A STUDY ON THE MECHANISM OF INTERCELLULAR ADHESION

Effects of Neuraminidase, Calcium, and Trypsin on the Aggregation of Suspended HeLa Cells

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ABSTRACT

Aggregation of suspended HeLa cells is increased on removal of cell surface sialic acid. Calcium ions promote aggregation whereas magnesium ions have no effect. The calcium effect is abolished by previous treatment of the cells with neuraminidase.

Trypsinization of the HeLa cells followed by thorough washing diminishes the rate of mutual cell aggregation. Subsequent incubation with neuraminidase restores the aggregation rate to the original value before trypsin treatment. Cells which had acquired a greater tendency for aggregation after removal of peripheral sialic acid lose this property when subsequently treated with trypsin. Calcium ions have no aggregative effect on trypsinized cells.

In contrast to HeLa cells, aggregation of human erythrocytes was not increased after treatment with neuraminidase or on addition of calcium.

The results with HeLa cells are interpreted as follows: (*a*) Trypsin-releasable material confers adhesiveness upon the cells. (*b*) The adhesive property of this material is counteracted by the presence of cell surface sialic acids. (*c*) Calcium ions exert their effect by attenuating the adverse effect of sialic acid.

INTRODUCTION

Intercellular adhesion is fundamental biological property in multicellular organisms and is believed to play a key role in growth, morphological differentiation, and metastasis (1, 2). Mutual adhesivity can be seen as a necessary condition preceding and facilitating the instalment of specialized intercellular contacts.

Cell surface sialic acids are responsible for a significant part of the negative surface charge of many mammalian cells (3). The observation that normal and malignant cells with their different adhesive behavior show differences in the extent to which sialic acids are exposed at the electrokinetic shear plane (4–7) may serve as an indication for a role of sialic acid in mutual cell adhesion. Sauter et al. (8) observed "agglutination" of human leukemic myeloblasts after neuraminidase treatment. Vicker and Edwards (9) demonstrated that the aggregation of freshly trypsinized baby hamster kidney (BHK) cells was enhanced in the

presence of neuraminidase. In contrast, Kemp (10, 11) found that the presence of neuraminidase decreased the aggregation of embryonic chick muscle cells harvested by trypsinization. Measurement of the effect of neuraminidase on the reaggregation of trypsin-dissociated neural retina cells did not yield reproducible results (12).

There are reasons to believe that the aggregative behavior of trypsinized cells differs from that of untreated cells. Treatment with trypsin often gives rise to the appearance of a slimelike extraneous material embedding or entrapping cells (13, 14). After thorough washing, the cells are less adhesive than before trypsin treatment, a common experience first observed by Moscona (15). It was shown that trypsin releases glycopeptides from cells (16–19) and degrades membrane glycoproteins (20–22). Trypsin also causes rearrangements in the localization of peripheral glycoproteins (23, 24).

In the present experiments the aggregation between suspended HeLa cells is measured by a sensitive new method (25) permitting measurement of relatively weak binding forces between cells. The cells are treated with neuraminidase for a short period and then are washed before the aggregation measurements. The results are related directly to the amounts of sialic acid released by the enzyme.

MATERIALS AND METHODS

HeLa Cells

We used HeLa cells grown in spinner culture with modified (26) Basal Medium Eagle 0405. The cells were harvested in the stationary phase of cell growth. The cell concentration in the culture medium was evaluated with the Burker hemacytometer. The cells were washed three times with NaCl 0.9%. 60 ml of washing solution were used per 200×10^6 cells. In the case in which cells were treated subsequently with neuraminidase, the washed cells were collected and resuspended in a concentration of approximately 25×10^6 cells/ml, in a solution pH 7.4 of the following composition: 8.0 g NaCl, 0.2 g KCl, 0.26 g $CaCl_2 \cdot 2\ H_2O$, 0.1 g $MgCl_2 \cdot 6H_2O$, 0.05 g $NaH_2PO_4 \cdot H_2O$, 1.0 g $NaHCO_3$. In the present paper, this solution is referred to as Ringer's solution.

Neuraminidase Treatment

7-ml aliquots of the homogenized suspension prepared as described above were shaken for 15 min at 37°C after addition of 1-ml acetate buffer 0.05 M, pH 5.5, containing different concentrations of α-neuraminidase (3:2:1:18) (from *Vibrio cholerae*, Serva, Heidelberg, Germany). The final pH of the mixture was 7.25. The sialic acid released in the supernatant solutions was assayed according to the Warren (27) method with introduction of a correction for the absorbance due to released glycoproteins (25). The values were expressed as percentages of the amount liberated after incubation with 170 enzyme units, the highest enzyme concentration used. The latter amount was found to be a 100% value since repeated treatments of cells with 170 units for 1 h instead of 15 min never led to an increase of the sialic acid release. One unit of enzyme activity is defined as that quantity that splits off 1 μg of sialic acid in 15 min at 37° C from human serum α-glycoprotein.

The amounts of sialic acid liberated after 15 min or 1 h were considered to be the maximal releasable amounts. It was found that this amount varied for cells harvested at different days from the spinner culture.

After enzyme treatment the cells were washed twice with NaCl 0.9%. 50 ml of washing solution were used per 175×10^6 cells.

Trypsin Treatment

In some experiments the cells were treated with 0.1% trypsin in Ca- and Mg-free Ringer's solution (pH 7.5; osmolality brought to 288 mosmol/kg by addition of NaCl), either before or after incubation of the cells with neuraminidase (crystalline trypsin, Sigma Chemical Co., St. Louis, Mo.: from bovine pancreas, type I; crude trypsin, Difco Laboratories, Detroit, Mich., 1:250). After incubation at 37°C for 15 min in the shaker system, the cells were washed with NaCl 0.9%.

The Lowry et al. (28) method for protein determination was used as a measure for the amount o material released into the medium by the action of trypsin. Human serum albumin was used for the preparation of a calibration curve. In order to determine the amount of sialic acid bound to released glycopeptides, aliquots of the supernate were exposed to 170 neuraminidase units for 30 min at 37°C.

