The Linkage of Sialic Acid in the Ehrlich Ascites-Carcinoma Cell Surface Membrane

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1. The sialic acid content of fresh and fixed Ehrlich ascites cells was determined by incubation with neuraminidase and analysis of the supernatants. 2. The content of sialic acid was also determined on ultrasonically disrupted cells either with or without prior neuraminidase treatment, and the location of sialic acid in the cell is discussed. 3. The sialic acids, cleaved from cells by neuraminidase, were identified chromatographically. 4. Proteolytic enzymes were used to isolate from cells a mucopeptide containing sialic acid and galactosamine in almost equimolar proportions. 5. The nature of the carbohydrate-amino acid bond in the mucopeptide was investigated by alkaline hydrolysis. 6. A suggestion is made about the particular amino acids involved in the sugar-peptide bond.

The importance of sialic acid as a charge-determining constituent of many animal tumour cells is now firmly established. Dorfman & Cifonelli (1958) suggested that sialic acid might occur quite generally at the surface of mammalian cells. Since that time precise work has been performed on a number of cell types, the results of which tend to confirm this theory (Fuhrmann, Granzer, Kubler, Reuff & Ruhenstroth-Bauer, 1962; Ben-Or & Doljanski, 1960; Cook, Heard & Seaman, 1962).

The high electrophoretic mobility of many tumour cells has often been linked with the 'social behaviour' associated with malignancy. Purdom, Ambrose & Klein (1958) linked the electrophoretic mobility of MCIM sarcoma to the invasiveness of this tumour. Forrester, Ambrose & Stoker (1964) noted a possible increase in the amount of sialic acid-containing material on the surface of hamster kidney fibroblasts after transformation with polyoma virus. At the time their work was published it was thought that the new properties of the cell appearing after the malignant transformation might in some way be due to the increased amount of mucosubstances on its surface. Clearly, however, before any direct correlation may be made between the electrophoretic mobility or cell surface charge and biological properties of a cell, a full knowledge of the molecules determining the charge on the cell surface is required. Indeed, a thorough understanding of the social behaviour of

cells may only result from an extensive study of all components on the cell surface. With this in mind, an investigation was begun into the nature of the linkage of sialic acid, already known to be an important factor contributing to the charge on the tumour cell membrane.

The Ehrlich ascites tumour was chosen on account of its ready availability in large amounts and for the ease with which cell suspensions, free of contamination from mucoprotein material, can be obtained. It has disadvantages such as a general heterogeneity and contamination with other cell types that are difficult to remove. However, it is a more convenient tumour system to study than other better-defined systems, which are only available in small quantities.

MATERIALS AND METHODS

Solutions

Physiological saline used for washing cells was a solution of 0.85% NaCl in water distilled once in Pyrex glassware. Formaldehyde solutions for fixation purposes were prepared by dilution of 40% solution with NaCl solution. The sialic acid, used throughout these investigations as a standard, was that described as *N*-acetylneuraminic acid by Sigma Chemical Co., St Louis, Mo., U.S.A. On examination by paper chromatography, as detailed below, it ran as a single compound with the expected R_F value.

Galactosamine hydrochloride, also used as a standard, was obtained from British Drug Houses Ltd., Poole, Dorset. It was chromatographically homogeneous. Neuraminidase was obtained from Behringwerke A.-G., Marburg, Lahn, Germany, in solution in 0.9% NaCl buffered with acetate at pH 5.5 and containing 0.1% of CaCl₂.

Crude trypsin (British Drug Houses Ltd.) was used as a

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routine for the proteolytic studies, but a crystalline salt-free (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) preparation was also employed on occasion. A preparation of ficin, described as crystalline, was obtained from L. Light and Co. Ltd., Colnbrook, Bucks.

Analytical techniques

Sialic acid. The method of Warren (1959) was used throughout the work. The correction deduced empirically for absorption due to substances other than sialic acids was generally applied.

Hexosamine. A modification of the Elson-Morgan method reported by Rondle & Morgan (1955) was employed; 1 cm. spectrophotometric cells were used. Galactosamine hydrochloride standards and blanks were run in parallel with the test samples.

Amino acids. An EEL automatic amino acid analyser was used after samples had been hydrolysed for 24 hr. with 6 N-HCl under nitrogen in sealed tubes at 105°.

