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HUMAN BLOOD-GROUP MN AND PRECURSOR SPECIFICITIES: STRUCTURAL AND BIOLOGICAL ASPECTS*

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ABSTRACT

The human blood-group MM and NN antigens carry 2 to 4 immunodominant groupings per repeating subunit and differ only by one sialic acid residue per immunodominant group. This residue covers in the MM antigen the β-D-galactopyranosyl group that is terminal in the N immunodominant structure and that, together with a terminal α-linked *N*-acetylneuraminic acid residue, is responsible for N specificity. M specificity was readily converted into N specificity by mild acid treatment. N structure is the immediate biochemical precursor of M structure, and M and N antigenic specificities are not determined by two allelic genes as believed hitherto. The NN antigen was inactivated by β-D-galactosidase as well as by removal of *N*-acetylneuraminic acid. Some of the reactivities of the NN antigen, lost upon β-D-galactosidase treatment, reappeared on subsequent partial *N*-acetylneuraminic acid removal. The structure uncovered by complete sialic acid depletion of MN antigens is the Thomsen–Friedenreich T antigen, the specificity of which is determined by β-D-galactopyranosyl groups. β-D-Galactosidase treatment transformed the T antigen into one possessing Tn activity. The significance of blood-group MN active substances extends to human breast cancer, where MN antigens were found in benign and malignant glands, but some of their precursors in cancerous tissue only.

INTRODUCTION

The MN glycoproteins are the major antigens of the second human blood-group system[1,2]. The first accurate information on the chemical basis of the MN specificities was the finding of their inactivation by neuraminidases[3,4]. There is only one report[5] on the overall chemical composition of an isolated blood group N-active terminal fragment with a carbohydrate part consisting of the structure α-NeuNAc→D-Gal→(D-Gal)→D-GalNAc→R†. Blood-group MM and NN antigens are glycoproteins having closely similar structures and potent inhibitors of hemagglutination by

*Dedicated to Professor Michael Heidelberger in honor of his 87th birthday.

†Abbreviation: NeuNAc, *N*-acetylneuraminic acid. The genotypes of the antigens are given throughout.

influenza viruses[5–9]. Recently, it was demonstrated[10] that M specificity is readily transformed to N at pH 2.0 and 56°. The N chemical structure appears to be the immediate biochemical precursor of M, and the allele to the M gene is amorph[10–12]. Thus, the M and N antigenic specificities are not determined by two allelic genes, as believed hitherto[13,14]. The molecular size of the aggregates[15] and perhaps the ε-amino groups[16] of these glycoproteins have been shown to be important for the extent of the biological activity.

MATERIAL AND METHODS

Anti-reagents, erythrocytes, suspending solution, and enzymes. — All anti-blood group reagents employed were saline-agglutinating and have been described before[3,5], except human anti-T and anti-Tn reagents which were both obtained from persons having blood-group A_1B; the anti-Tn reagent was used after exhaustive absorption with neuraminidase-treated O erythrocytes. *Phaseolus limensis*[17], *Dolichos biflorus*[18], and *Arachis hypogaea*[19] seed extracts were also used.

Human O,MM and O,NN erythrocytes were obtained, stored, washed, and employed as described previously[3]. The diluent and erythrocyte-suspending solution was aqueous 0.1M sodium chloride containing 50mM phosphate buffer at pH 7.3 (buffered saline). T-activation of O cells with RDE was performed as described by Springer and Ansell[3]. Tn red cells, from donors carrying this rare antigen, were donated by Dr. W. D. Bowman (Car. Lip.) and Dr. G. Leonard (Cla. Ric.). We accomplished, for the first time, the artificial activation of Tn specificity by a degradative procedure on O red cells: After neuraminidase treatment, the cells were treated twice with *E. coli* β-D-galactosidase, under the conditions described earlier[10], except that 1,200 U of enzyme in 1.25 ml of the appropriate buffer, made isotonic with sodium chloride, were added to 0.25 ml of packed red cells and the total incubation time was 18 h. All enzymes employed have been described before[5,10], except bovine testicular β-D-galactosidase kindly given by Dr. Jourdian[20].