Measurement of Cell Aggregation

The enzyme-treated cells were suspended in 100 ml of a solution prepared as follows: 8.0 g NaCl, 0.3 g KCl, 0.05 g $NaH_2PO \cdot H_2O$, 0.025 g KH_2PO_4, 1.0 g $NaHCO_3$, and 3 g Tris were dissolved in 1 liter of distilled water. The pH of this solution was adjusted to pH 7.5 by addition of Na-acetate buffer 0.5 M, pH 4.0. The osmolality of 310 was reduced

to 288 by addition of distilled water. In some experiments explicitly mentioned in the Results, crystalline $CaCl_2 \cdot 2H_2O$ or $MgCl_2 \cdot 6H_2O$ were added in concentrations varying from 0.5 to 3.0 mM.

Before the proper measurement, the cell suspensions were allowed to stay in a water bath at 37°C for 15 min. Then they were inverted several times in order to ensure that the cells were distributed homogeneously throughout the suspension.

For the measurement of aggregation we developed a method based upon the effect of size on the distribution of suspended particles in laminar flow conditions (25). The apparatus (Couette viscosimeter, VT 180, Gebr. Haake, Berlin) consists of a concentric pair of stainless steel cylinders separated radially by a gap of ca. 11 mm. The inner cylinder is rotated at a constant velocity of 11.7 rpm. The space between the cylinders is filled with the cell suspension to a height of ca. 70 mm (i.e., approximately 90 ml of the suspension). The outer cylinder is surrounded by a water mantle kept at 37°C by a circulation thermostat.

After 20 min of rotation, 2 ml was aspirated from the top layer of the suspension (depth ca. 5 mm) without stopping the motor, and the cell concentration in the sample was compared to that of the homogeneous suspension before rotation. Hence only relative cell concentration values were needed. For reasons of convenience and accuracy cell concentrations were substituted by protein concentrations determined by the Lowry et al. (28) method after lysis of the cells by saponin (0.01%). Experiments showed that the degree of cell aggregation was proportional to the decrease of the relative protein concentration (the ratio of the protein concentrations determined after and before rotation), in the top layer of the suspension (25).

Cell Viability

Viability was assessed by the trypan blue exclusion test. HeLa cells taken from the stock spinner culture had a viability of 88–92%. After measurement of aggregation the viability ranged from 76 to 84%. Diminution of viability during rotation of the suspension was about 3%.

Human Erythrocytes

CELL PREPARATION AND NEURAMINIDASE TREATMENT: Fresh human blood treated with EDTA was used. The red blood cell concentration was determined using an electronic particle counter (Celloscope 401, AB Lars Ljungberg & Co, Sweden). After centrifugation the pellets containing approximately 15×10^9 cells were washed three times with 50 ml of NaCl 0.9% and then were suspended in 16 ml of Ringer's solution. 2 ml of acetate buffer solution pH 5.5, 0.05 M containing varying amounts of neuraminidase up to 170 enzyme units, were added. The final pH of the mixture was 7.3. Incubation took place with shaking during 30 min at 37°C. Erythrocytes also were incubated in the absence of neuraminidase in the same circumstances.

After centrifugation, sialic acid assays were performed on the supernates and the pellets were washed twice with NaCl 0.9%.

AGGREGATION MEASUREMENT: The pellets were suspended in 100 ml of the same solution as that used for HeLa cells. Measurement of aggregation was performed exactly as described for HeLa cells except for the introduction of hemoglobin determinations instead of protein determinations for measuring the relative cell concentrations in the top layer of the suspensions. Hemoglobin was assayed by the cyanide method (Merckotest 3317, E. Merck AG, Darmstadt, Germany). The reagent solution of the commercial set was made 1.67 times more concentrated than prescribed. 3.0 ml of this solution were added to 2.0 ml of erythrocyte suspension. A calibration curve was prepared.

RESULTS

Effect of Cell Surface Sialic Acids

For these experiments four different cell batches taken at four different days from the spinner culture were used. Fig. 1 indicates that cell aggregation increases on removal of sialic acid from the cell surface.

Effect of Calcium Ions

From Fig. 1 it can be seen that the extent of aggregation is increased by the addition of calcium, only if sialic acid is not removed by neuraminidase. Fig. 2 demonstrates the influence of varying calcium concentrations on the aggregation of cells preincubated for 15 min either in the absence of enzyme or in the presence of 170 neuraminidase units i.e., approximately 1 enzyme unit per 10^6 cells (see Materials and Methods).

Fig. 3 combines the results of the preceding experiments. The fact that good coincidence exists between the end points of the curves indicates that the measurements are accurate and reproducible.

Adsorbed Neuraminidase

Adsorbed neuraminidase is known to cause a decrease in the net surface negativity especially

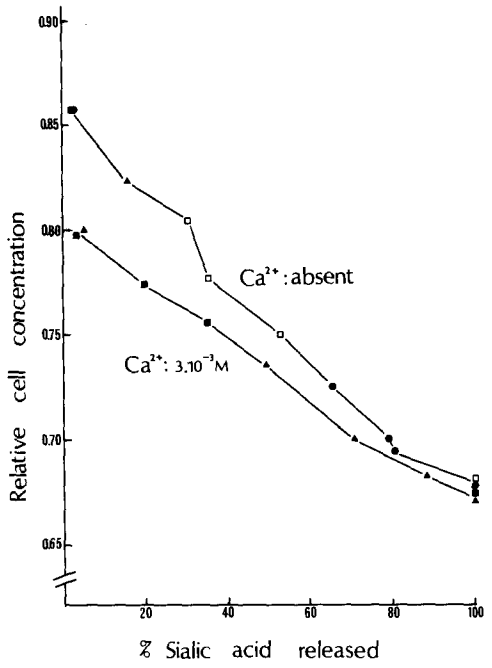

FIGURE 1 Effect of neuraminidase pretreatment on the aggregation of HeLa cells in absence of Ca^{2+} or in presence of 3.0 mM Ca^{2+}. *Ordinate:* Cell concentration in the top layer of the suspension after 20 min of rotation, divided by the homogeneous concentration at time zero. *Abscissa:* amount of sialic acid released by the enzyme, expressed in percent of the maximal amount of sialic acid releasable by neuraminidase. Maximal releasable amounts of sialic acid expressed in nmoles/10^6 cells were: □, 1.10; ●, 1.35; ■, 1.08; ▲, 1.07. Cell concentrations in the homogeneous suspensions: □, 1.86 × 10^6 cells/ml; ●, 2.03 × idem; ■, 1.81 × idem; ▲, 1.77 × idem.

