Sialic Acid Content of the Erythrocyte and of an Ascites Tumor Cell of the Mouse

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SUMMARY

The sialic acid content of the mouse erythrocyte was compared with that of the Ehrlich ascites carcinoma cell. Neuraminidase treatment of intact tumor cells yielded 36 times more sialic acid than was released from the erythrocyte on a per cell basis. If this were all located on the surface, it may be calculated that the density of sialic acid on the Ehrlich ascites carcinoma cell is about 4 times greater than that on the erythrocyte. Trypsin released a sialic acid-containing fragment from both types of cells, the sialic acid being in the “bound” form. The sialic acid released by trypsin treatment of Ehrlich ascites tumor cells was only about one-sixth of that released by neuraminidase. By contrast, approximately equal amounts were liberated from the erythrocytes by these two enzymes. The erythrocyte sialic acid-containing substance was nondialyzable and was not precipitated by trichloroacetic acid. N-acetylmuramidase constituted approximately 70 per cent and N-glycolynmuramidase the remaining 30 per cent of the sialic acid of the tumor cell as determined by paper chromatography. In the erythrocyte N-acetylmuramidase constituted almost all the sialic acid, N-glycolynmuramidase being undetectable in this cell.

The sialic acids are a family of substances formed by the condensation of N- or O-substituted mannosamine with phosphoenol pyruvate (17, 23). They are strongly acidic compounds whose terminal carboxyl group has a pK of about 2.6 (19). Sialic acids have been found on the erythrocyte membranes of a number of vertebrate species (5, 7, 12). Viral or bacterial neuraminidases, enzymes which cleave the a-ketosidic linkage between a terminal sialic acid group and a sugar or sugar derivative (9), can liberate free sialic acid from such red cell membranes (5, 7). The surface of the mammalian erythrocyte is negatively charged with a reported isoelectric point of about pH 4.0 (18). It seemed possible that sialic acid might be responsible for the negative charge of the EAT cell. In this study the concentration of sialic acid in the mouse EAT cell was determined and compared with that of the mouse erythrocyte. While this investigation was under way, Wallach and Eylar reported on the presence of sialic acid in the mouse EAT cell (21). The present study corroborates their findings and presents further data relating to the subject.

MATERIALS AND METHODS

Blood was obtained by cardiac puncture from 6- to 8-month-old female Swiss mice. Heparin was used as anticoagulant. Blood was pooled from a number of animals (up to fifteen), depending on the amount of blood needed. The blood was centrifuged at 1,400 × g for 20 minutes at 4° C., after which the plasma and buffy coat were removed. The erythrocytes were washed 3 times with cold 0.15 M NaCl and then made up to approximately the original volume. When “ghosts” were to be

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prepared, the washed cells were centrifuged again at 1,700 \( \times g \) for 45 minutes, and the supernatant fluid was removed. The ghosts were prepared from 1- or 2-ml aliquots of the packed red cells by the method of Tishkoff et al. \( (19) \). Tumor cells were obtained 7–8 days after the intraperitoneal injection of 3-month-old female Swiss mice with 0.3 ml of a suspension of a hyperdiploid EAT. The tumor had been previously propagated at 7-day intervals. Any ascitic fluid grossly contaminated with red cells was discarded. The EAT cells were centrifuged at 100 \( \times g \) at 4\( ^\circ \) C., and the supernatant plasma and the few red cells at the top of the EAT cell column were removed and discarded. The cells were then washed 3 times with 4 volumes of Krebs-Ringer bicarbonate, pH 7.4, containing 11 mmoles glucose. At the end of the last wash the cells were returned to their original volume with buffer.

**Sialic acid determination.**—Sialic acid was measured by the method of Warren \( (22) \), except that 0.5-ml aliquots of sample were used with a commensurate doubling of the volume of periodate added. The reaction product followed Beer-Lambert's law within the limits defined by Warren. Crystalline N-acetyl neuraminic acid\(^a\) (NANA) was used as a reference standard with each sialic acid assay. No correction was made for the N-glycolyl neuraminic acid in EAT cells or for the trace amount of an unknown sialic acid found in erythrocytes.