Hemagglutination and hemagglutination inhibition tests. — These tests, including positive and negative controls and standards, were performed and interpreted as described earlier[3,5]. Red-cell agglutination was read on the microscope by 3 individuals about 90 min after addition of 0.5% red-cell suspension. Inhibitory activities are given as mg or μmol/ml before addition of red cells and serum.

Preparation of MM and NN antigens. — This preparation has been described earlier[5]. Rejected or recently outdated, banked group O blood, less than 3 weeks old, was typed by standard procedures and without regard to the secretor status of the donors. Serological results throughout were verified by at least two individuals. Stroma was prepared by aqueous lysis at pH 5.3. The stroma was extracted with aqueous 45% phenol, in the presence of an electrolyte and at 23°, as described earlier[5]. The active material was found in the aqueous phase and was isolated by fractional ultracentrifugation and ethanol fractionation, followed by gel chromatography[5].

Preparation of T antigen. — The T antigen was uncovered on isolated O,MM and O,NN antigens by either neuraminidase or acid hydrolysis, as described earlier[3,5,11].

Quantitative analytical procedures. — These procedures were the same as those previously described[5,10]. In addition, the D-galactose content was determined with D-galactose dehydrogenase[21], after hydrolysis of the antigen with M hydrochloric acid for 8–10 h at 100°. *N*-Acetylhexosamines were determined by the method of Gatt and Berman[22]. Spectrophotometric measurements were performed with a Beckman DU spectrophotometer equipped with a Gilford 220 attachment. The free acetyl content was measured by g.l.c.[23] and the pyruvic acid content according to Lieberman *et al.*[24].

RESULTS AND DISCUSSION

Properties of the isolated red-cell antigens in relation to their MN specificities. — The *Vicia graminea* inhibitory activity of the NN antigens was uniformly higher than that of MM antigen preparations. Earlier enzyme studies had shown that the immunodominant structure responsible for reaction with the *V. graminea* reagent was a terminal, nonreducing β-D-galactopyranosyl group[5]. We, therefore, assessed the role of β-D-galactose groups in blood-group N specificity with 2 different, cross-reacting precipitating reagents, which possessed anti-β-D-galactose specificity, on six different MM and six different NN preparations. The two most active MM and NN preparations differed qualitatively in their activity towards some of the precipitating heterologous reagents. The most highly active MM antigen preparations (towards isologous anti-M sera) did not precipitate with anti-pneumococcus Type XIV serum, whereas the most highly active NN preparations did; this suggests the presence of more accessible terminal β-D-galactose groups in NN substance, since the β-D-galactopyranosyl group is the immunodominant structure in the interaction with this serum[25,26]. MM antigens of lesser homologous activity did precipitate anti-pneumococcus Type XIV antiserum, and less N-active NN antigens precipitated more antibody than the highly active ones, which indicated some degradation of the less active MM as well as of the NN antigens during their isolation. The precipitability of both antigens was much increased after removal of the *N*-acetylneuraminic acid groups. *Arachis hypogaea* extracts, which also have anti-β-D-galactose specificity[19], precipitated only *N*-acetylneuraminic acid-free MM and NN antigens.

Precipitation of *N*-acetylneuraminic acid-free MM and NN antigens by anti-pneumococcus Type XIV serum was inhibited by methyl β-D-galactopyranoside and other β-glycosides of D-galactose, most effectively by lacto-N-tetraose (<0.3 μmol/ml). Oligosaccharides having a subterminal 2-acetamido-2-deoxy-D-galactose residue were not available, and α anomers did not inhibit. Precipitation of *N*-acetylneuraminic acid-free MN antigens by *Arachis hypogaea* extracts was best inhibited by β-D-(1→4)-linked disaccharides. Thus, β-D-Gal*p*-(1→4)-GlcNAc and lactose gave >75% inhibition at a hapten concentration of 5mM, while over twice that amount of β-D-

Gal*p*-(1→3)-D-GlcNAc was needed to produce the same effect. The corresponding tetrasaccharides, lacto-N-tetraose and lacto-N-neotetraose, were less active on a molar basis than the disaccharides, but again the one having a terminal (1→4)-linked D-galactose group showed the greater inhibiting power. Melibiose was inactive, as were 2-acetamido-2-deoxy-D-galactose, 2-acetamido-2-deoxy-D-glucose, D-glucose, and L-fucose. D-Galactose possessed a trace of activity. We found no terminal hexosamine groups on the intact O,MM and O,NN antigens with the heterologous reagents, but the *N*-acetylneuraminic acid-free antigens precipitated strongly with reagents possessing anti-2-acetamido-2-deoxy-α-D-galactopyranosyl specificity.

