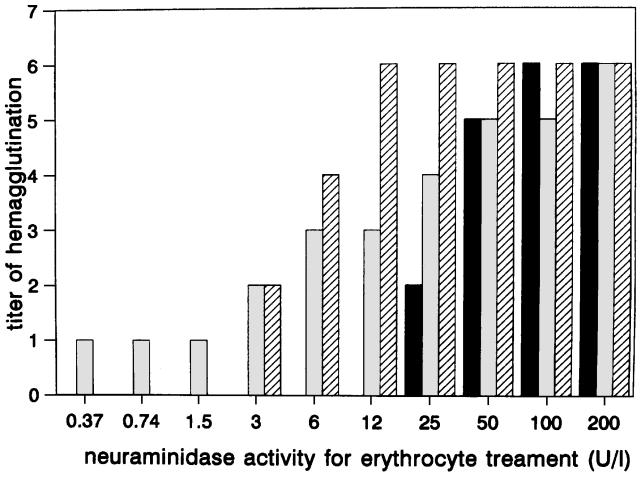


Figure 2. Binding of mcabs to human erythrocytes treated with increasing activities of *Vibrio cholerae* neuraminidase. The haemagglutination titre is given as two-fold serial dilutions of mcab. Mcabs were used in standard concentration for diagnostic purposes, giving a titre of six two-fold dilutions against human erythrocytes, which had previously disialylated with 0.2 U ml^{-1} VCN. The data are presented as the identical result of three to five measurements. ■ I-C4, ▨ III-Y12, □ II-Q9.

Clinical investigation of the diagnostic value of mcabs

The number of mcabs binding to the erythrocyte membranes of patients increased with increased severity of haemolytic disease (Table 2). In all 32 patients even a minimal release of Neu5Ac was detected by mcab II-Q9. The erythrocytes of 22/32 children (69%) with ages that ranged from newborn to 18 years reacted exclusively with this antibody, in addition to the PNA control reagent (group I). They developed haemolytic anaemia and mild to moderate thrombocytopenia, but none of them showed symptoms of life-threatening toxin action or an impairment of renal function. In 10/32 children (31%), however, the alteration in erythrocytes was not confined to simple T-antigen polyagglutinability. The erythrocytes of five patients showed additional binding of mcab III-Y12 (group II): three preterm infants and one newborn suffering from septicaemia and necrotizing enterocolitis caused by undefined infectious agents in two cases, and *Clostridium perfringens* or *Klebsiella pneumoniae* in one case each, and a boy aged 12.3 years with endocarditis of unknown origin. All these children developed haemolytic anaemia, thrombocytopenia, and cardiovascular signs of toxin action. Treatment required antibiotics and surgical exstirpation of the focus of infection. In two of the infants, intensive care including exchange-transfusion was necessary. Erythrocyte reactivity with all three mcabs was detected in another group of five children: two male toddlers with pleuropneumonia caused by *Streptococcus pneumoniae*, one newborn developed necrotizing enterocolitis caused by *Clostridium perfringens*, one pre-term infant suffered from *Serratia marcescens* septicaemia, and one 4.3-year-old boy who had symptoms of sinusitis and otitis caused by *Klebsiella pneumoniae* complicating the state of immunosuppression after heart transplantation. These patients developed life-threatening HUS (group III). Treatment included antibiotics, surgical intervention, blood exchange transfusion, and, in some cases, haemodialysis. The clinical state improved within 1–5 days according to the time course of disappearance of the marker red cells reacting with mcab I-C4. Haemolytic anaemia was observed over a period of 12–14 days as a sign of life-span reduction of partially desialylated erythrocytes reacting with PNA and mcabs II-Q9 and III-Y12. The results of these follow-up studies provided: (1) the mcab II-Q9 for the detection of a toxic release of Neu5Ac from the erythrocyte membrane (group I); and (2) the mcabs III-Y12 and I-C4 for the demonstration of a higher degree of sialic acid removal, reflecting systemic toxin action that appeared to predict the development of haemolytic uraemic syndrome (groups II and III).

Sensitivity and specificity

Using the combined haemagglutination of patient erythrocytes by both mcabs III-Y12 and I-C4 as a test for haemolytic uraemic syndrome, the test sensitivity was 1.0 and specificity 0.88. The positive predictive and negative predictive values were 0.7 and 1.0, respectively, while accuracy was 0.91. Haemolytic anaemia was found during the course of disease in 81% of the cases investigated (26/32) without any significant difference between group I and group II/III ($p = 0.23$). However, the development of thrombocytopenia ($p < 0.0005$) and haemolytic uraemic syndrome ($p = 0.0002$) during the clinical course were significantly associated with the agglutination of patients'

Table 2. Agglutination of patient erythrocytes by monoclonal antibodies during the course of different degrees of severity of haemolytic disease.

Severity of haemolytic disease	*No. of patients*	*Binding of mcabs*	*Haemolysis*	*Thrombocytopenia*	*HUS*
I. Mild	22	II-Q9	16/22	2/22	0/22
II. Moderate	5	II-Q9 + III-Y12	5/5	5/5	2/5
III. Severe or life-threatening	5	II-Q9 + III-Y12 + I-C4	5/5	5/5	5/5

For parameters of mcab binding and definition of severity of haemolytic disease see methods.

Table 3. Time course study for reaction pattern of monoclonal antibodies during neuraminidase-associated haemolytic disease in a newborn suffering from necrotizing enterocolitis caused by *Clostridium perfringens*.