In a typical experiment (a) total sialic acid, \( \beta \) sialic acid released by neuraminidase, and \( \gamma \) sialic acid released by trypsin were determined. All glassware was siliconized. One-half ml of the packed erythrocytes and 1 ml of EAT cell suspension were pipetted into a 50-ml Erlenmeyer flask. The red cell pipette was rinsed with three 0.5-ml aliquots of 0.15 \( M \) \( \text{NaCl} \) to insure quantitative delivery of the packed erythrocytes. Neuraminidase prepared from Cholera vibrio\(^b\) \( (0.5- \text{to} \ 8.0-\text{ml aliquots}) \) or trypsin\(^c\) three times recrystallized \( (1-8 \text{mg.}) \), dissolved in 1 ml of Krebs-Ringer phosphate, pH 7.6, was added to cell suspensions. Control flasks without added enzyme were included in each experiment. The cells were incubated at 37\( ^\circ \) C. in a Dubnoff shaker for 1 hour. Following incubation, the contents were centrifuged, and the supernatants were stored at 4\( ^\circ \) C. until the concentration of sialic acid was determined. All experiments were carried out on triplicate aliquots of cells.

The total sialic acid in mouse red cells was determined on aliquots of ghosts equivalent to 1 or 2 ml of packed erythrocytes. Three ml of 0.1 \( N \) \( \text{H}_2\text{SO}_4 \) was added to 1 ml of ghosts, and the contents were then hydrolyzed at 80\( ^\circ \)–85\( ^\circ \) C. for 1 hour. After hydrolysis the ghosts were centrifuged, and the supernatant, together with two 3-ml 0.1 \( N \) \( \text{H}_2\text{SO}_4 \) washings, were saved for sialic acid measurement. In preliminary studies it was found that 1 hour of hydrolysis was not sufficient for maximum recovery of red cell sialic acid. Therefore, a second 1-hour period of hydrolysis was routinely performed, the supernatants of both hydrolysis periods being combined for sialic acid determination.

EAT cell homogenates were prepared by freeze-thawing whole cells 3 times. The measurement of total sialic acid on these homogenates by acid hydrolysis was not feasible, owing to the presence of interfering substances in the sialic acid determinations. This problem was also noted by Wallach and Eylar \( (21) \). The interfering substances could not be eliminated by resin column chromatography on Dowex 50W-X8 and Dowex 2-X8 \( (19) \) or by extraction with a number of organic solvents. The use of the optical density readings at 5392 and 5692 m\(\mu\) instead of the 5392 and 5492 m\(\mu\) readings as suggested by Warren \( (22) \) and adopted by Wallach \( (21) \) did not materially improve the accuracy of the results. When cell homogenates were incubated with neuraminidase at 37\( ^\circ \) C. for 1–2 hours, materials absorbing light at 5392 m\(\mu\) were not released. Therefore, only the sialic acid released by neuraminidase treatment of cell homogenates could be measured. For the determination, homogenates from 1 ml of EAT cells were mixed with 1 ml of Krebs-bicarbonate buffer pH 7.4 and 1 ml of the neuraminidase solution. After incubation for 1 hour at 37\( ^\circ \) C., the mixture was centrifuged, and the free sialic acid was determined on the supernatant. In the estimation of total volume of supernatant, 100 per cent of the packed EAT cell mass was assumed to be due to intracellular water.

Duplicate 0.1-ml aliquots of packed red cells were diluted with 100 ml of isotonic saline. The EAT cells were diluted with 3 per cent acetic acid. Both were counted in a hemacytometer. Hematocrits and tumor cell volumes were measured in duplicate. No correction was made for the volume of trapped fluid in either of the cell types. The cal-

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\(^a\) Obtained from Dr. E. Eylar. Additional material obtained from General Biochemicals Inc. was also found to be satisfactory.

\(^b\) Behringwerke, West Germany. Enzyme was dissolved in 0.15 \( M \) \( \text{NaCl} \) solution containing 9 \( m \) \( \text{CaCl}_2 \) and 0.05 \( m \) \( \text{Naacetate buffer, pH 6.5.} \) One ml of the enzyme preparation contained 100 units of enzyme activity, one unit being equal to the amount of enzyme that would liberate 1 \( \mu g \) of sialic acid from a glycoprotein substrate during a 15-minute incubation period at 37\( ^\circ \) C.

\(^c\) Nutritional Biochemicals and Sigma Chemical Co.
cellulated mean corpuscular volumes (MCV) were found to be 54 cu. $\mu$ for the erythrocyte and 1800 cu. $\mu$ for the EAT cell. According to a modification of the theorem of Pappus (16) and a value of 6.7 $\mu$ for cell diameter (10), the erythrocyte membrane area was estimated as 85 sq. $\mu$. The surface area of the EAT cell was calculated as 780 sq. $\mu$, assuming the cell to be spherical.