Transformation of M into N specificity and its chemical basis. — We have isolated earlier N-specific haptens from MM antigens[27] and transformed MM antigens into antigens possessing only N specificity, whereas the reverse transformation was not possible[10,28]. The chemical basis for this transformation was not established.

We have now observed that the *N*-acetylneuraminic acid release at pH 2.0 and 56° from MM antigens (average *N*-acetylneuraminic acid content, 14.16%) was, during the first 8 h of hydrolysis, 130–150% of that from NN antigen preparations that had an average *N*-acetylneuraminic acid content of 13.78%. After 12 h, the NN glycoproteins retained twice as much *N*-acetylneuraminic acid as did the MM substances. No other sugar, pyruvic acid, or acetyl groups were released. These findings, as well as similar, though less pronounced ones, after *Vibrio cholerae* neuraminidase treatment, indicate that part of the *N*-acetylneuraminic acid residues in the MM antigen are bound differently than in the NN antigen.

The left-hand side of Table I shows the association of loss of M activity with progressive decrease of *N*-acetylneuraminic acid content on acid hydrolysis of the MM antigen. After 2 h, the M activity had decreased by 90% as measured with most anti-M sera (SM-662 is listed as example), while ~50% of the total *N*-acetylneuraminic acid content had been released. However, there are at least two different types of human anti-M sera, for, as determined with some sera (*e.g.*, Ortho-66-1359), M activity decreased much more slowly; up to 12% of the original M activity was demonstrable on prolonged hydrolysis while the loss of *N*-acetylneuraminic acid was >90%. All rabbit anti-M sera were inhibited similarly to the human anti-M sera described last.

The right-hand section of Table I shows the remarkable influence of mild acid hydrolysis on the specific inhibitory effect of the MM antigens on anti-N reagents. Some individual anti-N sera, to which the intact MM antigens had virtually no affinity, as well as some sera that were inhibited by native MM antigens showed up to a 16-fold increase in their reactivity with the MM antigens that had lost part of the *N*-acetylneuraminic acid residues. Maximal reactivity increase occurred between 1 and 4 h of hydrolysis, before the T-activity became demonstrable (see later), and when the content of antigen-bound *N*-acetylneuraminic acid was 2/3–1/3 of that of the original. It will be shown, however, that the majority of the *N*-acetylneuraminic acid residues of the MN macromolecules are not involved in the M and N immunodominant groups.

TABLE I

EFFECT OF HYDROLYSIS AT 56° AND pH 2.0 ON *N*-ACETYLNEURAMINIC ACID CONTENT AND SEROLOGICAL SPECIFICITY OF THE BLOOD-GROUP MM ANTIGEN[a]

Hydrolysis time (h)	*NeuNAc decrease (%)*	*Inhibition decrease of anti-M serum (-fold)*[b]			*Inhibition increase of anti-N reagent (-fold)*[b]		
		Human		*Rabbit*	*Human*[c]	*Rabbit*[c]	V. graminea
		SM-662	*Ortho 66-1359*				
1	14.1	3	2	0–2	>3–>8	1.5	2
2	50.0	15	3	0–2	>4–>8	4	4–8
4	67.8	80	4	2–3	>4–16	3	4–16
8	85.3	95	8	2–5	>3	nil	4–8
12	91.6	>150	10	3–7	nil	0.5	4

[a]Average of duplicate tests in three different experiments. [b]Compared to untreated antigen. [c]Average, of 7 human and 5 rabbit sera.

N activity of the MM antigens, as measured with human sera, rose to 20–100% of that of native NN antigen preparations. Rabbit anti-N sera were inhibited by MM antigens, which had been hydrolyzed for 2–4 h, to virtually the same extent as by NN antigen; except serum Wien. 112 which exhibited only a marginal increase.