Preparation of tumour-cell suspensions

C-White mice were killed 8–10 days after intraperitoneal transplantation with 0.2 ml. of a suspension of Ehrlich ascites-tumour cells. This tumour was originally obtained from Professor Klein and has since been maintained at the Institute of Cancer Research for many years. It is the hyperdiploid (near tetraploid) strain. Cells were obtained from mice by peritoneal puncture. Haemorrhagic tumours were discarded. Tumours from several mice were pooled and collected in saline containing 0.01 M-EDTA. The addition of the chelating agent prevented coagulation of the tumour that otherwise often resulted, due probably to the relatively large amount of fibrin present in the tumour (Wheatley, 1963). Cells were centrifuged at 2000 rev./min. in an MSE bench centrifuge for 4 min. and resuspended in 10 vol. of saline after removal of the supernatant. This washing procedure was repeated three times.

Fixation of cells

Washed cells were suspended in approx. 50 vol. of 3% formaldehyde saline solution and were left to fix at room temperature, with occasional shaking, for up to 3 weeks. After fixation cells were washed four or five times in saline.

Disruption of cells

Washed cells were broken up with a Mullard ultrasonic generator. Suspensions in appropriate media were positioned with the meniscus just below the probe of the generator which was then tuned to maximum noise. The tube containing the suspension was surrounded with ice-water to prevent excessive heating, with concomitant destruction of labile biological components. Between 10 and 15 min. of such treatment was sufficient to destroy completely the integrity of all the cells as observed by light-microscopy.

Alkaline treatment of fixed cells

Washed fixed cells (2 vol.) were treated with saturated Ba(OH)₂ solution (10 vol.) at 80° and kept at that tempera-

ture for 15 min. The cells were removed by centrifugation and the supernatant neutralized with 1 N-H₂SO₄. After removal of the precipitated BaSO₄ a sialic acid assay was performed directly on the supernatant. A similar assay was then performed on the supernatant after acidifying it to 0.1 N-H₂SO₄ and hydrolysing at 80° for 1 hr. A hexosamine assay was also performed after hydrolysis with 1 N-HCl in a sealed tube for 2 hr. at 105°.

Enzymic treatment of cells

Neuraminidase. Washed cells were diluted with buffered saline (0.85% NaCl–0.2 M-acetate buffer, pH 5.5; 4:1, v/v) including 0.1% calcium acetate. Enzyme solution was then added to this suspension (enzyme/cell ratio, 100 units/g. wet wt., where 1 unit of activity is defined as the amount of enzyme that releases 1 μ g. of *N*-acetylneuraminic acid in 15 min. at 37° from a suitable substrate in the appropriate medium). Cells were incubated at 37° for up to 90 min., samples of the suspension being removed at intervals. Each sample was first centrifuged and then deproteinized by the addition of an equal volume of 10% (v/v) trichloroacetic acid. Sialic acid assays were performed on the clear supernatants after removal of the precipitates. Fixed cells were treated in exactly the same way.

Normal washed cells, after treatment with neuraminidase and two further saline washes, were subjected to ultrasonic disruption. The suspension of cell debris was then treated with neuraminidase again for 1 hr. After protein precipitation sialic acid was assayed. Untreated washed cells were also subjected to these procedures.

Proteolytic enzymes. Crude trypsin was shaken with Krebs-Ringer phosphate, pH 7.2, to form a fine suspension and then mixed with a cell suspension in the same medium. The final concentration of enzyme varied between 2 and 10 mg./ml. in different experiments. A clear enzyme solution was obtained when crystalline trypsin was used. Cells were incubated at 37° for up to 3 hr. The viability of cells was tested at various stages by exclusion of Lissamine Green. After incubation, the cell-free supernatants were deproteinized with trichloroacetic acid (final concentration 5%). The sialic acid content was then determined on the clear supernatant directly, or after prior dialysis, freeze-drying and hydrolysis with 0.1 N-H₂SO₄ at 80° for 1 hr. Suitable control incubations of cells without enzyme and enzyme without cells were also included. The effect of ficin was also tried. Ficin was activated as described by Lisowska (1962).