Chromatographic identification of sialic acid substances.—Red cell ghosts from 15 to 20 ml. of packed human or mouse red cells were hydrolyzed with 0.1 N $\text{H}_2\text{SO}_4$ at 80° C. as previously described. The ghosts were then spun down, and the supernatant was neutralized with 0.1 m $\text{Ba(OH)}_2$. After removal of the $\text{BaSO}_4$ precipitate, the supernatant was passed through a cation exchange resin Dowex 50 W-X8, 50-100 mesh in the Tris (hydroxymethylaminomethane) cycle, so arranged that the contents would drip onto an anion exchange resin, Dowex 2-X8, 50-100 mesh in the acetate cycle (19). The sialic acid was eluted from the latter column with 45 ml. of 1 m ammonium acetate, pH 4.5. The eluted material was lyophilized and redissolved in 2–3 ml. of water.

Purification of EAT cell sialic acid as described above yielded impurities in the final eluate which prevented resolution of sialic acid on paper chromatograms. However, it was found that preliminary dialysis of the homogenates removed much of the interfering substances. Therefore, EAT cell homogenates (equivalent to 10–15 ml. of centrifuged cells) were dialyzed for 24 hours at 4°–6° C. against 1800 ml. of water, following which the homogenate was incubated with 3 ml. of neuraminidase at 37° C. for 3 hours. The supernatant was then applied to the resin columns as described. Ascending paper chromatography (Schleicher and Schuell paper No. 9048-B) was performed on erythrocyte and EAT cell eluates in the following solvents: (a) $n$-butanol-acetic acid-water (6:6:3:5), and (c) $n$-butanol-$n$-propanol-0.1 N HCl (1:2:1). Standards were applied to each paper strip. In addition to the crystalline material, NANA was prepared from human erythrocytes and plasma (19). An $N$-glycolyneuraminic acid standard was prepared from bovine plasma (15). After chromatography, the strips were dried and then sprayed with an orcinol-trichloroacetic reagent (13) to locate the sialic acid spots.

RESULTS

Mouse erythrocytes.—Acid hydrolysis of the mouse erythrocyte ghosts yielded $0.3924 \pm 0.056 \mu$ mole of sialic acid/ml of packed cells or $0.019 \pm 0.004 \mu$ mole/10^9 cells. Neuraminidase treatment of intact erythrocytes liberated only 69 per cent (mean of eight determinations) of the amount of sialic acid obtained from the hydrolyzed ghosts (Table 1). The amount of sialic acid released could not be increased by using higher concentrations of the enzyme or increasing the time of incubation. It was of interest that, in contrast to human red cells, the mouse erythrocyte did not agglutinate when suspended in homologous plasma following the neuraminidase treatment.

Treatment of the erythrocytes with trypsin liberated a substance which did not react directly in the Warren procedure but which did following acid-hydrolysis. The fragment split off by trypsin was undialyzable (Visking tubing) but was not precipitated by 10 per cent trichloroacetic acid. The amount of sialic acid recovered from this fragment was slightly greater than that obtained by neuraminidase treatment (Table 1).

Chromatographic analysis of the free sialic acids obtained from the erythrocytes in three different solvent systems is shown in Table 2. Two distinct spots were obtained with an orcinol spray. Substance A, which when eluted was found to contain...
96 per cent of the total Warren-reaction chromogen, had an \( R_f \) similar to NANA in all solvents. The other spot (Substance X) moved more rapidly than both NANA and N-glycolyneuraminic acid in all three systems but was not further identified.

**Ehrlich ascites tumor cells.**—Measurement of total sialic acid from acid-hydrolyzed EAT cells was not possible owing to technical difficulties (see "Methods"). The sialic acid released from the cell homogenates by neuraminidase was equal to \( 0.287 \pm 0.037 \) \( \mu \)moles/ml of packed cells or \( 0.485 \pm 0.100/10^6 \) cells. Only slightly less was recovered when the intact EAT cells were exposed to the neuraminidase (92 per cent of that obtained from the homogenates, see Table 1). Thus, the quantity of sialic acid released from the EAT cells by neuraminidase was some 96 times greater than that released from the erythrocytes on a per cell basis.

Tryptic digestion of the EAT cells produced a nondialyzable fragment which, as in the case of the erythrocytes, did not react directly in the Warren procedure unless first acid-hydrolyzed. However, by contrast with the red cells, the trypsin treatment of EAT cells yielded far less sialic acid than did the neuraminidase (Table 1).