Hydrolysis for 2–6 h of NN antigen at pH 2.0 and 56° resulted in an 8-fold or greater decrease of the inhibitory activity toward six of nine human anti-N sera, and all five rabbit anti-N sera, frequently after a brief initial increase. *V. graminea* activity increased 2–8-fold, and no M or other blood-group activities appeared.

Glycosidase action on MM and NN antigens. — The action of neuraminidase on MN antigens is well known[3,4], but not that of other glycosidases. Of the galactosidases tested now, α-D-galactosidase had no effect, but both β-D-galactosidases destroyed blood-group N specificity, after an 8-day incubation at 37°, to about 80% as measured in seven different experiments twice with four different human and four different rabbit sera. *V. graminea* activity was destroyed by ~90%. Decrease of N activity after two incubations with β-D-galactosidase was generally >95%; up to 3.10 mol of β-D-galactose were released per antigen subunit (mol. wt. ~50,000). The enzyme had no effect on M or influenza-virus inhibitory activities. Release of D-galactose from MM antigens was insignificant. The N-specific substance obtained by mild acid hydrolysis of the MM antigen was inactivated by β-D-galactosidase in the same way as native NN antigen.

In related experiments, NN antigen preparations, which had lost >80% of their N, *V. graminea*, and horse anti-pneumococcus Type XIV activities after repeated β-D-galactosidase treatment, were treated at an acidic pH of 2.0 for 0–12 h at 56°. *V. graminea* and pneumococcus Type XIV reactivities reappeared to approximately original levels and some reactivity with *Arachis hypogaea* extract appeared *de*

novo. The results are depicted in Table II, which shows the decrease of the β-D-galactopyranosyl-dependent activities upon β-D-galactosidase treatment and the reappearance of most of it on removal of *N*-acetylneuraminic acid. Surprisingly, the O,NN antigen did reacquire some N specificity with two of six human anti-N sera, but with none of the rabbit anti-N sera employed. While a D-galactose group uncovered by acid hydrolysis is required for this surprising N reactivation, *N*-acetylneuraminic acid groups are also required for significant N activity, as measured with unfractionated human anti-N sera, for we found it only when ~50% of the original *N*-acetylneuraminic acid content was still present. These results indicate that the *N*-acetylneuraminic acid group required for both human N, as well as M specificities is linked to a subterminal β-D-galactose residue.

TABLE II

EFFECT OF REMOVAL OF *N*-ACETYLNEURAMINIC ACID ON β-GALACTOSIDASE-INACTIVATED O,NN ANTIGEN

Antigen	*Hydrolysis conditions*	*Change of serological acitivity (-fold)*		
		V. graminea *extract*	*Human anti-N serum*	*Horse anti-pneumoc. XIV serum*
O,NN	β-Galactosidase[a]	>100↓	>32↓	8↓
β-Galactosidase-inactivated O,NN[b]	Hydrochloric acid[b]	>64↑	4↑[c]	16↑

[a]Two treatments with β-galactosidase resulted in a decrease of D-galactose of 10% of the total D-galactose content of the antigen. [b]Treatment for 4 h at 56°, pH 2.0, resulted in a decrease of *N*-acetylneuraminic acid of 36% of the total NeuNAc content of the antigen. [c]With 2 of 6 human and none of 2 rabbit antisera.

Neither α- nor β-*N*-acetyl-D-hexosaminidases had any effect on the MN specifities, and there was no significant hexosamine release. However, α-hexosaminidase released from *N*-acetylneuraminic acid-free MM and NN antigens 1.4–1.7 mol of *N*-acetylhexosamine/subunit of antigen. These residues apparently are linked to parts of the MN macromolecule that do not possess the MN specific structures. These observations agree with the just described immunochemical findings. Analysis by the Stoffyn and Jeanloz procedure[29] showed that the released hexosamine was exclusively 2-acetamido-2-deoxy-D-galactose, possibly α-D-linked directly to the peptide core. No hexosamine was released from *N*-acetylneuraminic acid-free MN antigens by β-*N*-acetyl-D-hexosaminidase.

