Cell-Surface Mucosubstances from Trypsin Disaggregation
of Normal and Virus-Transformed Lines of
Baby-Hamster Kidney Cells

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Cell disaggregation by trypsin solubilizes significantly less mucosubstance from the surface of polyoma-virus-transformed baby-hamster kidney cells than from the same non-transformed cell line. The mucosubstance, which consists of both acid mucopolysaccharides and mucoproteins, also differs qualitatively in the two cell lines.

Mucosubstances on the cell surface have multiple roles, e.g. cell adhesion (Roseman, 1970), growth control (Kornfeld & Kornfeld, 1969) and recognition (Watkins, 1966; Roth *et al.*, 1971). When cells in tissue culture are transformed by tumour-causing viruses, changes occur in the surface mucosubstances with the loss of cell-contact properties (Stoker, 1972; Burger, 1971). Mucolipids of the virus-transformed cells have been shown to be incomplete in their complement of sugar residues (Hakomori & Murakami, 1968; Mora *et al.*, 1969). The activities of individual glycosyltransferases are also deficient in virus-transformed cell lines (Cumar *et al.*, 1970; Kijimoto & Hakomori, 1971). In contrast with the mucolipids, an increase in the molecular size of a mucoprotein released by trypsin from the surface of transformed cells has been found (Buck *et al.*, 1970). This has been shown for four species of cells, hamster, mouse, chick and human, transformed by RNA viruses and DNA viruses. This change in molecular size of the mucoprotein has since been shown to be due to an increase in the amount of sialic acid, and there is a corresponding increase in the sialyltransferase activity of the transformed cell (Warren *et al.*, 1972). In this context it is noteworthy that earlier work involving histological (Defendi & Gasic, 1963) and cell-electrophoresis studies (Forrester *et al.*, 1962) pointed to an increase in the surface sialic acid in polyoma-virus-transformed BHK cells.*

Incubation with crystalline trypsin provides a satisfactory method for isolating fragments of cell-surface mucosubstances and, in particular, the mucoproteins (Snow & Allen, 1970). Comparison of the mucosubstances liberated by trypsin disaggregation of monolayers of non-transformed and transformed cells is particularly worth while. Trypsin is a specific method for disaggregating cells, and cell adhesion is deficient in at least some transformed cells (Coman,

1944; Edwards *et al.*, 1971). The specific agglutination of virus-transformed cells by plant lectins can be produced in the non-transformed cells by treatment with trypsin (Burger, 1971; Ben-Bassat *et al.*, 1971). One explanation for this effect of trypsin is that, in contrast with the transformed cells, the agglutination sites in the non-transformed cells are masked by surface material that can be removed by the enzyme (Burger, 1971). In view of these chemical and biological changes on the surface, it was decided to determine the total mucosubstances removed by trypsin from normal and virus-transformed cells. In the present work we have isolated and measured the amounts of bound hexosamines and bound sialic acids removed by trypsin from the surface of BHK cells and the same line transformed by polyoma virus (Py BHK cells). We have used these hexosamine and sialic acid contents as an index of the surface mucosubstances.

Experimental

The radioactivity experiments, tissue-culture methods and colorimetric determinations are described in Snow & Allen (1970) and Snary & Allen (1971, 1972).

Tissue culture. The cells used in all experiments were from a permanent cell line of BHK C13 21 cells and the corresponding polyoma-virus-transformed cells from the same clone (Py BHK C13 21 cells) (Edwards *et al.*, 1971). The cells were grown in 500ml medical flats containing 50ml of growth medium or in roller culture with 2.5-litre bottles containing 150ml of growth medium. The medium used was the Glasgow modification of Eagle's medium (obtained from Biocult Laboratories, Glasgow, U.K.) and was supplemented with the following: glutamine (0.584g/l), serine (0.021g/l), glycine (0.015g/l), streptomycin (0.1g/l), penicillin (0.317g/l), sodium pyruvate (0.1g/l), *n*-butyl *p*-hydroxybenzoate (0.2mg/l) and calf serum (10%, v/v). Before dis-

