AN AUTOSOMAL DOMINANT GENE REGULATES THE EXTENT OF 9-O-ACETYLATION OF MURINE ERYTHROCYTE SIALIC ACIDS

A Probable Explanation for the Variation in Capacity to Activate the Human Alternate Complement Pathway*

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Erythrocytes of some animal species are lysed by human serum as a result of activation of the alternate pathway of complement. This process may be modulated by the sialic acid content of the erythrocytes in the low levels of sialic acid favor lysis, whereas high levels of this sugar impede lysis (1–3). The sialic acid residues appear to function by favoring access of the β1H protein to bound complement convertase of the alternate complement pathway (C3bBb),[1] a process that promotes decay-dissociation of the C3 amplification convertase, thereby preventing amplification of the alternate complement pathway (1, 2). Nydegger et al. (4) have reported that the sialic acid content of murine erythrocytes is determined by an autosomal locus, and that the resultant variation in surface sialic acid can explain the differences in susceptibility among inbred strains to lysis by human complement. In that study, the sialic acid content of the erythrocyte ghosts was determined by the thiobarbituric acid method of Warren (5), following mild acid hydrolysis. However, it was subsequently demonstrated that murine erythrocyte sialic acids may contain O-acetyl groups, which are known to interfere with the estimation of total sialic acid by the Warren method (6). We postulated that the genetic trait responsible for the differences in susceptibility of murine erythrocytes to lysis by human complement might be the degree of O-acetylation of the sialic acid residues rather than the absolute content of sialic acid. The data presented in this paper are consistent with this proposal.

Materials and Methods

Inbred strains of mice were purchased from The Jackson Laboratory (Bar Harbor, Maine). Recombinant inbred strains (B × D) were provided by Dr. Donald Shreffler of Washington University (St. Louis, Mo.). These were originally obtained from Dr. Ben Taylor of The Jackson

Laboratory. *Vibrio cholerae* neuraminidase was from Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif. Butylated hydroxytoluene (BHT) was from Sigma Chemical Co. (St. Louis, Mo.), and 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) was from Eastman Kodak Co. (Rochester, N. Y.). AG analytical grade ion-exchange resins were obtained from Bio-Rad Laboratories (Richmond, Calif.) and trimethylsilylimidazole (TSIM) from Pierce Chemical Co. (Rockford, Ill.). 3% OV-17 on Gas-Chrom Q, 100–200 mesh, was from Applied Science Laboratories (Waltham, Mass.). *N*-acetylneuraminic acid (NeuNAc) and *N*-glycolylneuraminic acid (NeuNGl) were from Sigma Chemical Co. Authentic 9-*O*-acetyl-*N*-acetylneuraminic acid (9-*O*-Ac-NeuNAc) was kindly provided by Dr. Roland Schauer, Kiel, Federal Republic of Germany. All other chemicals were of reagent grade.

Preparation of Erythrocyte (RBC) Ghosts. Blood was collected by orbital sinus puncture into phosphate-buffered saline (PBS) that contained 20 U of heparin/ml. After centrifugation at 1,500 g at room temperature for 15 min, the buffy coat, plasma, and upper one-fifth of the packed cells were removed, and the remaining RBC were washed twice with cold PBS. The cells were then hemolysed with 15 vol of ice-cold 10 mM Tris buffer, pH 7.4, with 1 mM EDTA. The ghosts were sedimented at 10,000 g (4°C) for 10 min and washed repeatedly until the stroma were free of any pink tinge. A final wash was made in distilled water. 10 μl of 1% BHT in ethanol was added to 20 ml of the final water wash if storage was necessary before analysis.

Determination of Total Sialic Acid Content. Samples of RBC ghosts (200 μl) were incubated in 0.1 N NaOH fro 45 min on ice to remove the *O*-acetyl groups of the sialic acid residues (6). The pH was then adjusted to 1.0 with HCl, and 2 μl of 1% BHT in ethanol was added to inhibit lipid peroxidation during the subsequent acid hydrolysis. The samples were then hydrolyzed at 80°C for 75 min. The released sialic acid was determined by the thiobarbituric acid (TBA) procedure of Warren (5).