T and Tn specificities of O,MM and O,NN antigens. — No T activity was demonstrable on the "intact" isolated MM and NN glycoproteins with either human antiserum or *Arachis* anti-T lectin. No enzyme other than neuraminidase uncovered the T antigen, as did acid treatment. Human anti-T serum was completely inhibited by 0.01 mg of T antigen, by 15 mg of asialoganglioside, which has a terminal β-D-

Gal*p*-(1→3)-D-GalNAc structure, and by 50 μmol of β-D-Gal*p*-(1→3)- or (1→4)-D-GlcNAc; lactose partially inhibited, whereas melibiose, methyl α- and β-D-galactopyranosides, D-galactose, D-mannose, 2-acetamido-2-deoxy-D-galactose, D-glucose, and 2-acetamido-2-deoxy-D-glucose were inactive; β-D-galactose is the most important residue for the human anti-T combining site, but it does not possess the entire complementarity, as it does with *Arachis* anti-T as shown by Uhlenbruck *et al.*[19].

Isolated, fully activated T antigen possessed no Tn activity in inhibition assays with Tn cells and properly absorbed human antiserum and *Dolichos* anti-Tn lectin; the reaction was also negative with these reagents and A_1 red cells. β-D-Galactosidase treatment of isolated T antigen and of neuraminidase-treated red cells produced Tn, as measured with these two reagents but not with the *Phaseolus* extract. This observation agrees with previous findings for the naturally occurring Tn[30,31], as does our finding that 2-acetamido-2-deoxy-D-galactose was a strong inhibitor of β-D-galactosidase-produced Tn specificity. We also found that, on a molar basis, ovine submaxillary mucin (OSM) had >50,000 times the inhibitory activity of 2-acetamido-2-deoxy-D-galactose when measured with human anti-Tn, and >1,000 times when measured with anti-Tn from *Salvia sclarea*, provided that the *N*-acetylneuraminic acid residues of OSM had been removed. The extremely high anti-Tn inhibitory activity of *N*-acetylneuraminic acid-free OSM, which carries[32] only 2-acetamido-2-deoxy-D-galactose α-linked to Ser and Thr, permits the speculation that the 2-acetamido-2-deoxy-D-galactose residue, in the MN antigens, that confers Tn specificity may also be α-linked to Ser, or to Thr, or to both.

As described earlier, the transformation of MM antigen into an antigen that reacts like the NN antigen was accompanied chemically by a partial loss of *N*-acetylneuraminic acid groups and the uncovering of subterminal β-D-galactose residues, detected by enzymic and immunochemical assays. NN antigen in turn was inactivated specifically by β-D-galactosidase treatment as well as by removal of *N*-acetylneuraminic acid residues, since both terminal β-D-galactosyl and terminal *N*-acetyl-α-neuraminosyl groups are required for full N specificity. The structure uncovered by the complete removal of *N*-acetylneuraminic acid residues from M and N immunodeterminants is the T antigen of Thomsen and Friedenreich[3,33]. β-D-Galactosidase transformed the T antigen into one possessing Tn activity.

Interpretation of our major chemical and immunochemical findings on the immunodominant structures of the M-, N-, and their immediate precursor specificities is summarized in Fig. 1. The N structure thus has a position in the MN system closely akin to that ascribed to the H(O) structure in the ABH(O) system[34]. The *N*-acetylneuraminic acid group required for N specificity is linked to a D-galactose residue (see earlier); this normally subterminal D-galactose residue in turn is probably linked to the same or to an adjoining, subjacent sugar residue in a similar way as the terminal, nonreducing D-galactose group in the intact NN antigen.

Thus, the findings on red-cell MM and NN antigens indicate that the human blood-group MM and NN antigens differ in their carbohydrate structure only by one *N*-acetylneuraminic acid residue per immunodominant group (Fig. 1). In contrast to

the classical theory, the indirect product of the N blood-group gene is the immediate precursor of that of the M gene, and the allele to the M gene is an amorph[10–12].