The possibility that the trypsin in the EAT cell experiments was inhibited or inactivated by some cellular product was considered. To determine whether this were the case, the supernatants were removed following the EAT cell digestion. The supernatant fluid was then incubated with an aliquot of red cells, and the sialic acid released compared with that obtained with a similar aliquot of red cells incubated with fresh trypsin. The trypsin liberated sialic acid of the erythrocyte. The differences in total sialic acid may be even greater if the EAT cells contain gangliosides (11) or nucleotide-sialic acid compounds (3), substances whose sialic acid may not have been released by the neuraminidase treatment of cell homogenates. The increased sialic acid found in the EAT cell could merely be due to the greater membrane area of the much larger EAT cell. However, when these values are expressed in these terms (using the estimated areas of 780 sq. \( \mu \) for the EAT cell and 85 sq. \( \mu \) for the erythrocyte), the sialic acid released by neuraminidase from the intact EAT cell was still 4 times greater than that liberated from the erythrocyte. Errors incurred in the counting of the cells, in the estimation of the membrane area of both cell types, and differences in the amount of trapped extracellular fluid in the hematocrit and tumor cell

### TABLE 2

**The \( R_f \)'s of Sialic Acid Substances Isolated from Mouse Erythrocytes and Ehrlich Ascites Tumor Cells Separated in Three Different Solvent Systems**

<table>
<thead>
<tr>
<th>Sialic Acid Substance</th>
<th>Solvent System</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>See-butanol-acetone-acetic acid-water</td>
</tr>
<tr>
<td>( N )-acetylmuramic acid</td>
<td>0.46-0.49</td>
</tr>
<tr>
<td>( N )-glycolyneuraminic acid</td>
<td>0.30-0.41</td>
</tr>
<tr>
<td>Erythrocyte:</td>
<td></td>
</tr>
<tr>
<td>Substance A</td>
<td>0.47-0.48</td>
</tr>
<tr>
<td>Substance X</td>
<td>0.58-0.53</td>
</tr>
<tr>
<td>Ascites tumor cell:</td>
<td></td>
</tr>
<tr>
<td>Substance A</td>
<td>0.46-0.48</td>
</tr>
<tr>
<td>Substance B</td>
<td>0.39-0.41</td>
</tr>
</tbody>
</table>
volume measurements probably do not appreciably alter the above values.

Despite the much greater density of negatively charged sialic acid groups on the EAT cell membrane, the isoelectric point of this cell has been reported as about pH 4 (18) in contrast to that of about pH 2 found for the mammalian erythrocyte (1, 8). In this regard, it is pertinent to note that 33 per cent of the total erythrocyte sialic acid could not be liberated by neuraminidase. A similar situation has been noted for the horse erythrocyte (7). It is possible that this sialic acid, possibly linked to a ganglioside (11), may contribute to the negative charge of the erythrocyte. However, this fraction of sialic acid represents a relatively small number of negatively charged groups and could not account for the observed findings. It has been observed that the anionic polymer, polyxylenylphosphate, is bound to the EAT cell, presumably by positively charged groups on the membrane (18). Since the isoelectric point is determined primarily by the net charge on the cell surface, variability in the concentration of cationic groups may account for the fact that the reported isoelectric point in EAT cells is higher than the mammalian erythrocyte.

Another consideration is the possibility, raised by Wallach and Eylar (21), that not all the sialic acid liberated by neuraminidase is situated on the surface of the cell. The amount of sialic acid so released in their study was similar to that found by us and was greatly in excess of that which could be accounted for on the basis of the change in electrophoretic mobility. In view of the proposed dynamic continuity of the surface with the ergastoplasmic membranes of the cell (2), Eylar and Wallach suggested that a considerable pool of sialic acid might be present in the interior of the cell at any one time which, although not contributing to the surface charge, would ultimately be exposed to the action of the enzyme.

In contrast to neuraminidase, the sialic acid released by trypsin was not free but was part of a nondialyzable fragment. Similar observations were made by Mäkela and co-workers (14) following trypsin treatment of human red cells. On the other hand, Eylar and Madoff found the trypsinnized fragment to be a glycopeptide with a minimum molecular weight of 4,000—5,000 (6). A highly asymmetrical molecule of this size might not dialyze through Visking tubing. However, owing to the differences in methods of preparation, it is also possible that the substance reported by these authors represents a group detached from a larger parent substance. Cook et al. (4) also concluded that the trypsinnized fragment was a small peptide, since it was not precipitated by trichloroacetic acid and the acid-soluble fraction could be separated by paper chromatography. The trypsinnized, nondialyzable fragment of mouse erythrocytes was also not precipitated by trichloroacetic acid, but this is compatible with the properties of a glycopeptide.

In view of the similar amount of sialic acid liberated by both neuraminidase and trypsin from mouse erythrocytes, it seems reasonable to suppose that the sialic acid liberated by neuraminidase is an end group on a large membrane protein, one of whose peptide bonds can be split by trypsin. In sharp contrast to the erythrocyte, trypsin treatment of EAT cells only split off about one-sixth as much sialic acid liberated by neuraminidase. These results suggest that most of the sialic acid of the EAT cell is located on proteins or substances which do not contain trypsin-sensitive sites. The possibility that such sites are present, but are protected from enzyme attack by neighboring molecular groups, cannot be excluded.

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