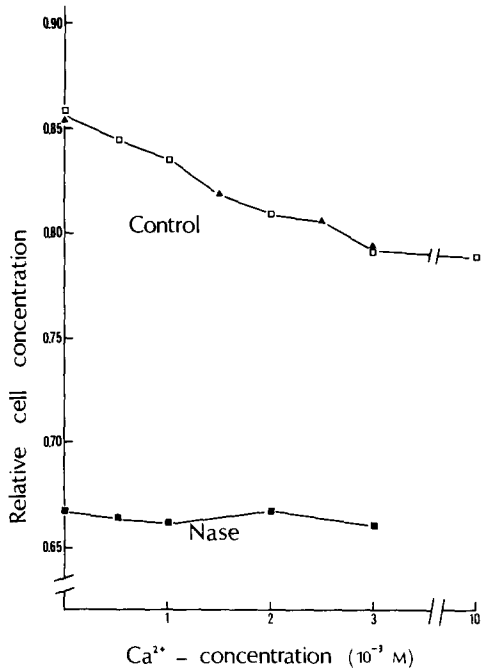

FIGURE 2 Effect of Ca^{2+} on the aggregation of HeLa cells which were either pretreated with 170 neuraminidase units (Nase) or were preincubated in absence of enzyme (Control). *Ordinate:* Same as in Fig. 1. *Abscissa:* Ca^{2+} concentration in the medium. Maximal releasable amounts of sialic acid expressed in nmoles/10^6 cells: □, 1.03; ▲, 1.11; ■, 1.27. Cell concentrations in the homogeneous suspension: □, 1.77 × 10^6 cells/ml; ▲, 1.82 × idem; ■, 1.83 × idem.

when high enzyme concentrations are used (29). Hence, it could be argued that neuraminidase remaining on the cell surface after washing had an influence on the enzyme effect observed. In order to test this, HeLa cells were treated with neuraminidase (170 units/approximately 175 × 10^6 cells) and were washed twice with NaCl 0.9%, as described in Materials and Methods. Sialic acid released was 1.28 nmol/10^6 cells. Two 95-ml suspensions containing equal cell amounts were prepared for measurement of the aggregation rate. To one suspension, 5 ml containing 850 neuraminidase units in acetate buffer were added. The other suspensions was rotated after addition of buffer solution alone. No difference in aggregation rate was observed.

Effect of Magnesium Ions

Addition of magnesium up to a concentration of 3 mM had no effect on the aggregation of intact cells or on cells pretreated with neuraminidase (Fig. 4).

Effect of Pretreatment with Trypsin on the Neuraminidase and Calcium Effects

Cells incubated with 0.01% crude trypsin were clumped together completely after 15 min of incubation. The vial with 0.1% crude trypsin contained dissociated cells together with aggregates. The cells were washed once with NaCl 0.9% and then divided into two portions one of which was subjected to treatment with 170 neuraminidase units. The results of the aggregation measurements (Table I, batch A) show that trypsinization increased the rate of aggregation

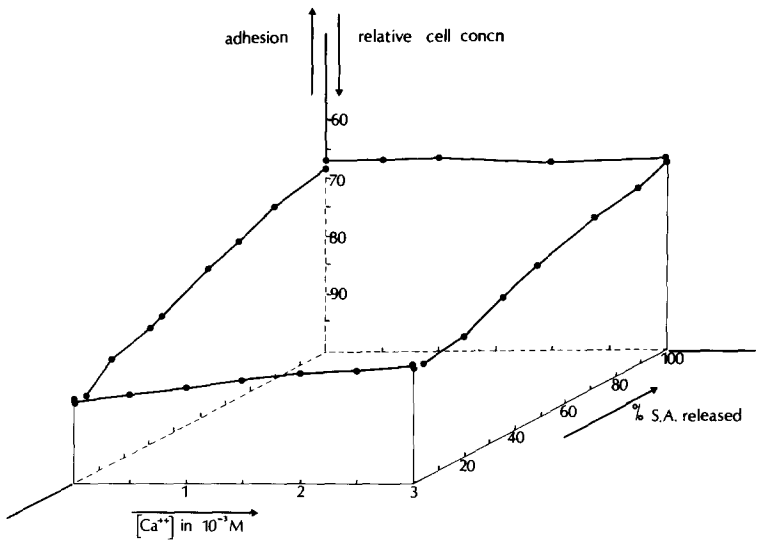

FIGURE 3 HeLa cell aggregation as a function of cell surface sialic acid (S.A.) and concentration of calcium ions. Fig. 3 is a combination of the data from Figs. 1 and 2.

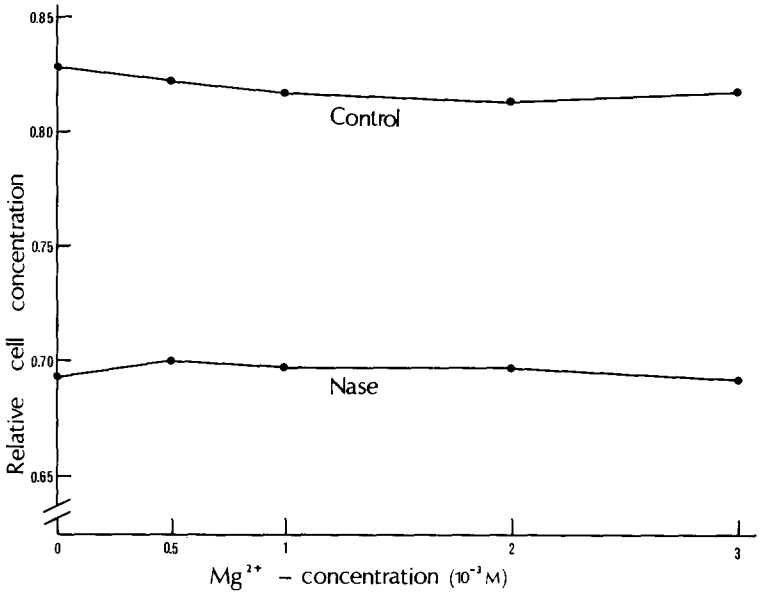

FIGURE 4 Effect of Mg^{2+} on the aggregation of HeLa cells. (See legend of Fig. 2.) Maximal releasable amounts of sialic acid in nmoles/10^6 cells: ● (Nase), 1.12; ● (Control), 1.18. Cell concentrations in the homogeneous suspensions: ● (Nase), 1.85 × 10^6 cells/ml; ● (Control), 1.83 × idem.