Estimation of the Extent of O-Acetylation of the Sialic Acid Residues. For the reasons outlined in the Results, it was not possible to obtain conditions that allowed an exact determination of the extent of *O*-acetylation of the sialic acid residues. The following conditions were chosen because they allowed the best approximation of this value; these conditions were strictly adhered to throughout the study. Duplicate samples of ghosts (200 μl) were adjusted to pH 2.0–2.1 with HCl, 2 μl of 1% BHT was added, and the samples were hydrolyzed at 75°C for 75 min. Two similar samples were first subjected to de-*O*-acetylation in 0.1 N NaOH for 45 min on ice and then brought to pH 2.0–2.1 with HCl and hydrolyzed as above. The released sialic acid was then measured by the Warren method (5). The increment in color formation observed when base treatment preceded acid hydrolysis was used to express the degree of *O*-acetylation of the sialic acids in the following way: *O*-acetylation index = a − b/a × 100, where a equals sialic acid after base treatment and acid hydrolysis, and b equals sialic acid after acid hydrolysis only (6). In every assay, an RBC ghost sample from the high acetylation strain BUB/BnJ was included so that the other values could also be expressed relative to this strain (arbitrarily set at 1.00). This was done to control for slight day-to-day fluctuations in the assay.

Purification of Sialic Acids. Sialic acids from bovine submaxillary mucin and RBC ghosts were isolated according to the method of Schauer (7).

Analysis of Sialic Acids by Gas-Liquid Chromatography. The procedure of Schauer (7) was followed with a few modifications. The purified sialic acids from bovine submaxillary mucin and authentic 9-*O*-Ac-NeuNAc were used as standards. Portions of each sample were pretreated with NH₄OH, pH 11.0, on ice for 2 h to de-*O*-acetylate the sialic acids. The NH₄OH was then removed by lyophilization. Direct trimethylsilylation of the samples was carried out with 15 μl of TSIM reagent at 65°C for 5 min. Aliquots of 3–5 μl were chromatographed on 1.6-m columns of 3% OV-17 (2.0 mm inner diameter) in a F and M 402 gas chromatograph with a temperature program from 200 to 250°C at 2°C/min. A correction factor of 2.16 for the decreased relative detector response of 9-*O*-Ac-NeuNAc compared with NeuNAc was made, based on the data of Casall-Stenzel et al. (8).

Analysis of 9-O-AcNeuNAc by the MBTH Assay. A modification of the MBTH procedure of Massamiri et al. (9) was used to estimate 9-*O*-Ac-NeuNAc content, because this derivative gave no color formation in the assay, whereas after de-*O*-acetylation, it reacted identically to NeuNAc. Hence, the increment in color formation with base treatment was used to measure

the relative amount of 9-*O*-Ac-NeuNAc in a mixture of purified NeuNAc and 9-*O*-AcNeuNAc. The following procedure was followed: 355 µl of PBS (pH 7.3) and 20 µl of 2 mM NaIO₄ were added to a sample that contained 5–20 nmole of total sialic acid in 25 µl of H_2O, and the reaction was allowed to proceed for 15 min on ice in the dark. Then, 90 µl of MBTH reagent (10 mM MBTH in 0.05 N HCl) was added, the mixture was vortexed, and allowed to stand at room temperature for 20 min. 90 µl of 5% $FeCl_3$ solution was added, the mixture shaken, and then left at room temperature for 15 min. The A_{625} was read against a similarly prepared blank solution and compared with a standard of 10 nmole NeuNAc. Similar 25-µl samples were also treated with 25 µl of 0.2 N NaOH on ice for 1 h, neutralized with 50 µl of 0.1 N HCl, and subjected to a similar analysis. The increment in color formation with base treatment was taken to represent the amount of 9-*O*-Ac-NeuNAc in the mixture.

Polyacrylamide Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate.   The method of Laemmli (10) was used with the following modification: The pH of the separatipn gel was changed to 8.0 to prevent possible de-*O*-acetylation of the sialic acids. With 200–300 µg of protein/gel and a 10% acrylamide concentration, relatively sharp resolution of Coomassie blue staining bands could be obtained. Gel slices (6 mm) were assayed for sialic acid content and *O*-acetylation as follows: For total sialic acid, the slices were incubated in 200 µl of 0.2 N NaOH on ice for 1 h. 45 µl of 2 N HCl and 135 µl of H_2O were added, and the mixture was incubated at 80°C for 75 min. 500 µl of water was then added, and after mixing, 750-µl aliquots were removed, concentrated, and assayed for sialic acid by the Warren method (5), using a scaled down modifications of the TBA procedure. The chromophor was extracted into 350 µl of cyclohexanone, and the absorbance was read in a 300-µl microcuvette. This procedure was capable of easily detecting 400 pmole of sialic acid. To estimate *O*-acetylation, parallel gel slices were suspended in 1 ml of water and adjusted to pH 2.0 with formic acid. After incubation at 75°C for 75 min, 730 µl was removed, evaporated to dryness three times to remove all traces of formic acid, and then the sialic acid content was determined. An estimate of the percent *O*-acetylation of the sialic acid in each gel slice was made as described above.

Determination of Protein.   The method of Lowry et al. (11) was used to estimate the protein content of the samples.