* Abbreviations: BHK cells, baby-hamster kidney cells; Py BHK cells, polyoma-virus-transformed BHK cells.

aggregation of the cells, the Eagle's medium was poured off and the cells were washed with 2×40 ml and 4×10 ml of Tris-buffered saline (for composition see Snow & Allen, 1970). The cells were disaggregated with 20 ml of 0.04% crystalline trypsin in Tris-buffered saline, with Ca^{2+} and Mg^{2+} present, at 37°C by using the method of Snow & Allen (1970). Twice the volume in the medical flats was used for harvesting the cells in roller culture.

For all experiments the cells were grown for 48 h, after which time the BHK cells had formed a confluent monolayer. The Py BHK cells were seeded at a lower cell density, so that after 48 h the non-contact-inhibited Py BHK cells had about the same cell density as the confluent monolayer of BHK cells.

Quantitative analysis of samples. After disaggregation, the cells were separated from the trypsin and liberated cell-surface material by centrifugation at $1500 g$ for 10 min. The cell pellet was resuspended in 2 ml of water, stored, frozen and analysed. The supernatant containing trypsin and cell-surface material was either freeze-dried and resuspended in 5 ml of water or concentrated by vacuum dialysis to a final volume of about 5 ml. The added trypsin, which was at the minimum concentration necessary to disaggregate the cells, interfered seriously with direct colorimetric determinations. The trypsin could not be separated from radioactive mucosubstances by ordinary gel filtration on Sephadex. Precipitation of the protein with 5% (w/v) trichloroacetic acid also precipitated 8–10% of the radioactive mucosubstance. Thus the use of trichloroacetic acid precipitation is not suitable for the quantitative determination of mucosubstances, and such methods of isolation (Onodera & Scheinin, 1971) will detect only a fraction of the total mucosubstance liberated from the cell surface.

The hexosamines, glucosamine and galactosamine, were liberated by hydrolysis in 8M-HCl for 3 h at 95°C (Swann & Balazs, 1966). The hydrolysates were dried down by rotary evaporation, washed twice with water and filtered. Separation of the hexosamines was accomplished by elution of the sample on a $14 \text{ cm} \times 1 \text{ cm}$ column of basic resin on a Locarte Mini amino acid analyser. The sample was dissolved in citrate buffer, pH 2.2 (Moore *et al.*, 1958), except that 0.01% (w/v) sodium octanoate was used in place of phenol.

Sialic acids were liberated by hydrolysis in 0.2M- H_2SO_4 at 80°C for 50 min, and measured by the thio-barbituric acid method (Aminoff, 1961). The sialic acids were separated from the hydrolysate by adsorption on a $1 \text{ cm} \times 3 \text{ cm}$ column of Amberlite CG-400 followed by elution with 0.3M-formic acid, 5 ml fractions being collected. The sensitivity of the thio-barbituric acid assay was increased fourfold by using 2 ml instead of 0.5 ml of the formic acid eluate. Before analysis of non-dialysed freeze-dried samples, buffer

salts were removed by gel filtration on Sephadex G-50, with water as the eluent. A less-viscous solution was obtained by precipitation with trichloroacetic acid (final concentration 5%, w/v) before gel filtration. The excluded volume, which contained all the sialic acid-positive material, was freeze-dried, pooled with the trichloroacetic acid precipitate and analysed for sialic acids.