of the cells. The small amount of "protein" released after neuraminidase treatment probably must be ascribed to spontaneous release of glycoproteins (30, 31) since similar protein amounts constantly were found also when cells were shaken in buffer solution alone. Subsequent removal of sialic acid by neuraminidase restored the aggregation rate to the original value of cells not treated with the enzymes.

It was suspected that the increased tendency for aggregation was due to incomplete washing out of trypsin-released surface material. The effects of neuraminidase treatment might be due not so much to removal of sialic acid but to the

TABLE I

Effect of Successive Treatment with Crude Trypsin and Neuraminidase on HeLa Cell Aggregation

	Cell batch A					Cell batch B				
	Control	Trypsin 0.1%		Trypsin 0.01%		Control	Trypsin 0.1%		Trypsin 0.01%	
Pretreatment of separate cell portions	I	II (a)	III (a) + (b)	IV (a)	V (a) + (b)	I	II (a)	III (a+b)	IV (a)	V (a)+(b)
(a) Cells: + trypsin for 15 min										
Supernate:										
(1) Proteins/10^6 cells*		0.016	0.016	0.011	0.012		0.028	0.030	0.072	0.076
(2) nmol S.A./10^6 cells‡ after 30-min treatment with 170 Nase units		0.156	0.279	0.105	0.197		0.250	0.244	0.353	0.256
Pellet: number of washings		1	1	1	1		3	3	3	3
(b) Resuspended cells: + 170 Nase units for 30 min										
Supernate:										
(3) Proteins/10^6 cells			0.010		0.009			0.008		0.008
(4) nmol S.A./10^6 cells			0.527		0.549			0.611		0.579
Pellet: number of washings		0	2	0	2		0	2	0	2
(5) (2)+(4) as percent of the max Nase-releasable amount (0.782 resp. 0.853 nmol S.A./10^6 cells for batches A and B)§		19.9	103.0	13.4	95.5		29.3	100.2	41.4	98.0
Aggregation rate										
Cell concn of homogeneous suspension (10^6 cells/ml)	1.84	1.85	1.85	2.02	1.96	2.34	2.48	2.37	2.26	2.40
(6) Proteins(lysis): homogeneous suspension	0.421	0.400	0.400	0.438	0.425	0.503	0.535	0.509	0.482	0.491
(7) Proteins(lysis): top layer after 20-min rotation	0.365	0.323	0.347	0.335	0.371	0.433	0.478	0.435	0.435	0.420
Relative cell concn. (7:6)	0.867	0.807	0.868	0.765	0.873	0.855	0.893	0.854	0.903	0.855

The cells from batch A were washed once after trypsin treatment whereas those of batch B were washed three times.

*Measured as "protein" by the Lowry et al. (28) method and expressed as milligrams albumin/10^6 cells.

‡S.A., sialic acid; Nase, neuraminidase.

§Determined in separate experiments.

fact that two additional washings were intercalated in this case in order to remove the second enzyme. In fact, an opposite trypsin effect was observed if the cells were washed three times after incubation with trypsin. Subsequent exposure to neuraminidase again resulted in an aggregation rate indistinguishable from that of the control cells which underwent no enzyme treatment (Table I, batch B).

Fig. 5 demonstrates that the effect of calcium is nearly abolished after treatment of the cells with crystalline trypsin. Trypsinization followed by neuraminidase treatment leads to the complete disappearance of the calcium effect. The cells were washed four times after trypsinization. Fig. 5 also shows the relative cell concentration before trypsin treatment. In the absence of subsequent incubation with neuraminidase, tryp-

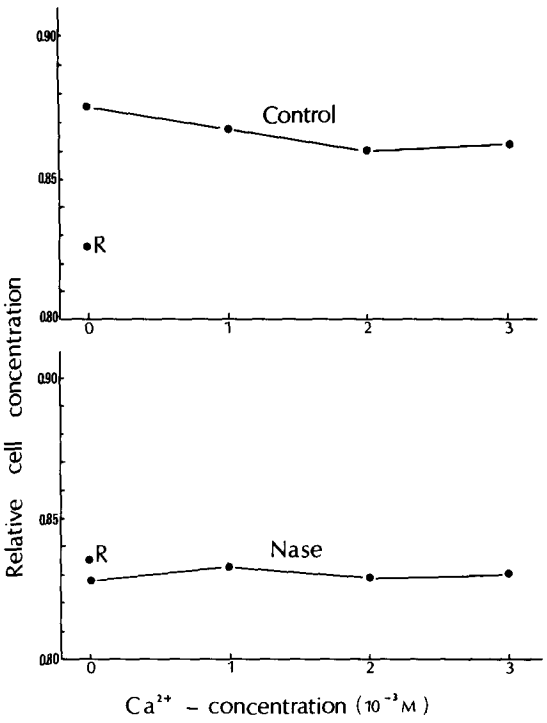

FIGURE 5 Effect of Ca^{2+} on cells pretreated with 0.1% crystalline trypsin. Trypsinized cells were either treated with 170 neuraminidase units (Nase) or were incubated in absence of enzyme (Control). R: intact cells, not treated with enzymes. Coordinates as in Fig. 2. Maximal releasable amounts of sialic in nmoles/10^6 cells: ● (Nase), 0.96; ● (Control), 1.15. Cell concentrations in the homogeneous suspensions: ● (Nase), 1.63 × 10^6 cells/ml; ● (Control), 1.55 × ID.