Results

Analysis of Variables in the Estimation of the Extent of O-Acetylation of Glycosidically Bound Sialic Acids.   Our initial experiments were designed to determine the best method for quantitating the extent of *O*-acetylation of the RBC sialic acid residues. The approach used took advantage of the fact that the *O*-acetylated species give a decreased color yield in the standard TBA procedure of Warren (5). Because *O*-acetyl esters are extremely labile at alkaline pH, pretreatment with base will result in maximum color formation (6, 12). This increment in color yield following alkaline treatment can be used to estimate the extent of *O*-acetylation of the sialic acid residues. The procedure can be carried out on free or bound sialic acids. In agreement with previous reports, we found that treatment of RBC ghosts with 0.1 N NaOH for 45 min at 4°C resulted in complete removal of *O*-acetyl groups from the bound sialic acids, yielding maximum color formation in the TBA assay. This increment has been previously used to obtain a de-*O*-acetylation index (6).

We found, however, that numerous variables prevented an accurate measurement of the absolute amount of *O*-acetylation of the sialic acid residues in the membrane preparations. Some of these are demonstrated in Fig. 1. The TBA assay of Warren requires prior cleavage of the glycosidic bond, which is conventionally achieved by acid hydrolysis at pH 1.0 or 2.0 for 1 h at 80°C. As shown in Fig. 1 A, hydrolysis at pH 1.1 at 75°C resulted in considerable de-*O*-acetylation (i.e., minimal increment in color yield with base treatment), whereas hydrolysis at pH 2.2 (Fig. 1 B) led to a selective release of non-*O*-acetylated species because these are more acid labile than
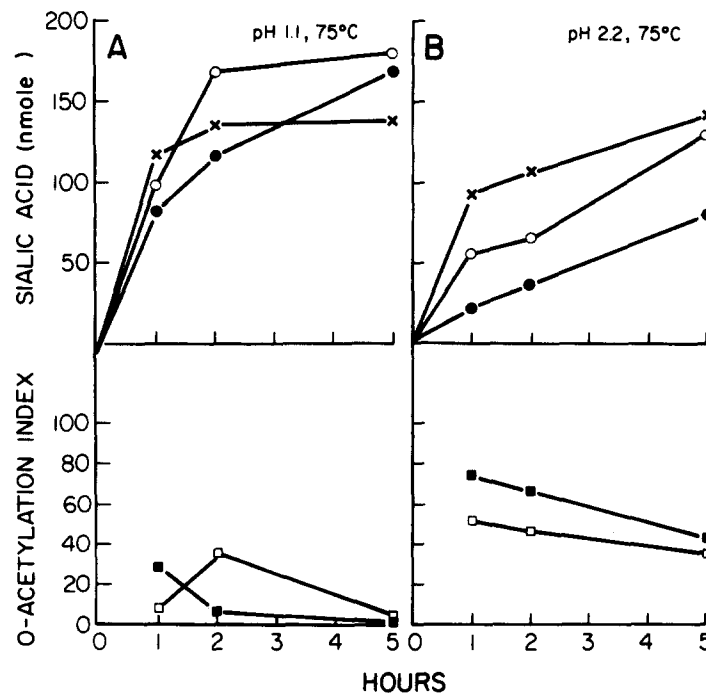
Fig. 1. Variables in the estimation of the degree of O-acetylation in the sialic acids of murine RBC. A pooled sample of RBC ghosts from strain C56BL/6J was used. Large aliquots were subjected to de-O-acetylation (0.1 N NaOH on ice for 45 min) before or after acid hydrolysis under several different conditions, in the range 0.2–0.005 N HCl. Similar samples were also subjected to acid hydrolysis alone. The actual pH of the samples was checked at each step. Equal samples were assayed in duplicate for color formation by the TBA reaction at several time intervals. Upper panels: Sialic acid measured by the TBA assay. (●) acid hydrolysis alone; (O) acid hydrolysis followed by base treatment; and (X) base treatment followed by acid hydrolysis. Lower panels: O-acetylation index for each time point and sequence = a − b/a X 100. (a equals sialic acid value when base treatment was before (■) or after (□) acid hydrolysis, b equals sialic acid value with no base treatment.)

the O-acetylated species (13, 14). With prolonged hydrolysis at pH 2.2, de-O-acetylation occurred as indicated by the decrease in the acetylation index (Fig. 1 B, bottom).

After several experiments similar to thse shown in Fig. 1, we were unable to find ideal conditions for the accurate estimation of the degree of O-acetylation in a given specimen. The conditions that we used (Materials and Methods) represented an attempt to release most of the sialic acid residues with a minimum of de-O-acetylation. Even though these conditions were rigidly adhered to throughout the study, there was some variation in the acetylation index during repeated assays, most likely because of factors such as slight pH differences and fluctuations in the temperature of the heating block. For this reason every assay for the acetylation index included a specimen from the high acetylation strain BUB/BnJ as an internal control. This value could be set to 1.0 and others related to it.