Gel filtration

A column of Sephadex G-50 was carefully prepared (3.5 cm. diam. $\times$ 25 cm.). The uniformity of the bed was tested by filtration of a haemoglobin solution through the column and the void volume was determined. Cell-free deproteinized supernatants of trypsin-treated cells were dialysed against running tap water overnight and freeze-dried. A little distilled water was used to redissolve the material, any insoluble matter being discarded at this stage. This solution was then passed down the gel column, the eluting solvent being distilled water and 2 ml. fractions being collected. The extinction at 260 m μ of each fraction was measured and the sialic acid and hexosamine content also determined.

Analysis of fractions containing sialic acid

Paper chromatography. Fractions containing sialic acid were pooled, freeze-dried and hydrolysed by refluxing with 2*N*-HCl for several hours. The hydrolysate was concentrated by vacuum distillation, and HCl was removed by evaporation to dryness followed by re-solution in water (these operations being repeated three times). The concentrated hydrolysate was examined in two separate solvent systems [butan-1-ol-acetic acid-water, 4:1:5, by vol. (organic phase), and pyridine-butan-1-ol-water, 4:6:3, by vol.]. The descending method with Whatman no. 1 paper was used. The air-dried chromatograms were developed in one of the three following ways. The chromatogram was either sprayed with 0.25% ninhydrin in butanol saturated with water and heated for 5 min. at 105°, or reducing sugars were revealed with AgNO₃ as in the modification of Trevelyan, Procter & Harrison (1950), or hexosamines were shown up with the Elson-Morgan reaction.

The sialic acids present in the supernatants of cells after treatment with neuraminidase were purified by the procedure described by Seaman & Uhlenbruck (1963). For chromatographic identification the solvent system used by Svennerholm & Svennerholm (1958) (propan-1-ol-butan-1-ol-0.1*N*-HCl, 2:1:1, by vol.) was employed and the spots were revealed with the thiobarbituric acid spray reagent of Warren (1960).

Pronase digestion

Pooled fractions containing sialic acid were incubated at 37° with Pronase (Calbiochem, Los Angeles, Calif., U.S.A.) at a concentration up to 10mg./ml. in the presence of 0.1% calcium acetate. Incubation times ranged between 1 and 24 hr. The Pronase digestion mixture after incubation and deproteinization was filtered on a Sephadex G-50 column. Sialic acid was assayed in fractions eluted with water and sialic acid-positive fractions were pooled, freeze-dried and analysed for amino acids.

RESULTS

Fig. 1 shows the release of sialic acid (calculated as *N*-acetylneuraminic acid) from viable and

Fig. 1. Release of free sialic acids from Ehrlich ascites-carcinoma cells on incubation with neuraminidase, as measured by the method of Warren (1959): ○, fresh washed cells; ●, formaldehyde-fixed cells. Cellular concentration was determined with a micro-haematocrit centrifuge.

formaldehyde-fixed cells. Both curves show a similar increase though the limiting amounts of released sugar differ by a factor of two-thirds. Prior disruption of viable cells yielded no more sialic acid, and prior enzyme treatment removed all the sialic acid leaving none to be released after disruption.

Chromatography of sialic acids liberated from the cells by neuraminidase showed one spot which corresponded exactly with the *N*-acetylneuraminic acid standard (R_f 0.45), and another fainter spot with R_f 0.34, the value expected from *N*-glycolylneuraminic acid. A sample of this compound was not available for use as a standard. No estimation of the relative proportions of the two sugars could be made, though visual inspection showed that the intensity of the spot corresponding to *N*-acetylneuraminic acid was much greater than the other.

Table 1 shows the result of the action of proteolytic enzymes. The amount of released sialic acid may be compared with that released by neuraminidase from the intact cell. No detectable amount was present in any of the control incubations.

Study of the ultraviolet-absorption pattern of the dialysed supernatant of trypsin-treated cells revealed a maximum at about 260mμ. Thus the extinction of fractions obtained by gel filtration of this material was measured at that wavelength. These results together with those of sugar assays on comparable runs are shown in Fig. 2. It is clear that the strongly ultraviolet-absorbing material is not associated with the peptide containing sialic acid. The sialic acid and first hexosamine peaks were found to be almost coincident. The void volume of this column was approx. 30ml., i.e. bound sialic acid was eluted in a volume equal to, or a little greater than, the void volume.