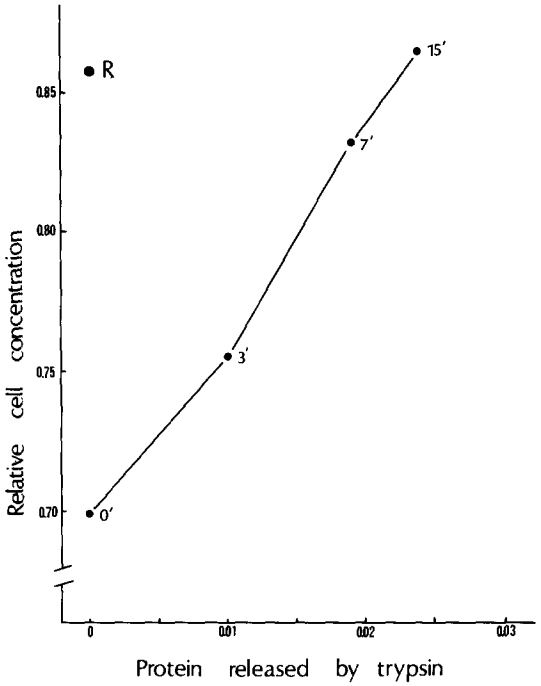

FIGURE 6 Aggregation after treatment with 0.1% crystalline trypsin of cells which previously had been incubated with 170 neuraminidase units. R: intact cells, not treated with enzymes. *Abscissa:* material released in supernate on trypsinization, measured as "protein" by the Lowry et al. (28) method and expressed as milligrams albumin/10^6 cells. In parentheses, time in minutes of exposure to trypsin. Sialic acid released by neuraminidase: 1.16 nmol/10^6 cells = maximal releasable amount. Cell concentration in the homogeneous suspension: 1.60 × 10^6 cells/ml.

sinized cells show a decreased aggregation rate. Trypsinization followed by incubation with neuraminidase yielded an aggregation rate which was almost indentical to that of nonenzyme-treated cells.

Trypsin Treatment of Cells Previously Incubated with Neuraminidase

HeLa cells preincubated with neuraminidase were exposed to the action of crystalline trypsin solutions for different periods. The cells were washed four times with NaCl 0.9%. Fig. 6 shows that the increased adhesiveness after neuraminidase treatment is progressively abolished with increasing amount of surface material released by trypsin.

Aggregation of Human Erythrocytes

There was no indication of an influence of sialic acid removal on the aggregation of erythrocytes. Measurements were performed in the absence of calcium or in the presence of 3 mM calcium (Table II). Table III led us to conclude that calcium ions did not significantly enhance the aggregative tendency of the erythrocytes.

In general, measurements with erythrocytes were not so accurate as was the case with HeLa cells. Also, duplicate experiments show that different lots of erythrocytes displayed different relative cell concentrations in the top layer of the suspension.

DISCUSSION

Before going into a discussion of a possible mechanism, we wish to examine the molecular basis

TABLE II

Effect of Sialic Acid Removal on the Aggregation of Human Erythrocytes

	Sialic acid removed	Relative cell concn in top layer
	nmol/10⁹ cells	
In the absence of calcium		
Exp. 1. 131 × 10^6 erythrocytes/ml of homogeneous suspension	0.7	0.951
	11.3	0.953
	24.3	0.954
	49.3	0.945
	44.6	0.943
	68.7	0.949
Exp. 2. 112 × 10^6 erythrocytes/ml of homogeneous suspension	0.9	0.920
	23.2	0.922
	38.1	0.912
	55.0	0.919
	49.2	0.920
	67.1	0.895
In presence of 3 mM calcium		
165 × 10^6 erythrocytes/ml of homogeneous suspension	1.6	0.891
	1.6	0.890
	26.1	0.893
	42.9	0.892
	60.0	0.893

TABLE III

Effect of Calcium Ions on the Aggregation of Human Erythrocytes not Pretreated with Neuraminidase

	Ca concn	Relative cell concn in top layer
	mmol	
Exp. 1. 143 × 10^6 erythrocytes/ml of homogeneous suspension	0	0.946
	0.75	0.958
	1.50	0.952
	2.50	0.956
	3.50	0.945
Exp. 2. 139 × 10^6 erythrocytes/ml of homogeneous suspension	0	0.886
	0.50	0.895
	1.00	0.911
	2.00	0.899
	3.00	0.918

for the main effects of neuraminidase, trypsin, and calcium on the rate of mutual adhesion of HeLa cells. These effects are: (*a*) After neuraminidase, adhesion rate is increased (Fig. 1). (*b*) After trypsin, adhesion rate is decreased (Table I, batch B; Fig. 6). (*c*) The presence of calcium ions increases the adhesion rate of cells not pretreated with neuraminidase (Fig. 2).

In comment to (*a*) we mention that Nordling and Mayhew (32) showed that neuraminidase was taken up intracellularly by Roswell Park Memorial Institute (RPMI) no. 41 cells after 15–20 min of incubation. Sialic acid was liberated from internal organelles. Although it was suggested (33) that penetration also occurred in L cells, evidence indicates that this does not necessarily apply to other cell types (34, 35).

In view of the short incubation time (15 min) with neuraminidase and the preferential localization of sialic acid in the plasma membranes of HeLa cells (36) it seems highly probable that most of the sialic acid found by us in the supernatant solution originated from the cell surface. Another argument in support of this, is the abolishment of the calcium effect after neuraminidase treatment, and the disappearance of the neuraminidase effect by subsequent treatment of the HeLa cells with trypsin. Because it may be assumed that the principal action of calcium and trypsin must be situated at the cell periphery, these results clearly indicate involvement of cell surface sialic acid in cell aggregation. A logical conclusion is that the chief effect of neuraminidase must be ascribed to the release of peripheral sialic acid residues.