This O-acetylation index significantly underestimates the absolute amount of O-acetylation in mouse RBC sialic acids. This is because 9-O-AcNeuNAc, which is the major O-acetylated species (see below), gives a 50% color reaction in the TBA assay before de-O-acetylation (7).

Comparison of Total and O-Acetylated Sialic Acid in RBC of Various Murine Strains. As
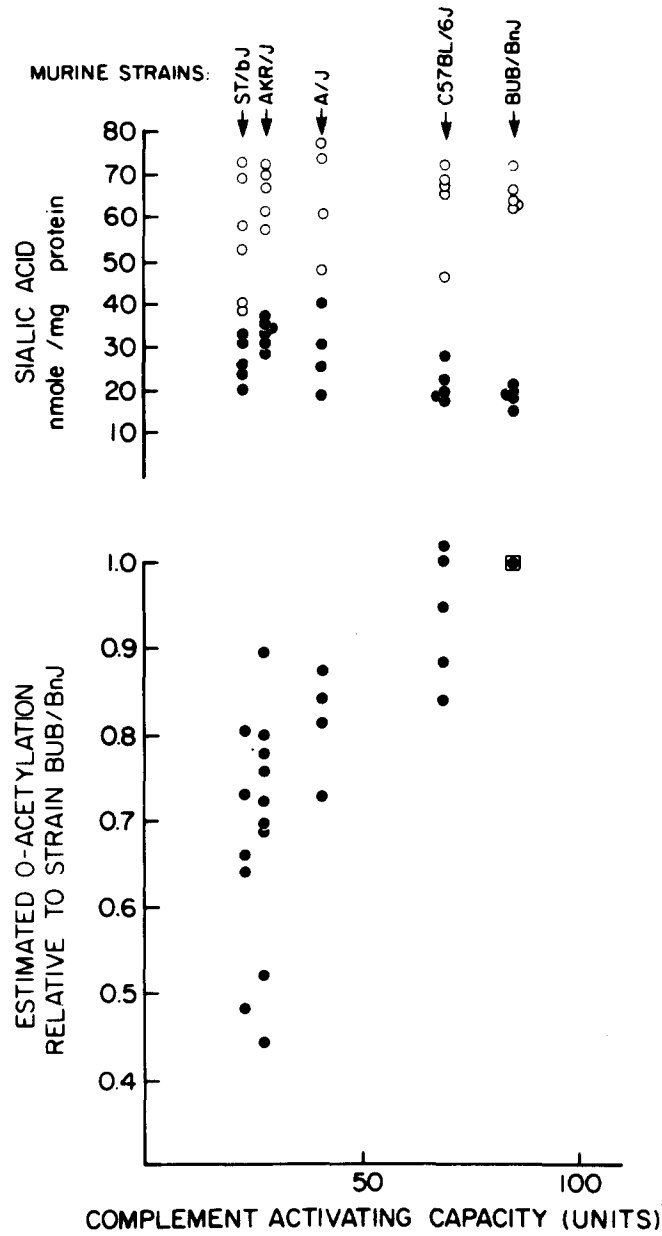
FIG. 2. Sialic acids of murine RBC ghosts: relationship to capacity of intact RBC to activate the human alternate pathway of complement (data of Nydegger et al. [4]). Upper panel: Sialic acids measured by the TBA assay with (○) and without (●) prior base treatment. Lower panel: Degree of *O*-acetylation of sialic acids relative to strain BUB/BnJ. Calculated from the data in the upper panel.

TABLE I

Sialic Acids of Murine RBC: Relationship of O-Acetylation to Complement Activating Capacity

Murine strain	Complement activating capacity*	Sialic acid content	O-acetylation index	O-acetylation relative to strain BUB/BnJ
	U	nmol/mg protein		
BUB/BnJ	85 ± 1	65 ± 4 (5)	67 ± 7 (12)	1.00
C57BL/6J	69 ± 6	64 ± 10 (5)	68 ± 5 (5)	0.98 ± 0.08
A/J	42 ± 5	68 ± 17 (4)	56 ± 5 (4)	0.81 ± 0.07
DBA/2J	33 ± 2	64 ± 8	49 ± 7 (4)	ND
AKR/J	28 ± 3	70 ± 4 (6)	46 ± 7 (10)	0.73 ± 0.16
ST/bJ	26 ± 4	52 ± 14 (5)	47 ± 8 (5)	0.67 ± 0.12

All values are mean ± SD. Figures in parentheses represent total number of experiments. The actual data points are shown in Fig. 2. ND, not done.
* Data of Nydegger et al. (4).

shown in Fig. 2 and Table I, the quantity of sialic acid per milligram of membrane protein was similar in all the strains studied. In contrast, the extent of O-acetylation of the sialic acid residues was significantly different among the strains and bore a direct relationship to the capacity of the RBC to activate the human alternate pathway of complement (Table I). This relationship held whether the data were expressed as the acetylation index or as O-acetylation relative to strain BUB/BnJ.

