INTERCELLULAR ADHESIVENESS AND NEURAMINIDASE EFFECT FOLLOWING RELEASE FROM DENSITY INHIBITION OF CELL GROWTH

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Summary: Mutual cell adhesion increases when HeLa cells growing in suspension culture are released from density inhibition of growth. Neuraminidase treatment considerably enhances the adhesiveness of density inhibited cells but produces only a small effect on cells at low density.

Removal of cell surface sialic acids by neuraminidase has been shown (1) to increase the mutual adhesiveness of HeLa cells which were harvested from stationary suspension cultures. Bound sialic acids were assumed to counteract the adhesive properties of surface macromolecules.

Here we describe measurements on the aggregation rate of HeLa cells which were harvested at different densities from asynchronously growing suspension cultures.

Experimental

The cells were grown in spinner vessels using modified (2) Basal Medium Eagle supplemented with 10 % calf's serum. The spinner vessels had a volume of 2 liter and contained 600 ml of suspension. Rotation was at 110 r.p.m.

The occurrence of density inhibition of growth was established in the following manner. Cells taken from dense cultures (approximately 2.0×10^6 cells/ml) were centrifuged and the pellets were resuspended at various densities in 400 ml fresh BME medium unto which 200 ml medium was added in which cells had already grown.

After 24 hr of culture the increase in cell density was determined. Evaluation occurred by repeated countings in a Bürker hemacytometer. Figure 1 indicates that there was a significant inhibition of cell growth above cell densities of 1.8 x 10^6 cells/ml.

In another experiment (Figure 2), a suspension containing 1.9 x 10^6 cells/ml was diluted to 0.5 x 10^6 cells/ml by addition of fresh BME medium. This low density suspension was distributed among three spinner vessels which afterwards were harvested after respectively 4 hr, 24 hr and 48 hr. After harvest each suspension was washed three times with physiological saline at 4°C and then was divided into two portions one of which was treated with α-neuraminidase from Vibrio Cholerae for 15 min at 37°C (purchased from Serva, Heidelberg, Germany). One enzyme unit was used per 10^6 cells. The incubation medium and the further preparations of the suspensions were the same as those described elsewhere (1). The conditions of the incubation were such that maximal liberation of the enzyme-releasable sialic acid was assured. Released sialic acid was determined by the Warren method (4) with a slight modification (3). Measurement of the aggregation rate took place on cells suspended in a Tris-Saline-Phosphate (T.S.P.) buffer at pH 7.5 and a temperature of 37°C. The method has been published in detail (3,1).

For the measurement of the electrophoretic cell mobility the washed cells were suspended in the same T.S.P. buffer as that used for the measurement of the aggregation rate. The method of Bangham et al. (5) was followed. Use was made of a cylindrical microelectrophoresis apparatus (Apparatus Mk II, Rank Brothers, Bottisham, England). Measurements were carried out at 25°C at 4V per cm. The direction of the current was reversed after each determination. The mean mobility was calculated from thirty different determinations.

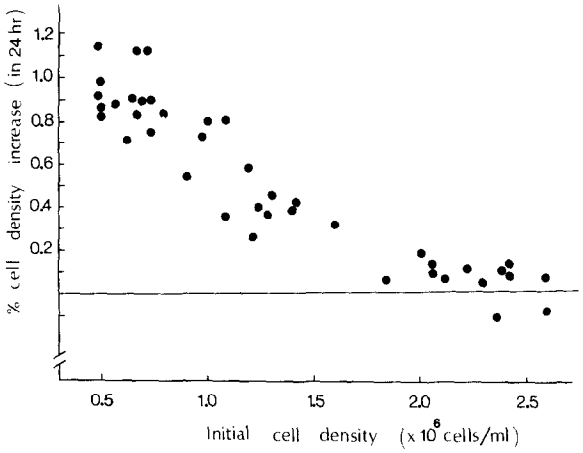

Figure 1. Growth of HeLa cells in spinner culture as a function of the initial cell density at 0 hr. In abscissa the initial cell density is expressed as 10^6 cells per ml of medium. The ordinate shows the percentage increase in cell density after 24 hr of culture i.e. $(\text{cell concn})_{24\ hr} - (\text{cell concn})_{0\ hr}/(\text{cell concn})_{0\ hr}$.