Concerning (*b*), there are strong indications that the presence of complex carbohydrates at the cell periphery is necessary for intercellular adhesion to occur. It was shown that addition of L-glutamine to the growth medium was necessary for adhesion in a variety of cell types (37, 38). The L-glutamine requirement appears to stem from its participation in the formation of aminosugars which subsequently are incorporated into certain complex carbohydrates. It was mentioned earlier that trypsin causes release of glycopeptides. Proteins of erythrocyte membranes are not accessible to the action of trypsin (21). Trypsin seems to merely enhance the process of spontaneous release of glycoproteins from the surfaces of embryonic chick cells (31). Glycolipids remain firmly bound after trypsin treatment (39). Sialic acid moieties are resistant to release from glycolipids by neuraminidase (40, 41). Glycolipid sialic acid in HeLa cells represents but one-tenth of the amount of sialic acid bound to glycoproteins (42). The latter remarks are relevant in view of the connection found by us between the neuraminidase and trypsin effects. For the above reasons we consider it very probable that the

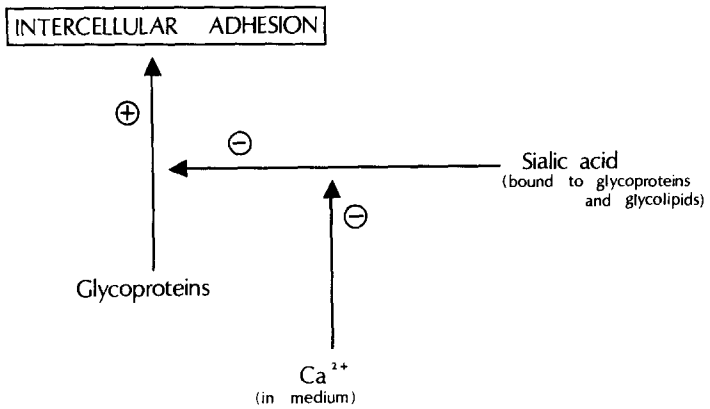

FIGURE 7 The sites of action of the various factors influencing intercellular adhesion. Arrow: regulatory effect. ⊕, increase; ⊖, decrease.

present results must be interpreted by the assumption that glycoproteins and not glycolipids were involved.

As for (*c*), evidence for a direct interaction of calcium ions with cell surface sialic acids comes from experiments with erythrocytes (43) and liver plasma membranes (44), which indicated that a considerable portion of membrane-bound calcium is actually bound to sialic acid residues. It was reported (45) that dissociation of HeLa cells with EDTA became more difficult after previous neuraminidase treatment.

Support for an adhesive role of sialoglycoproteins comes from our viscosimetric experiments (46) performed on concentrated mucin solutions, the main organic components of which are glycoproteins. Viscosity was considered to be due in a large part to intermolecular interaction between glycoproteins which could be the formation of associative bonds or physical entanglement of the molecular network. The effects of neuraminidase and calcium were interrelated in the same way as described in the present paper.

It should be appreciated that our results cannot be explained by the assumption that proteins are released during rotation of the suspension. Release of proteins would lead to an increase of the protein ratio determined after lysis of the samples and therefore would decrease the magnitude of the effects.

Other results of our experiments are: (*d*) A larger neuraminidase effect is obtained with intact cells than with trypsin-treated cells (Fig. 1, Table I, batch B). (*e*) There is a strong trypsin effect after previous removal of sialic acid (Fig. 6). On intact cells the trypsin effect is small (Table I, batch B). (*f*) The calcium effect is abolished after removal of surface sialic acid (Fig. 2).

The diagram (Fig. 7) illustrates the essence of our working hypothesis. It is assumed that calcium ions counteract the effect of sialic acid and that sialic acids counteract the adhesive effect of the glycoprotein molecules. The relationship between calcium and sialic acid is based on the fact that whereas the presence of surface-bound sialic acid is necessary for the calcium effect to occur, the reverse is not true: calcium ions are not needed for the effect of sialic acid removal. Trypsin treatment is assumed to influence the calcium effect indirectly (see Fig. 5) because it degrades the molecules which serve as the sites of action for the sialic acids.

In the case of the glycoprotein–sialic acid relationship, it is realized that the experiments do not prove unequivocally that sialic acids exert their effect in an indirect way i.e., by modification of the adhesive properties of membrane glycoproteins as illustrated in Fig. 7. Support for the *indirect* effect comes from our results summarized above in points (*d*) and (*e*). The residual neuraminidase effect on cells previously treated with trypsin can be plausibly explained by the assumption that trypsin might not have removed all adhesive material from the cell periphery. However, because actually we were not able to prove this, account has to be taken of the possibility that sialic acids might have a *direct* effect on intercellular adhesion. Such effect seems only possible if it is assumed that negatively charged carboxyls from sialic acid contribute to the formation of a potential energy barrier (47, 1) counteracting close approach between cells. Armstrong

(48) found that there was no direct relation between cell surface charge and adhesion rate. Whereas arguments exist for the assumption that sialic acid charges on erythrocytes largely are responsible for the existence of the long-range electrostatic repulsive forces between these cells (49, 50), there are indications which imply a more intricate mechanism with other cell types. Kojima and Maekawa (51, 52) found no reduction in the surface charge of island-forming strains of rat ascites hepatoma cells after neuraminidase treatment. By contrast, a considerable reduction was obtained with free cell-type strain cells. The same observation was made with other cell types such as solid and ascites forms of sarcoma 37 cells (53) and solid and ascites forms of MC_1M_{ss} cells (54). Neuraminidase only reduced the surface charge of the ascites cells. In all cases mentioned, comparable amounts of sialic acids were released from the adhesive and the nonadhesive cell types. These findings suggest the possibility of a transition from exposed sialic acids (contributing to the net surface charge) into sialic acids in cryptic positions. Neuraminidase is supposed to release exposed and cryptic residues at approximately the same rate.

Other considerations corroborate the thesis that the ratio of exposed to cryptic sialic acids might be an important factor for adhesive cell behavior. Taking a value of 1.0 nmol sialic acid/10^6 HeLa cells, and assuming that intracellular release of sialic acid by neuraminidase is negligible, we calculate that approximately 600 $\times$ 10^6 residues must be present at the surface of a single HeLa cell. Since the total surface charge of HeLa cells was found (55) to be 3.9 $\times$ 10^6 electronic charges per cell of which only 45% can be ascribed to sialic acid charges (44), it follows that less than 0.4% of the total number of surface sialic acids are in an exposed position. This situation on HeLa cells stands in strong contrast to that on erythrocytes, a typical nonadhesive cell type. The surface charge of human erythrocytes was found to be nearly completely due to *N*-acetylneuraminic acid, and cryptic and exposed sialic acids are present in approximately equal amounts (56). Despite these facts, no increase in erythrocyte aggregation after neuraminidase treatment was observed in the present investigation. Absence of a neuraminidase effect cannot be ascribed to a different type of sialic acid bound on erythrocytes as compared with HeLa cells, since it was established (57) that also in HeLa cells only *N*-acetylneuraminic acid is present.