Characterization of the O-Acetylated Acid Species. Sialic acids may be O-acetylated at positions 4, 7, 8, and 9 (7). Consequently, the differences between the murine strains in the extent of O-acetylation of the sialic acid residues could reflect either quantitative differences in the amount of a particular O-acetylated species, or qualitative differences in the types of O-acetylated species. For instance, strains with high degrees of O-acetylation could have two types of O-acetylated sialic acids, whereas strains with low percentages of O-acetylated species could have only one of these types. To resolve this point, sialic acids were released by mild acid from C56BL/6J, A/J, and ST/bJ RBC ghosts, purified, and analyzed by gas-liquid chromatography (GLC). Some examples of the scans of the GLC analyses are shown in Fig. 3. All of the strains had, in addition to NeuNAc, a peak that eluted with an R_{NeuNAc} identical to standard 9-O-Ac-NeuNAc (Fig. 3 A and B). This peak disappeared after mild base treatment (Fig. 3 C) as expected for an O-acetylated species. The small peak that corresponded in location to 7-O-acetyl-N-acetylneuramininic acid (7-O-Ac-NeuNAc) was not affected by base treatment and, therefore, does not represent an O-acetylated sialic acid.

Because it is known that the relative detector response (RDR) to 9-O-Ac-NeuNAc is less than that to an equimolar amount of NeuNAc, we used the correction factor reported by Casal-Stenzel et al. (8) when computing the percentage of 9-O-Ac-NeuNAc in each sample. The corrected and uncorrected values for each strain are presented in Table II along with the acetylation index for the starting RBC ghosts. The estimate of the percentage of 9-O-Ac-NeuNAc derived from the GLC analysis follows that predicted by the acetylation index. The values are somewhat lower than expected, most likely because of some de-O-acetylation that is known to occur during purification of the sialic acid species (7). We also used the MBTH procedure described by Massamiri et al. (9) for estimating the percent 9-O-Ac-NeuNAc in purified sialic acid preparations. This assay depends upon the quantitative release by periodate of
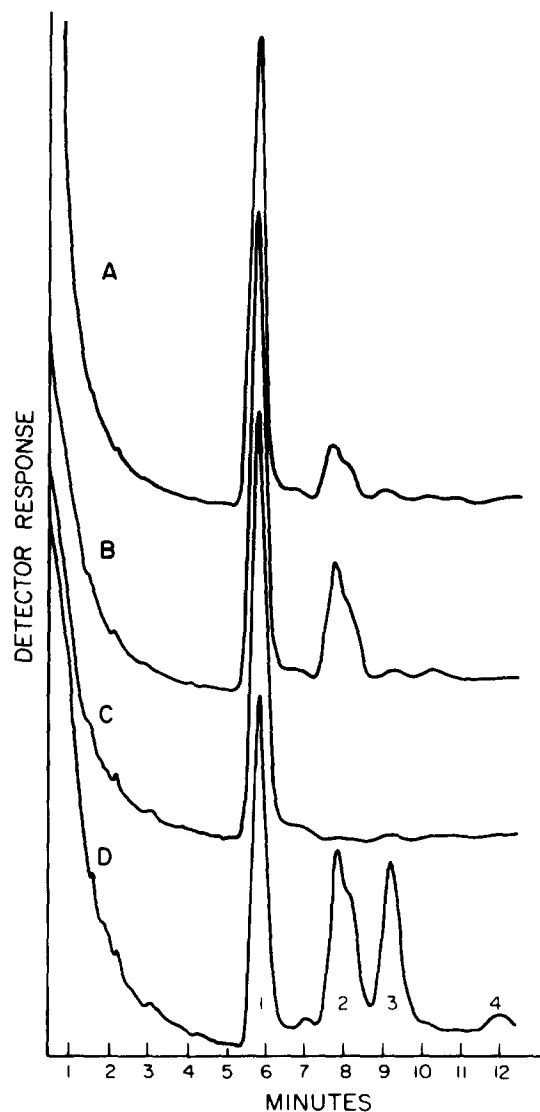
FIG. 3.   Identification of O-acetyl substitution in sialic acids of murine RBC ghosts. GLC of purified sialic acids from murine RBC ghosts and bovine submaxillary mucin on OV-17 (3%). Temperature program started at 200°C at 2°C/min (Materials and Methods). A, sialic acids from strain ST/bJ. B, sialic acids from strain C56BL/6J. C, sialic acids from strain A/J, de-O-acetylated with base treatment. D, sialic acids from bovine submaxillary mucin. Peak 1, NeuNAc; 2, 9-O-Ac-NeuNAc; 3, 7-O-Ac-NeuNAc; 4, NeuNGl.