Results and discussion

Before quantitative analysis it was necessary to demonstrate that all the cell-surface mucosubstance accessible to trypsin had been solubilized. This was studied by measuring the release of the non-diffusible radioactive mucosubstance from cells grown in the presence of [$1\text{-}^3\text{H}$]glucosamine. Acid hydrolysis and chromatography showed that radioactivity from [$1\text{-}^3\text{H}$]glucosamine was incorporated by the BHK cells or the Py BHK cells into the non-diffusible cellular material solely as bound glucosamine, galactosamine and sialic acid residues. This radioactivity is therefore a monitor of the cellular mucosubstances, namely mucoproteins, mucolipids and acid mucopolysaccharides. Incubation for 12 min with 0.04% trypsin at 37°C completely disaggregated BHK cells and Py BHK cells, radioactively labelled from [$1\text{-}^3\text{H}$]glucosamine, and solubilized 23–25% of the non-diffusible cellular radioactive mucosubstance. No additional radioactive material was solubilized by further incubation with trypsin for 30 min at 37°C , showing that there had been complete release of trypsin-accessible mucosubstance in the first 12 min. Under these conditions trypsin releases only material from the cell surface, there being negligible release of intracellular material (Snow & Allen, 1970). With 0.005% trypsin, the minimal amount necessary to increase the agglutination of BHK cells by lectin (Burger, 1969), 12.7 and 10.7% of the total cellular radioactive mucosubstance was released from BHK cells and Py BHK cells respectively. This indicates that there is no selective effect of low trypsin concentration on the BHK cells, although only half the accessible mucosubstance is solubilized under these conditions and the cells are not disaggregated.

Quantitative analysis of the amount of mucosubstance solubilized by trypsin was assayed by measuring the hexosamine (glucosamine and galactosamine) (Table 1) and sialic acid (Table 2) contents. Whereas the hexosamine content is a measure of the amount of acid mucopolysaccharides and mucoproteins, the sialic acid content is a measure of the amount of mucoproteins only (Gottschalk, 1972). Mucolipids are not released by trypsin treatment of the cells (Buck *et al.*, 1970). The presence of acid mucopolysaccharides in the trypsin-solubilized mucosubstances was detected by the presence of uronic acid (determined colorimetrically) and ester

Table 1. *Analysis of glucosamine and galactosamine in material released by trypsin from the surface of BHK cells and Py BHK cells*

The conditions for treatment with trypsin are described in the text under 'Experimental'. The samples were freeze-dried before analysis. The cell volume was measured (haematocrit) and the surface area calculated from it. Each value is the average of at least four analyses.

Cell line	Protein/cell (ng)	Hexosamine (μmol)		
		Trypsin-released cell-surface material		Trypsin-treated cells (per mg of cell protein)
		(per mg of cell protein)	(per cm^2 cell surface area)	
BHK	0.55	0.00791	7.15×10^{-5}	0.0192
Py BHK	0.38	0.00563	4.50×10^{-5}	0.0173
Ratio of glucosamine to galactosamine				
		Trypsin-released cell-surface material		Trypsin-treated cells
BHK		3.8		14.3
Py BHK		2.6		9.5

Table 2. *Analysis of sialic acid in material released by trypsin from the surface of BHK cells and Py BHK cells*

The cells were treated with trypsin as described in the text under 'Experimental'. The trypsin-released material was either vacuum-dialysed to about 5 ml final volume or freeze-dried before analysis. The trypsin-treated cells were analysed directly. *N*-Acetylneuraminic acid was used as a standard in the thiobarbituric acid reaction (Aminoff, 1961). No correction was made for the small amount of *N*-glycolylneuraminic acid present (Snow & Allen, 1970). The cell volume was measured (haematocrit) and the surface area calculated from it. Each value is the average of at least four analyses.

Cell line and preparation method	Sialic acid (μg)		
	Trypsin-released cell-surface material		Trypsin-treated cells (per mg of cell protein)
	(per mg of cell protein)	(per cm^2 cell surface area)	
Freeze-drying			
BHK	0.78	7.05×10^{-3}	2.60
Py BHK	0.62	4.96×10^{-3}	2.08
Vacuum dialysis			
BHK	0.34	3.17×10^{-3}	—
Py BHK	0.53	4.24×10^{-3}	—

sulphate measured as incorporated $^{35}\text{SO}_4^{2-}$ (Snary & Allen, 1972). The non-diffusible ^{35}S was distributed as follows: growth medium plus washings, 59.3%; trypsin-released cell-surface mucosubstance, 8.3%; cells 32.5%. The lower ratio of glucosamine to galactosamine in the trypsin-solubilized mucosubstance from both BHK cells and Py BHK cells (Table 1) could also be due to a high content of the acid mucopolysaccharides predominantly containing galactosamine (e.g. chondroitin sulphate).