With regard to the potential-energy barrier hypothesis, it seems paradoxical that neuraminidase strongly promotes aggregation of cells on which almost all sialic acids are in a cryptic position, whereas the enzyme has no effect on cells with a large percent of exposed sialic acid residues. Other arguments against involvement of sialic acid in long-range electrostatic repulsion have been presented by Vicker and Edwards (9).

One possible explanation is that the presence of charged sialic acid residues may have an influence on the adhesive properties of glycoproteins. Due to mutual repulsion between the carboxyl charges, sialic acid residues may confer rigidity to the molecular shape of glycoproteins (58) and therefore are likely to impair adhesive pairing between peripheral glycoproteins of adjacent cell surfaces. Also repulsion between sialic acid residues located on different glycoproteins may induce a configurational change resulting in an impaired contact between adhesive sites on these macromolecules. The phenomenon can be ascribed to short-range electrostatic forces in distinction to the long-range electrostatic forces mentioned above. Mutual association between glycoproteins on the same cell surface will impair their reactivity towards glycoproteins of adjacent cells. Therefore, for the mechanism to be fully operative in mutual cell adhesion it must be considered advantageous that glycoproteins on the same cell surface are spatially isolated from each other. Evidence for this comes from the work of Capaldi (59).

A high concentration of sialic acids at the outer cell periphery (exposed position) will be most effective in counteracting the formation of adhesive bonds on first contact between cells. The strength of intercellular adhesion might be regulated by the extent to which sialic acid residues pass from an exposed into a cryptic position. Hence, adhesivity can become progressively stronger after instalment of the first contacts because exposed sialic acids are displaced to other sites by short-range electrostatic repulsion. A hint that calcium ions possibly are involved in this process comes from the observation (60) that instalment of adhesive contacts between HeLa cells in the presence of calcium ions, is accompanied by

the disappearance of the binding affinity of Hale's stain towards surface sialic acids.

A comparison between the effects of different cations on intercellular adhesion (48, 61) made it highly improbable that the action of calcium could be explained as a lowering of the zeta potential at the cell surface. Phospholipids and sialic acid residues were found to be the most important if not exclusive binding sites for calcium in liver plasma membranes (44). There are indications (62, 63) that calcium ions might mediate binding between the polar groups of phospholipids and the sialic acid groups of glycoproteins. Such binding implies conversion of exposed sialic acids into cryptic positions. Other possible roles of calcium leading to the same issue are neutralization of fixed charges in the deeper regions of the cell coat and enhancement of the degree of cross-linking between polycarboxylic substances (64).

We realize that the proposed schema is far from complete and that e.g., the nature of the adhesive bonds between glycoproteins has been left open. The interpretation of the results, given by us, seems to fit better into the theory advanced by Parsegian and Gingell (65) than into the hypothesis of Roseman (2) in which a glycosyltransferase-substrate binding was proposed. In the former theory which in fact is an extension of the lyophobic colloid theory (66), (a-specific) electrodynamic attractive forces between sugar residues, and electrostatic repulsive forces between fixed charges are thought to be the factors governing intercellular adhesion. The strength of electrostatic repulsion is assumed to be regulated chiefly by the thickness of the glycoprotein coat and by the extent to which mobile counterions neutralize the fixed charges of the coat.

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REFERENCES

1. CURTIS, A. S. G. 1962. *Biol. Rev. (Camb.).* **37**:82.
2. ROSEMAN, S. 1970. *Chem. Phys. Lipids.* **5**:270.
3. COOK, G. M. W. 1968. *Biol. Rev. (Camb.).* **43**:363.
4. RUHENSTROTH-BAUER, G., G. F. FUHRMANN, E. GRANZER, W. KUBLER, and F. RUEFF. 1962. *Naturwissenschaften.* **49**:363.
5. FORRESTER, J. A., E. J. AMBROSE, and J. A. MACPHERSON. 1962. *Nature (Lond.).* **196**:1068.
6. FORRESTER, J. A., E. J. AMBROSE, and M. G. P. STOKER. 1964. *Nature (Lond.).* **201**: 945.
7. VASUDEVAN, D. M., K. BALAKRISHNAN, and G. P. TALWAR. 1970. *Int. J. Cancer.* **6**:506.
8. SAUTER, C., J. LINDENMANN, and A. GERBER. 1972. *Eur. J. Cancer.* **8**:451.
9. VICKER, M. G., and J. G. EWDARDS. 1972. *J. Cell Sci.* **10**:759.
10. KEMP, R. B. 1968. *Nature (Lond.).* **218**:1255.
11. KEMP, R. B. 1970. *J. Cell Sci.* **6**:751.
12. COLLINS, M. F., K. D. HOLLAND, and R. SANCHEZ. *J. Exp. Zool.* **183**:217.
13. EASTY, G. C., D. M. EASTY, and E. J. AMBROSE. 1970. *Exp. Cell Res.* **19**:539.
14. STEINBERG, M. S. 1963. *Exp. Cell Res.* **30**:257.
15. MOSCONA, A. 1952. *Exp. Cell Res.* **3**:535.
16. COOK, G. M. W., D. H. HEARD, and G. V. F. SEAMAN. 1960. *Nature (Lond.).* **188**:1011.
17. LANGLEY, O. K., and E. J. AMBROSE. 1964. *Nature (Lond.).* **204**:53.
18. KORNFELD, S., and R. KORNFELD. 1969. *Proc. Natl. Acad. Sci. U. S. A.* **63**:1439.
19. CODINGTON, J. F., B. H. SANFORD, and R. W. JEANLOZ. 1970. *J. Natl. Cancer Inst.* **45**:637.
20. BUCK, C. A., M. C. GLICK, and L. WARREN. 1970. *Biochemistry.* **9**:4567.
21. HUBBARD, A. L., and Z. A. COHN. 1972. *J. Cell Biol.* **55**:390.
22. SANTER, V., R. E. CONE, and J. J. MARCHALONIS. 1973. *Exp. Cell Res.* **79**:404.
23. PHILIPS, D. R. 1972. *Biochemistry.* **11**:4582.
24. TILLACK, T. W., R. E. SCOTT, and V. T. MARCHESI. 1971. Abstracts of Papers, 11th Annual Meeting. The American Society for Cell Biology. 305.
25. DEMAN, J., and E. BRUYNEEL. 1973. *Exp. Cell Res.* **81**:351.
26. MILLER, A. O. A. 1967. *Arch. Biochem. Biophys.* **122**:270.
27. WARREN, L. 1959. *J. Biol. Chem.* **234**:1971.
28. LOWRY, O. H., N. J. ROSEBOROUGH, A. L. FARR, and R. J. RANDALL. 1951. *J. Biol. Chem.* **193**:265.
29. WEISS, L. 1973. *J. Natl. Cancer Inst.* **50**:3.
30. HUGHES, R. C., B. SANFORD, and R. W. JEANLOZ. 1972. *Proc. Natl. Acad. Sci. U. S. A.* **69**:942.
31. KAPELLER, M., R. GAL-OZ, N. B. GROVER, and R. DOLJANSKI. 1973. *Exp. Cell Res.* **79**:152.
32. NORDLING, S., and E. MAYHEW. 1966. *Exp. Cell. Res.* **44**:552.
33. GLICK, M. C., C. COMSTOCK, and L. WARREN. 1970. *Biochim. Biophys. Acta.* **219**:290.