Day of disease	*Haemoglobin (g dl^{-1})*	*Platelet count [per nl]*	*Creatinine [mmol l^{-1}]*	*Diuresis [ml per kgbw per h]*	*Reaction of mcab II-Q9*	*Reaction of mcab III-Y12*	*Reaction of mcab I-C4*
1	17.7	228	1.0	<1	ND	ND	ND
2	10.3	100	1.6	1.3	13	7	7
3	13.3	172	1.5	2.2	9	7	4
4	13.9	76	1.4	6.3	4	5	0
5	13.9	28	0.8	6.8	5	5	0
6	12.4	17	0.8	3.8	4	4	0
7	11.5	28	0.5	5.8	4	4	0
8	10.9	30	0.5	7.3	4	4	0
9	9.6	84	0.4	6.7	4	4	0
10	ND	ND	ND	6.2	4	4	0
11	6.5	91	0.4	6.2	4	4	0
12	11.0	105	0.4	6.2	3	3	0

Reaction of monoclonal antibodies: haemagglutination titre in steps of two-fold serial dilutions. ND, not determined.

erythrocytes by mcabs III-Y12 and I-C4 in groups II and III.

Case study

Table 3 summarizes an impressive example of a time course study of haemolytic disease (Grade III severity) and the mcab reactivity during 14 days of treatment. The clinical course was characterized during the first 2 days by septicaemia, necrotizing enterocolitis with presence of blood in the stool, gut perforation, and peritonitis, haemolytic anaemia, renal insufficiency, respiratory distress, hypocalcaemia and bradycardia. After 3 days of intensive care, treatment with antibiotics, surgical intervention and colectomy, as well as daily blood transfusions, stabilization began on the fourth day of disease. Within 4 weeks altered erythrocytes were eliminated from the circulation and after one additional transfusion on day 11 the child fully recovered.

Discussion

The use of mcabs II-Q9, III-Y12 and I-C4 in haemagglutination tests can be of considerable value in the follow up of haemolytic disease caused by microbial neuraminidase. These antibodies detected epitopes of asialoglycophorin A, which were accessible only after cleavage of different amounts of sialic acid. The complex oligosaccharide, N-glycosidically linked to the amino acid 26 of the glycophorin A molecule [32, 33], is known to contain mannose. This sugar seems to be included in the binding site of all three mcabs. Beyond that, the mcab I-C4 showed strong specificity for the β-D-galactose(1-3)-*N*-acetyl-D-galactosamine sequence of the T-antigen, which is linked O-glycosidically to 15 serine or threonine residues of glycophorin A [34, 35].

The mechanism for the rapid elimination of erythrocytes with T-antigen from the circulation during infectious diseases was first suggested to be due to either a naturally occurring serum antibody [12, 16, 36] or a receptor-mediated specific phagocytosis by Kupfer cells, hepatocytes, or mononuclear spleen cells [37–39]. Further investigations, however, provided evidence against the T-antigen as the main and sole cause of haemolysis. The mode of *in vivo* destruction of neuraminidase treated erythrocytes varied depending on the amount of Neu5Ac release [40]. Aminoff *et al.* found a 15% decrease in Neu5Ac during senescence of human erythrocytes, limiting the survival of these cells [41]. A 10–20% release of Neu5Ac from membranes is sufficient to initiate the binding of erythrocytes to macrophages (Schauer *et al.* [1]). Phagocytosis was observed after removal of more than 30–40% Neu5Ac after treatment with 10 U l^{-1} neuraminidase [1]. In animals, the application of similar amounts of neuraminidase caused a disease that was similar to the HUS in young children: *ie* rapid destruction of erythrocytes and platelets [41], alterations of arterial endothelial cells, adhesion of platelets, and thrombotic changes. After removal of more than 60% of the Neu5Ac, the half-life of human erythrocytes *in vivo* is reduced to less than 2 h [1]. The acute intravascular haemolysis was supposed to be caused by alterations in the membrane charge [42], or mediated by the alternative complement pathway [43]. During the incubation of erythrocytes with viral or bacterial neuraminidases, an activation of the alternate complement pathway can be demonstrated [44].

In the follow-up studies of the 32 children, the reaction pattern of the mcabs seemed to reflect the severity of the clinical state of those patients suffering from the *in vivo* action of microbial neuraminidase. Further investigations showed antibody binding that depended on the amount of sialic acid released from membranes. The reactivity of these

reagents is therefore assumed to vary according to the presumed mode of erythrocyte destruction during the course of the haemolytic disease. Mcab II-Q9 bound to erythrocytes treated with minimal amounts of neuraminidase releasing in less than 10% sialic acid. Mcab III-Y12 agglutinated erythrocytes only after their incubation with an enzyme activity of more than 1.5 $U l^{-1}$, causing a decrease of almost 25% in the Neu5Ac content of membranes [1]. The clinical course of patients, whose erythrocytes showed binding of this antibody, seemed to confirm that if more than 10–25% of the Neu5Ac was removed this caused erythrocytes to be bound by macrophages producing significant haemolytic anaemia. For binding of mcab I-C4, the erythrocytes needed to be incubated with more than 12 $U l^{-1}$ of neuraminidase, this is known to cause a release of more than 45–50% of the Neu5Ac [1]. It is, therefore, suggested that the reactivity of mcab I-C4 is confined to erythrocytes, which become fragmented, haemolysed and eliminated from the circulation. This is probably due to the loss of membrane charge and the exposure of target antigens for binding of macrophages.

Despite the fact that the mechanism of cell destruction by microbial neuraminidases still remains to be clarified, the availability of a diagnostic tool for the determination of the extent of toxin action is of considerable value for a concomitant control during the clinical course of severe haemolytic disease in childhood. The reactivity of mcab I-C4 in particular can be assumed to predict the development of haemolytic uraemic syndrome and can provide clinicians with evidence of significant toxin action *in vivo*.

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