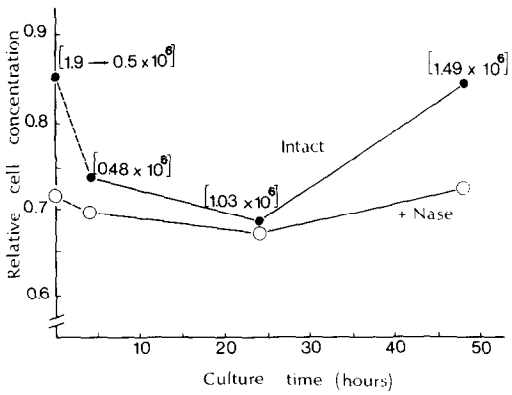

Figure 2. Measurement of the aggregation rate of HeLa cells which were harvested after different culture times (abscissa), after dilution to low density at 0 hr. The relative cell concentration (ordinate) in the top layer of a suspension rotated for 20 min is inversely related to the extent of aggregation (3). Between brackets : cell densities in the culture vessels. Nase : neuraminidase.

The cell viability at harvest was determined by the trypan blue exclusion test. The number of cells in mitosis was assessed by the crystal violet method (6). Counts were performed on 2.000 cells.

Results and Discussion

As already mentioned the occurrence of density dependent inhibition of cell growth can be inferred from Figure 1.

In Figure 2 the high relative cell concentration at 0 hr is indicative for the low adhesiveness of the high density cells with which the experiment was started. The adhesiveness was found to be increased at 4 hr and 24 hr of culture i.e. during the period of fast growth. During the second day the growth rate diminished. After 48 hr the cell density had increased considerably and the cells had become less adhesive. Desialylated cells displayed a higher aggregation rate than intact cells. The most pronounced neuraminidase effect was found with low adhesivity cells i.e. with high density cells at 0 hr and 48 hr of culture. The effect of neuraminidase, represented by the distance between both curves diminished during rapid growth and was nearly absent after 24 hr of culture.

Table I shows that there was no great variation between the sialic acid amounts which were released by neuraminidase at the different times of harvest. The amount found with high density cells represented about 85 % of the amount which could be liberated by treatment of the cells with 0.1 NH_2SO_4 at 80°C for 1 hr.
It has been demonstrated that neuraminidase from Vibrio Cholerae releases sialic acids from glycoproteins at the cell surface but does not attack sialoglycolipids (7,8). In HeLa cells the glycolipid bound sialic acid was found to represent about one tenth of the sialic acid amount bound to glycoproteins (9).

Measurements of the electrophoretic cell mobility give evidence for a small mobility increase following release from density inhibition of growth. (Table I). The reduction in mobility caused by neuraminidase, remained considerable during the first day of cul-

TABLE I

Additional information on the experiment described in Figure 1

	Culture time			
	0 hr	4 hr	24 hr	48 hr
n Mol sialic acid[a] per 10^6 cells	0.82	0.75	0.78	0.84
Electrophoretic mobility ($\mu m\ sec^{-1} V^{-1}\ cm$) 25°C				
1) Intact cells	0.91	0.98	1.08	0.97
(s.d.)	(0.14)	(0.12)	(0.15)	(0.17)
2) Desialylated cells[b]	0.64	0.74	0.72	0.71
(s.d.)	(0.15)	(0.13)	(0.17)	(0.10)
Cell viability at harvest	95 %	94 %	95 %	95 %
Cells in mitosis at harvest	3.9 %	5.2 %	4.0 %	2.9 %

a. maximal amounts of sialic acid releasable by neuraminidase.
b. cells from which the neuraminidase-sensitive sialic acids were removed.

ture indicating that sialic acids did not disappear from the electrokinetic shear plane. These results confirm earlier findings on other cell types (10,11,12). A high cell viability was found at all times of harvest indicating that the decreased adhesiveness of dense cells was not due to dead or dying cells.

The method used for the measurement of the aggregation rate is based on the circumstance that the sedimentation rate of particles (cells and aggregates) augments with greater size. Microscopic examination against a calibrated grid showed that there was no significant variation in mean cell diameter due to neura-

minidase treatment. Therefore it appears highly probable that the enzyme effect observed in the experiment should be ascribed to an increase in intercellular adhesiveness.

The existence of a relation between adhesion and inhibition of cell growth has already been reported for monolayer cultures. Cells harvested from subconfluent cultures with E.D.T.A. were found to be more adhesive towards each other than cells from confluent monolayers (13). The present study provides evidence for a similar phenomenon occurring with suspension cultures. It indicates that sialic acids are involved in the regulation of mutual cell adhesion. As a possible mechanism we suggest that the increase in adhesiveness after release from density inhibition of growth is caused by a changed distribution of the sialic acids below the electrokinetic shear plane. By such interpretation a function is ascribed to those sialic acids which do not contribute to the net surface charge. In HeLa cells the latter condition is fulfilled by more than 99 % of the total cell surface sialic acids (1).

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