the 9-carbon of NeuNAc as formaldehyde. When this carbon is blocked, as in 9-O-Ac-NeuNAc, no reaction occurs, but a complete reaction can be obtained after de-O-acetylation with mild base. This increment in color formation following base treatment can be used to assay the amount of 9-O-Ac-NeuNAc in a purified mixture of NeuNAc and 9-O-Ac-NeuNAc. As shown in Table II, the values obtained with this method are in good agreement with those obtained by the GLC analysis. We conclude from these studies that 9-O-Ac-NeuNAc is the predominant O-acetylated species in both the high

TABLE II

Characterization of Sialic Acids Purified from Murine RBC Ghosts

Murine strain	Starting RBC ghosts		Purified sialic acids			
	Total sialic acid	Acetylation index	Total sialic acid recovered	Percent 9-O-Ac-NeuNAc		
				By GLC		By MBTH method
				Area	Corrected*	
	nmol		*nmol*			
C57BL/6J	956	59	651	30	47	39
A/J	1,314	50	1,177	21	35	25
ST/bJ	945	38	567	16	29	22

The techniques for purifying the sialic acids and for analyzing them by GLC and the MBTH method are described in Materials and Methods.

* The correction factor for the decrease in RDR of 9-O-Ac-NeuNAc with a 3% OV-17 column (1.6 m × 2 mm inner diameter) was taken from Casal-Stenzel et al. (8). RDR NeuNAc/RDR 9-O-Ac-NeuNAc = 2.16.

and low acetylation strains of mice.

Distribution of O-Acetylated Sialic Acids among the Glycoproteins of the Murine RBC. Another possible mechanism for the observed differences in the extent of O-acetylation of the sialic acid residues could be that there are differences in the glycoprotein content of RBC from the various murine strains, and that these glycoproteins have different percentages of O-acetylated sialic acid residues. To examine this possibility, RBC ghosts from BUB/BnJ and AKR/J were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) to separate the glycoproteins. The gels were then frozen and sliced, and the slices were tested directly for total sialic acid content and the extent of O-acetylation. As shown in Fig. 4, O-acetylated sialic acids were distributed throughout the gel, although to an increasing extent toward the cathodal end. The difference in the degree of O-acetylation between the high and low strains was clearly seen and was present in all sections of the gel.

Pattern of Inheritance of the Differences in O-Acetylation. To establish the pattern of inheritance of these differences in 9-O-acetylation of the RBC sialic acids, we studied the progeny of C56BL/6J (high acetylation) and DBA/2J (low acetylation) strains. As shown in Fig. 5, all samples from the B6.D2/F1J hybrids showed a high acetylation index, which suggested an autosomal dominant pattern of inheritance. To further substantiate this, and to attempt to map the locus involved, we studied 14 recombinant inbred strains (B × D) originally derived from B6.D2/F1J hybrids (15). Each of these strains was derived by systematic inbreeding from the cross of two individual B6.D2/F1J (15). As expected for an autosomal dominant trait (15), the B × D strains segregated into high acetylation (six) and low acetylation (eight) strains (Fig. 5). This provides evidence for a single autosomal dominant genetic locus.