Paper chromatograms, of the 2*N*-acid hydrolysate of fractions containing sialic acid, showed with ninhydrin a whole range of amino acids or peptides, but only one spot that reacted with reagents for reducing sugars or amino sugars. This spot had an R_f value in both solvents identical

Table 1. Release of sialic acid from Ehrlich cells with enzymes

Enzyme	Released sialic acid	
	(μmole/ml. of cells)	(% neuraminidase-sensitive)
Trypsin (crude)	0.220	84.5
Trypsin (crystalline)	0.191	73.5
Ficin	0.204	78.5
Neuraminidase	0.260	—

Fig. 2. Gel filtration of the sialomucopeptide obtained by trypsin treatment of Ehrlich ascites-carcinoma cells: ○, E_{260} mμ; □, sialic acid concentration determined by the method of Warren (1959) after 0.1 N- H_2SO_4 hydrolysis of individual freeze-dried fractions; ● hexosamine concentration obtained with method of Rondle & Morgan (1955), with a galactosamine standard.

Fig. 3. Release of free sialic acid from isolated mucopeptide: ○, with 0.1 N- H_2SO_4 at 80°; ●, with 100 units of neuraminidase/ml.

with that of the galactosamine standard and could not be separated from added galactosamine hydrochloride.

The molar ratio of galactosamine to sialic acid determined on the purified sialopeptide was found to be 1.18. Within experimental error this value may be equated with unity.

When fixed cells were treated with barium hydroxide no free sialic acid was detectable in cell-free supernatants. However, the supernatants contained bound sialic acid and hexosamine equivalent to about 36% of the amount that could be liberated by neuraminidase. Hexosamine and sialic acid were present in the supernatant in a molar ratio of 1.12, a value that again may be equated with unity.

Fig. 3 shows the release of sialic acid from the sialopeptide by enzymes and hydrolytic means. The amounts of the sugar obtained per mg. of

material by both procedures was essentially the same. The ease with which sialic acid is released from the molecule should be noted. Studies on the release of hexosamine showed that almost all is released within 2 hr., when the material is hydrolysed in a sealed tube with 1 N-hydrochloric acid at 105°.

Gel filtration showed that a small decrease in molecular size had been caused by Pronase digestion of the peptide. The amino acid composition was also changed. There were notably smaller amounts of valine, leucine, isoleucine, histidine, phenylalanine, tyrosine and methionine. The digested peptide consisted primarily of the amino acids aspartic acid, glutamic acid, proline, alanine, serine, threonine and glycine in approximate molar proportions 4:3:3:1:1:1:2. This may be compared with the amino acid composition of the undigested peptide reported elsewhere (Langley & Ambrose, 1964).

DISCUSSION

It is important to understand the extent to which results obtained in these studies *in vitro* may be extrapolated to the situation *in vivo*. Tumour ascitic fluids contain large quantities of sialoproteins. The efficiency of the washing procedure adopted is therefore of prime importance. Preliminary experiments involving estimations of total cell-surface sialic acid after a variable number of washes showed that the amount of sugar associated with the cell falls rapidly after the first wash and is constant after the third wash. There is thus no doubt that the present results refer to material that is very firmly attached to the washed

cell, and that probably constitutes structural elements within the cell membrane *in vivo*.

The results show that almost as much sialic acid may be obtained by enzyme treatment of viable cells as in treatment of a cell homogenate. Nearly all the cellular sialic acid is available to enzyme in the intact viable cell in a medium containing enzyme, i.e. it is associated with the cell surface membrane. The evidence obtained from fixing cells might indicate that some of these sialic acid residues are deep-seated within the membrane, if the action of formaldehyde is such that it prevents enzyme molecules from reaching all possible substrate sites, though it is possible that during fixation *N*-acetylneuraminic acid could have been changed chemically. Prolongation of fixation beyond 2 days, however, did not appreciably alter the sialic acid content of fixed cells. Certainly a change in cell size was not responsible for the lower values obtained with fixed cells, since cellular size was found, by cell counting and measurement of packed cell volumes of cell suspensions, to remain constant on fixation.