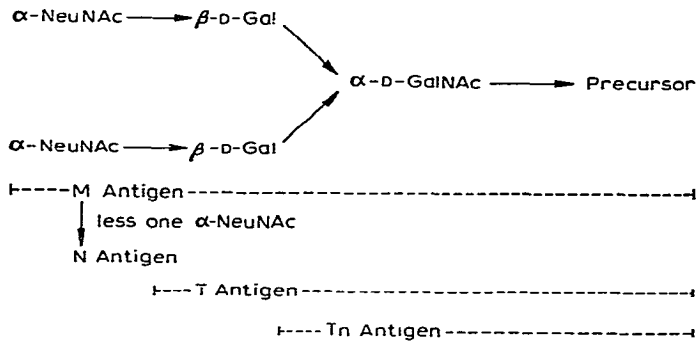

Fig. 1. Proposed structural relationship of blood-group MN and precursor specificities, as determined with human antisera.

The rather large release of *N*-acetylneuraminic acid from MM and NN antigens required to achieve transformation or inactivation (Table I) is readily explained, for calculation shows that 75 to 90% of the total terminal *N*-acetylneuraminic acid groups of the MN glycoproteins are not components of MN specific groupings. Approximately 25% of the total D-galactose residues are involved in MN immunodeterminant carbohydrate chains, but only one D-galactose residue per chain is in terminal position and only in the N structure (see Fig. 1).

While the scheme given in Fig. 1 is compatible with all our observations, it may be an oversimplification. Our findings do not firmly exclude that additional *N*-acetylneuraminic acid residues may be linked directly to the *N*-acetylneuraminic acid residues depicted in Fig. 1, though no published results favor this possibility. Proof that the two β-D-galactose residues are linked to the same sugar residue is missing; their linkage to adjacent monosaccharides cannot be excluded. The precipitating anti-reagents in this study were all heterologous and unsuitable for more than general structural information. The accurate determination of the complete immunodominant M- and N-specific structures is further complicated by the presence of other carbohydrate structures on the same macromolecule, such as the NeuNAc → GalNAc structure mentioned earlier, as well as by the paucity of M and N immunodominant groups in comparison to those on ABH(O) active substances[34].

The scheme outlined in Fig. 1 covers only the last 4 steps of an obviously longer pathway. It readily explains the occasional difficulty in identifying undiluted anti-N antisera, because of their reactivity with NN as well as MM erythrocytes that carry some still unconverted N substance[35]. It also explains the red-cell reactions of a presumably hemizygous propositus[36]: in contrast to ordinary MN antigens, a genetic block at which the precursor accumulates may have occurred early in the biosynthetic pathway, so that neither the product of the N nor that of the M gene (transferases)

can act for lack of a suitable substrate. The occurrence of the silent antigen M^k can be interpreted in a similar way[37]. The scheme in Fig. 1 is also in accord with the deductions of Sturgeon *et al.*[38] concerning the Tn antigen.

The importance of the MN substances and their precursors goes beyond their serologic, hematologic, forensic, and biochemical-genetic significance. We have recently found by adsorption studies that membranes from all 6 nonmalignant human breast glands, and from 3 primary *in situ* and 15 grossly malignant breast lesions tested possessed blood-group M and N activity, in accordance with their representation on red cells as measured with human antisera and *V. graminea* extracts. The Thomsen–Friedenreich T antigen was uniformly demonstrable with human anti-T serum in chemically untreated malignant but not in benign tissues. The reactions were strictly specific[39,40].

Anti-T activity is present in the sera of all mature humans tested; anti-T titers in many breast-cancer patients did not differ from titers of normal individuals or those having other diseases. Nevertheless, we found that among 112 breast-cancer patients, 42 (38%) had an anti-T titer score of 12 or less, as compared to 32 persons with such a titer score out of 280 (11%) who were either healthy or had other diseases, including some other malignancies; 25% of the breast-cancer patients had a score of 10 or less, as compared to 6% of the control population. All these persons had normal IgG, IgA, and IgM levels. The normal anti-T titer score, as determined in our laboratory[40,42], is 23–28.

Finally, the spontaneous TA3 carcinoma of the mouse carries *V. graminea* reactive blood-group N precursor substance[41], and the immunodominant structure of this glycoprotein is a precondition for the killing effect of anti-T antibodies, on the TA3 cancer, in conjunction with complement[42].

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Note added in proof: After this paper had been completed, the uncovering of A-like receptor sites on sialic acid-less MN glycoproteins by destruction of the D-galactose residues with periodate and high inhibitory activity of sialic acid-less OSM were reported[43].