To map this locus to a specific chromosomal site, we compared this data with the previously available information regarding the chromosomal mapping of other known genes among the B × D strains (15). Gene *d*, on chromosome 9, showed only 2 discrepancies in the 14 strains examined. The adjacent genes, *Sep-1* and *Mod-1* showed 3 and 4 discrepancies out of 14, respectively. This level of concordance was not found in any other region of the chromosomal map. These results make it very likely that
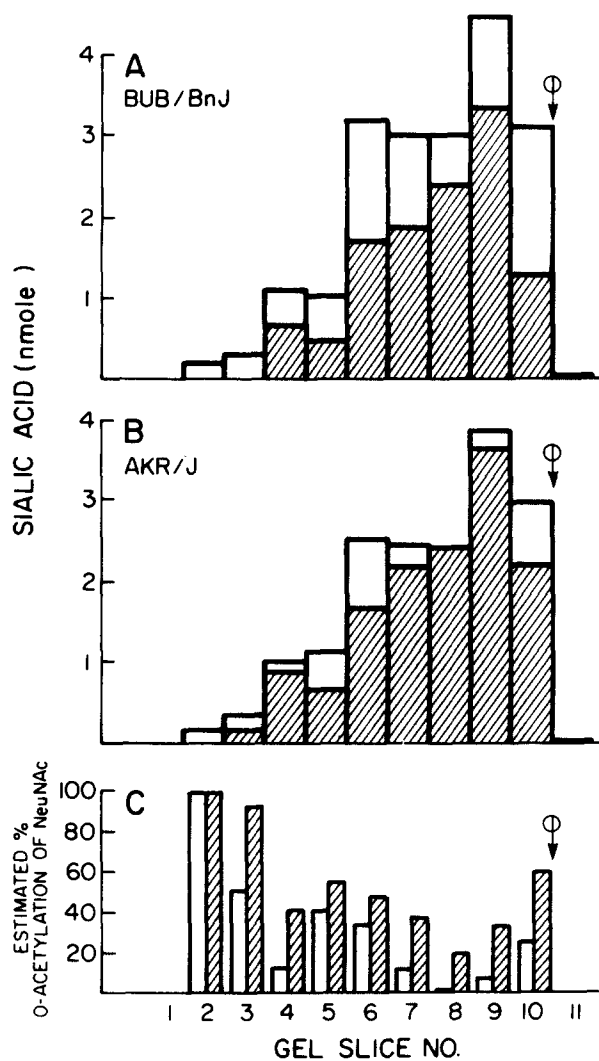
FIG. 4.   Distribution of the O-acetylated sialic acids among the murine RBC ghosts glycoproteins. RBC ghosts were prepared, extracted, and subjected to SDS-PAGE gel electrophoresis as described in Materials and Methods; 6-mm gels slices (~125-μl vol each) were analyzed for sialic acid content with and without prior base treatment. The sialic acids were released by acid hydrolysis and concentrated for analysis as described in Materials and Methods. $\mathbb{Q}$ indicates the position of the bromophenol blue marker. (A) 300 μg of ghost protein from strain BUB/BnJ that contained 22.4 nmol of sialic acids was applied to the gel. The sum of the sialic acids measured in all the slices was 20 nmol (89.3% recovery). (B) 300 μg of ghost protein from strain AKR/J that contained 20.1 nmol of sialic acid was applied to the gel. The sum of the sialic acids measured in all slices was 17.2 nmol (85.4%). The area within the hatched bars ▨ represents sialic acids measured in each gel slice after acid hydrolysis only. The clear area □ shows the increment in sialic acids measured after base treatment. (C) Estimation of the extent of O-acetylation of the sialic acids in the individual gel slices. This value represents the percent increment after base treatment and was calculated from the data in panels A and B. ▨, BUB/BnJ; □, AKR/J.
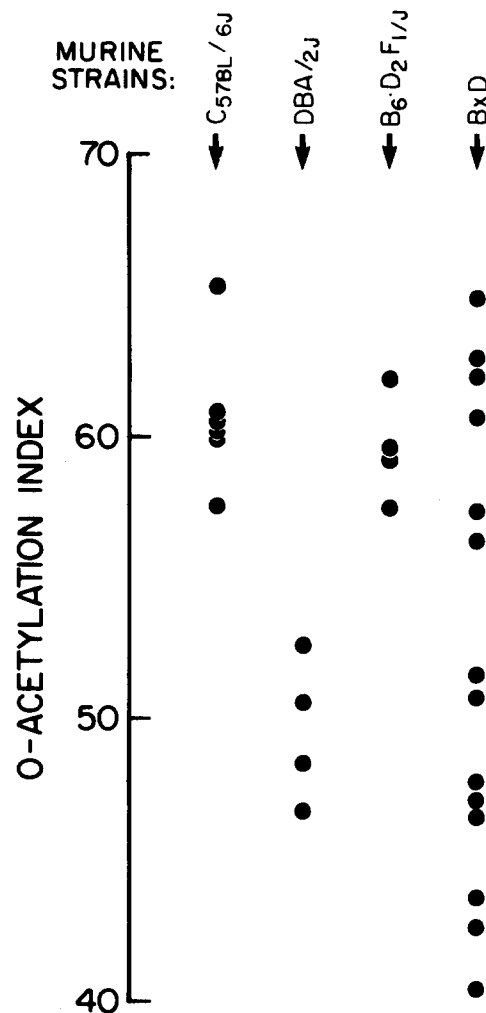
Fig. 5.   Distribution of high and low O-acetylation of RBC sialic acids among F₁ and stable RI (B × D) progeny of C56BL/6J and DBA/2J. Mean values of O-acetylation index of RBC sialic acids of murine strains as noted. RI strains (B × D) in order (from top to bottom) are B × D-1, B × D-11, B × D-28, B × D-27, and B × D-24 (high strains) and B × D-5, B × D-30, B × D-12, B × D-16, B · × D-25, B × D-21, B × D-18, and B × D-6 (low strains).

the gene modulating O-acetylation of sialic acid is located on chromosome 9, close to gene d.