34. RAY, P. K., and R. L. SIMMONS. 1973. *Cancer Res.* **33**:936.
35. MARCUS, P. A. J. M. SALB, and V. G. SCHWARTZ. 1965. *Nature (Lond.).* **208**:1122.
36. BOSMANN, H. B., A. HAGOPIAN, and E. H. EYLAR. 1968. *Arch. Biochem. Biophys.* **128**:51.
37. OPPENHEIMER, S. B., M. EDIDIN, C. W. ORR, and S. ROSEMAN. 1969. *Proc. Natl. Acad. Sci. U. S. A.* **63**:1395.
38. OPPENHEIMER, S. B. 1973. *Exp. Cell Res.* **77**:175.
39. HAKOMORI, S., C. TEATHER, and H. ANDREWS. 1968. *Biochem. Biophys. Res. Commun.* **33**:563.
40. KLENK, E. 1958. *In* The Chemistry and Biology of Mucopolysaccharides. G. E. Wolstenholme and M. O'Connor, editors. J. & A. Churchill Ltd., London. 296.
41. WEINSTEIN, D. B., J. B. MARSH, and M. C. GLICK. 1970. *J. Biol. Chem.* **245**:3928.
42. TU, S., R. E. NORDQUIST, and M. J. GRIFFIN. 1972. *Biochim. Biophys. Acta.* **290**:92.
43. LONG, G., and B. MOUAT. 1971. *Biochem. J.* **123**:829.
44. SHLATZ, L., and G. V. MARINETTI. 1972. *Biochim. Biophys. Acta.* **290**:70.
45. RUHENSTROTH-BAUER, G., G. F. FUHRMANN, W. KUBLER, F. RUEFF, and K. MUNK. 1962. *Z. Krebsforsch.* **65**:37.
46. DEMAN, J., M. MAREEL, and E. BRUYNEEL. 1973. *Biochim. Biophys. Acta.* **297**:486.
47. PETHICA, B. A. 1961. *Exp. Cell Res.* **8** (*Suppl.*): 123.
48. ARMSTRONG, P. B. 1966. *J. Exp. Zool.* **163**:99.
49. BROOKS, D. E., and G. V. F. SEAMAN. 1972. *Nature (Lond.).* **238**:251.
50. JAN, K., and S. CHIEN. 1973. *J. Gen. Physiol.* **61**:638.
51. KOJIMA, K., and A. MAEKAWA. 1970. *Cancer Res.* **30**:2858.
52. KOJIMA, K., and A. MAEKAWA. 1972. *Cancer Res.* **32**:847.
53. COOK, G. M. W., G. V. F. SEAMAN, and L. WEISS. 1963. *Cancer Res.* **23**:1813.
54. WALLACH, D. F. H., and M. V. DE PEREZ ESANDI. 1964. *Biochim. Biophys. Acta.* **83**:363.
55. SHERBET, G. V., M. S. LAKSHMI, and K. V. RAO. 1972. *Exp. Cell Res.* **70**:113.
56. EYLAR, E. H., M. A. MADOFF, O. V. BRODY, and J. L. ONCLEY. 1962. *J. Biol. Chem.* **237**: 1992.
57. HOF, L., and H. FAILLARD. 1973. *Biochim. Biophys. Acta.* **297**:561.
58. GOTTSCHALK, A. 1960. *Nature (Lond.).* **186**:949.
59. CAPALDI, R. A. 1973. *Biochem. Biophys. Res. Commun.* **50**:656.
60. MAREEL, M., L. DE RIDDER, and J. DEMAN. 1972. *Histochemie.* **32**:335.
61. MODJANOVA, E. A., and A. G. MALENKOV. 1973. *Exp. Cell Res.* **76**:305.
62. DEMAN, J., P. M. VAN VAERENBERGH, and P. JOOS. 1971. *Eur. J. Cancer.* **7**:317.
63. TOSTESON, M. T., F. LAU, and D. C. TOSTESON. 1973. *Nature (Lond.).* **243**:112.
64. HORVATH, C., and M. SOVAK. 1973. *Biochim. Biophys. Acta.* **298**:850.
65. PARSEGIAN, V. A., and D. GINGELL. 1972. *J. Adhesion.* **4**:283.
66. CURTIS, A. S. G. 1967. The Cell Surface: Its molecular role in morphogenesis. Academic Press Inc. Ltd., London.