Thiobarbituric acid will form a chromophore only with compounds that produce β -formylpyruvic acid on cleavage with periodate (Warren, 1960). Only 3-deoxy-2-oxo sugar acids and free sialic acids are therefore detected by the thiobarbituric acid spray reagent on chromatograms. The faster-moving spot in chromatograms of the supernatant of neuraminidase-treated cells is thus almost certainly the *N*-acetyl derivative of neuraminic acid. The same specificity of the reagent indicates the nature of the second component. It seems very probable that this component of R_f 0.34 was *N*-glycolylneuraminic acid, since the R_f for this derivative was found by Svennerholm & Svennerholm (1958) to be 0.34 when the same solvent was used in the descending technique. The faster component correspondingly had an R_f identical with that reported by these workers for *N*-acetylneuraminic acid. The two *N*-acetyl derivatives often appear together in Nature: indeed it is the exception rather than the rule to find a single derivative in biological material from the same source (Gottschalk, 1960). The finding that both *N*-acetylneuraminic acid and *N*-glycolylneuraminic acid are present in this tumour (the former predominantly) confirms the work of Miller, Sullivan & Katz (1963).

The first clue as to the nature of the linkage of sialic acid in the Ehrlich ascites cell has come from the use of proteolytic enzymes. In preliminary experiments not reported here, very little sialic acid was found to be extracted from the cells on leaching with chloroform-methanol (2:1, v/v) and it seems likely that most of the sialic acid in this tumour is associated with trypsin-labile proteins

rather than with lipids. Gray (1963) could show only minute amounts of glycolipids, which may have possibly contained sialic acid, in an extensive study of the lipids of this particular tumour. Partial purification of the sialopeptide obtained here was achieved by using the method of gel filtration. The coincidence of the sialic acid and the first hexosamine peak in the eluate is significant. Coupled with the value of the molar ratio determined for these sugars and the absence of other carbohydrate components, this suggests a direct link between them. Analysis of the supernatant of alkali-treated fixed cells showed that bound sialic acid and hexosamine were released in equimolar proportions, indicating that the sugars were linked together in a disaccharide unit and therefore not bound singly to the peptide chain. When linked to sialic acid galactosamine is found as the *N*-acetyl derivative. *N*-Acetyl groups would have been hydrolysed off in the procedure used for chromatographic investigation, and it seems possible that galactosamine is present in the cell *in vivo* as the *N*-acetyl derivative. The ready release of sialic acid from the peptide, both enzymically and hydrolytically, is suggestive of sialic acid being in a non-reducing terminal position. The suggestion is made therefore that the linkage of the sialic acid in the Ehrlich cell membrane is a terminal one in a disaccharide unit similar to that found in the submaxillary mucins (Fig. 4; Gottschalk & Graham, 1959).

When considering to which amino acids the carbohydrate residues may be attached, it is realized that few amino acids have the necessary functional groups capable of binding sugar. Of those present in the Pronase-digested peptide,

Fig. 4. Structural formula of α -D-sialylpyranosyl-*N*-acetylgalactosamine, after Gottschalk & Graham (1959). Ac, Acetyl.

aspartic acid and glutamic acid predominate over serine and threonine. The two former can give ester bonds with their carboxylic acid side chains whereas the latter can participate in an ether type of bond-formation. Treatment of fixed cells with weak alkali demonstrates the ready removal at pH 12.9 of significant amounts of disaccharide, a result which might indicate that an ester link exists between sugar and peptide, rather than a more resistant ether bond. Harbon, Herman-Boussier & Clauser (1963), however, in an investigation of such linkages in ovine submaxillary-gland mucoprotein concluded that the hydroxyl groups of serine and threonine were the chief points of attachment of sugar side chains. Their results were largely based on hydroxylaminolysis and the kinetics of alkaline hydrolysis of the mucoprotein. They did not exclude the possible presence of ester linkages to carboxylic amino acids (Harbon, Herman, Rossignol, Jollès & Clauser, 1964) even though much smaller amounts of these acids relative to serine and threonine were present in their material. In the material under present study carboxyl-containing amino acids predominate and it is suggested that aspartic acid and glutamic acid may be important in binding the disaccharide residues to the peptide chain. The possibility that a role may be played by serine and threonine cannot be ignored, however.

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