Discussion

The elegant studies of Fearon, Austin, and others have clearly demonstrated a role for surface sialic acid in permitting access of the β1H protein to bound C3bBb, thereby preventing amplification of the alternate pathway of complement (1, 2). Furthermore, these investigators showed by periodate degradation that it was the exocyclic hydroxyl groups on the 8 and 9 carbons of sialic acid that were vital to this function (1). It is reasonable to hypothesize that the presence of varying amounts of

O-acetylation in the 9-carbon position of surface sialic acids could result in varying degrees of activation of the alternate complement pathway. Our finding of a good correlation between the degree of 9-O-acetylation of the sialic acids of murine RBC and their capacity to activate the alternate pathway of human complement strongly supports this postulation. RBC from the strains with the highest degree of 9-O-acetylation are the most sensitive to complement lysis, presumably reflecting the impaired binding of the β1H protein.

Nydegger et al. (4) reported an inverse correlation between the quantity of sialic acid on the surface of murine RBC and the sensitivity to lysis by the alternate complement pathway. However, the interference caused by O-acetylation of the sialic acids in the conventional TBA assay could not be taken into account. We found no significant difference in the total quantity of sialic acids in RBC of several of these murine strains when base treatment was done before acid hydrolysis to eliminate the interfering O-acetyl groups.

We conclude that the autosomal locus, which determines sensitivity to complement lysis, regulates the extent of 9-O-acetylation of the RBC sialic acid residues rather than the quantity of RBC sialic acid. These findings, taken with those by Nydegger et al. (4) indicate that membrane-associated NeuNAc residues, with unmodified C7-9 polyhydroxylated side chains, are critical in preventing human alternative complement pathway activation. Our studies of the segregation of high and low O-acetylation in recombinant inbred (RI) strains supports control of this trait by a single autosomal dominant genetic locus on chromosome 9. However, the nature of the gene product of this locus is not evident from our studies. Because both high and low O-acetylation strains contain 9-O-Ac-NeuNAc, the gene product is unlikely to be the 9-O-acetylating enzyme. Many factors could potentially modulate the extent of 9-O-acetylation and de-O-acetylation of the sialic acid residues (for a review of the metabolism of O-acetylation of sialic acids, see Schauer [16]). The postulated gene product could act at any of these steps.

Our findings might also explain the previously described polymorphism in the electrophoretic mobility and the nonspecific agglutinability of murine RBC (17). With a few exceptions, these two characteristics segregate in a predictable manner in relation to the sensitivity to complement lysis and the polymorphism in the extent of O-acetylation of the sialic acid residues. Because surface sialic acid is the major determinant of the electrophoretic mobility of RBC (18), varying degrees of substitution at the 9 position could alter the net charge to a minor degree, which could be sufficient to explain the small differences observed in electrophoretic mobility. Likewise, because nonspecific agglutinability by polybrene is probably related to partial neutralization of surface charge, RBC with the greatest extent of O-acetylated sialic acid might be the most easily agglutinated by this reagent.

The finding that 9-O-Ac-NeuAc acid is the major sialic acid derivative in murine RBC agrees with the results of Howard and Schauer with BALB/c mice (R. J. Howard and R. Schaurer. Personal communication.). These investigators also detected small quantities of an unidentified di-O-acetyl species. We were unable to demonstrate the presence of several other O-acetylated species reported by Sarris and Palade (6).

A recent report by Howard (19) indicates that environmental factors, as well as genetic factors, may influence the O-acetylation of murine RBC sialic acid residues. Howard found that during *Plasmodium berghii* infection in BALB/c mice there appeared

to be a marked increase in the extent of O-acetylation of surface sialic as evidenced by the almost complete loss of periodate/NaB[^{3}H]$_4$ labeling of surface glycoproteins without any actual loss of the sialic acid residues. In preliminary experiments we have noted that repeated bleeding or hemolysis produced by phenylhydrazine appeared to result in increased O-acetylation in the low acetylation strains. The biological significance of these changes is as yet, unclear.

Summary

Nydegger et al. (4) have reported that the difference in susceptibility of erythrocytes from different inbred murine strains to lysis by the human alternate complement pathway is determined by an autosomal locus. We have found a good correlation between the degree of O-acetylation of the erythrocyte sialic acid residues and the susceptibility to complement lysis, whereas there was no correlation between total erythrocyte sialic acid content and complement sensitivity. The major O-acetylated species in all the murine strains is 9-O-acetyl-N-acetylneuraminic acid.

We propose that the autosomal dominant locus, which determines complement sensitivity, acts by influencing the extent of 9-O-acetylation of the erythrocyte sialic acid residues. By using recombinant inbred strains, we determined that this genetic locus is probably located on chromosome 9. The nature of the gene product remains unknown.

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