Significantly less mucosubstance and in particular

mucoprotein, measured as hexosamine and sialic acid contents and related to cell protein, was solubilized by trypsin disaggregation of Py BHK cells compared with BHK cells (Tables 1 and 2). The difference is even more marked when the results are expressed with respect to cell surface area. The surface area was calculated from the cell volume, assuming that the cells are spherical in suspension. The smaller size of the Py BHK cells has been previously documented (e.g. Inbar & Sachs, 1969). The results thus show that the amount of mucosubstance per unit surface area

of Py BHK cells is only 60–70% of that per unit surface area of BHK cells. This loss of mucosubstance could be due to fewer molecules per unit surface area and/or an incompleteness in their sugar chains with respect to the amino sugars. Such a decrease in the Py BHK cells, which may be reflected in one or several species of mucosubstance, could be responsible for changes in surface properties of these cells. Trypsin still releases substantial amounts of mucosubstance from the surface of the Py BHK cells, so if there is extra protease activity on the surface of the transformed cell (Burger, 1969) it can be releasing only less than half of the mucosubstance that is solubilized by trypsin. The amount of mucosubstance in the disaggregated Py BHK cells is also less than that in the disaggregated BHK cells (Tables 1 and 2).

On dialysis of the trypsin-solubilized cell-surface material there is a much greater loss of bound sialic acid from the BHK-cell preparation than from the Py-BHK-cell preparation. Thus, in contrast with the freeze-dried material, after exhaustive dialysis there is considerably more sialic acid (measured with respect to cell protein) in the trypsin-solubilized material from Py BHK cells than in that from BHK cells (Table 2). All the diffusible sialic acid was excluded on Sephadex G-50 and was therefore bound to relatively large molecules. The loss of sialic acid on dialysis was not prevented by previously heating (at 105°C for 20 min) or by treating the sample with trichloroacetic acid (final concentration 5%, w/v) and was therefore not due to neuraminidase activity. Thus the trypsin-sensitive sialic acid-containing mucoprotein can be separated into diffusible and non-diffusible fractions with more of the latter in the Py-BHK-cell preparation. This indicates that there are qualitative differences between the mucoproteins from Py BHK cells and those from BHK cells. A possible explanation of these differences is the finding that there is an increase in the sialylation of a mucoprotein fraction from a Pronase digest of the trypsin-solubilized material from Py BHK cells (Warren *et al.*, 1972). Such an increased sialic acid content could make the mucoprotein less diffusible. Chiarugi & Urbano (1972) have shown that a highly glycosylated mucoprotein present in BHK-cell membranes is absent from Py-BHK-cell membranes and is excreted into the medium by the Py BHK cells. This points to more than one difference between the mucoproteins of Py BHK cells and BHK cells. Other, less specific, changes in the mucoproteins of virus-transformed cells have also been shown (Onodera & Sheinin, 1970; Meezan *et al.*, 1969).

Ohta *et al.* (1968) have shown the total sialic acid content of BHK cells is higher than that of Py BHK cells by a factor of 1.6. In contrast, other workers (Kraemer, 1966) have found the total sialic acid content of BHK cells lower than that of Py BHK cells by a factor of 0.7. Our values for the total cellular

sialic acid content/mg of protein for BHK cells are 1.25 times that for the Py BHK cells (Table 2) and are in qualitative agreement with Ohta *et al.* (1968), in whose work the cells were harvested with EDTA and the sialic acid was determined without prior separation on a column.

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