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Cell Size and Mutual Cell Adhesion

II. Evidence for a Relation Between Cell Size, Long-Range Electrostatic Repulsion and Intercellular Adhesiveness During Density-Regulated Growth in Suspension

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Summary. The strength of the long-range electrostatic repulsion forces on HeLa cells is measured by agglutinative titration using low molecular weight polylysine (M.W. 11,000). Repulsion forces, found to be present on the smaller HeLa cells from density-inhibited suspension cultures, are weakened by incubation of the cells in hypotonic NaCl solutions. Repulsion forces, found to be absent on the larger cells from fast growing cultures, can be induced on these cells by incubation in hypertonic NaCl solutions. Both effects of anisotonicity are reversible, and disappear on restoration of the medium to normal tonicity. Induction of repulsion forces on fast growing cells is prevented by previous treatment of the cells with neuraminidase. Neuraminidase also abolishes repulsion on density-inhibited cells. It is proposed that alterations of the cell size, produced by anisotonicity or occurring during growth in isotonic suspension medium, affect mutual cell adhesiveness by modifying the strength of the repulsion forces generated by cell surface sialic acids.

Good evidence for the existence of long-range electrostatic repulsion (LRER) forces on cells, has been obtained recently with erythrocytes, a typical nonadhesive cell type (Brooks & Seaman, 1972; Jan & Chien, 1973). For adhesive cells, such as tissue cells, available evidence was less direct, although there are many indications that LRER forces exist and might be a controlling factor in intercellular adhesion (Pethica, 1961; Curtis, 1973; Jones, 1974).

In an earlier paper (Deman & Bruyneel, 1974), we introduced a general method based on agglutinative titration of suspended cells with low molecular weight poly-L-lysine molecules. Strong arguments were presented which indicated that LRER forces were the cause for inhibition of agglutination at low polylysine concentration. When applied to HeLa cells, LRER forces were found to be present on cells harvested from suspension cultures in which growth was density-inhibited (DI). The

repulsion forces were weaker on cells in moderate growth, and could not be detected on fast growing (FG) cells. Evidence that modulation of the repulsive force might regulate the strength of mutual cell adhesion came from the inverse relationship between LRER force and intercellular adhesiveness, observed at the different stages of cell growth. Also, desialylation of DI cells abolished the LRER forces, and enhanced intercellular adhesiveness towards a value approximately equal to that of FG cells (Deman & Bruyneel, 1975).

In the accompanying paper (Deman, Vakaet & Bruyneel, 1976), cell size alterations, induced by incubation of DI HeLa suspension cells in anisotonic NaCl solutions, were reported to bring about modifications in mutual cell adhesiveness. The effect on adhesion was assumed to be mediated by cell surface sialic acids. In the present investigation, we examine the effect of anisotonicity on the strength of the LRER forces generated by the sialic acids.

Materials and Methods

Most of the methods used in this study have been described in the companion paper (Deman *et al.*, 1976).

For the measurement of the electrostatic repulsive forces, HeLa cells after harvest were washed with 0.9% NaCl and then were divided into several portions. The portions which were not used immediately were stored as pellets at 5 °C with 0.9% NaCl on top of them.

Before measurement the cells were suspended in a NaCl solution of given tonicity, at 37 °C for 10 min. Poly-L-lysine·HBr, M.W. 11,000 (Sigma Chemical Co., St Louis, Mo.; lot 91C-5000) was added after incubation of the cells. Then the suspensions were made homogeneous by inverting the incubation flasks several times. The contents were transferred immediately into the Couette system for measurement of the agglutination rate. The cell densities during measurement were kept between 1.6 and 1.9×10^6 cells per ml of homogeneous suspension.

After 20 min of rotation in the apparatus, a cell sample was taken from the uppermost layer of the suspension. The relative cell concentration (R.C.C.) value was determined in the same way as described in the companion paper, for the measurement of the aggregation rate. An increase in agglutination rate is reflected by a decrease in R.C.C. value. It is emphasized that also an increase in aggregation rate might yield lower R.C.C. values, and that actually the R.C.C. values are determined by the combined effects of agglutination and aggregation.

In a previous paper (Deman & Bruyneel, 1974), where the method was presented in great detail, strong evidence was advanced for the assumption that the length of the initial plateau phase in the agglutination curve, i.e. the low-slope part of the curve, could be interpreted as a measure of the strength of the LRER forces between the cells. The decrease in R.C.C. value during that phase is ascribed to an increased tendency for aggregation, due to neutralization of the negative cell surface charges by the positive polylysine. The reduction of the electrostatic forces of repulsion enables the cells to come sufficiently close for the polylysines to cross-link them in the next phase of agglutinative titration.

Results

Density-Inhibited Cells

HeLa cells were harvested from three suspension cultures in which growth was density inhibited. After washing, the cells were incubated in NaCl 0.9, 0.6 and 0.5%, respectively. Subsequent agglutination of the cells with polylysine, M.W. 11,000, in isotonic NaCl, revealed the occurrence of a large plateau (i.e. low-slope) phase, indicating that strong LRER forces were present (Fig. 1). The repulsion effect was considerably weakened after hypotonic swelling of the cells.

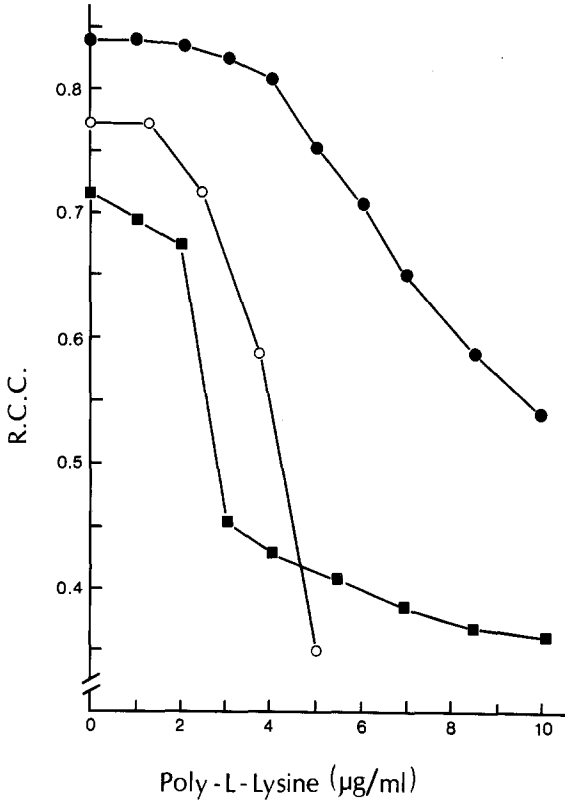

Fig. 1. Agglutination of HeLa cells from density-inhibited cultures, with poly-L-lysine, M.W. 11,000. Agglutination rate is inversely related to R.C.C. values (ordinate). Diminishment of the electrostatic repulsive force, by incubation in hypotonic NaCl solutions. ●—●: Harvest at 2.05×10^6 cells/ml, incubated in NaCl 0.9%, mean cell diam. 14.3 ± 1.6 μm; ○—○: harvest at 1.91×10^6 cells/ml (14.4 ± 1.5 μm), incubated in NaCl 0.6%, final mean cell diam. 15.4 ± 2.0 μm; ■—■: harvest at 1.90×10^6 cells/ml (14.1 ± 1.6 μm), incubated in NaCl 0.5%, final mean cell diam. 15.3 ± 1.8 μm

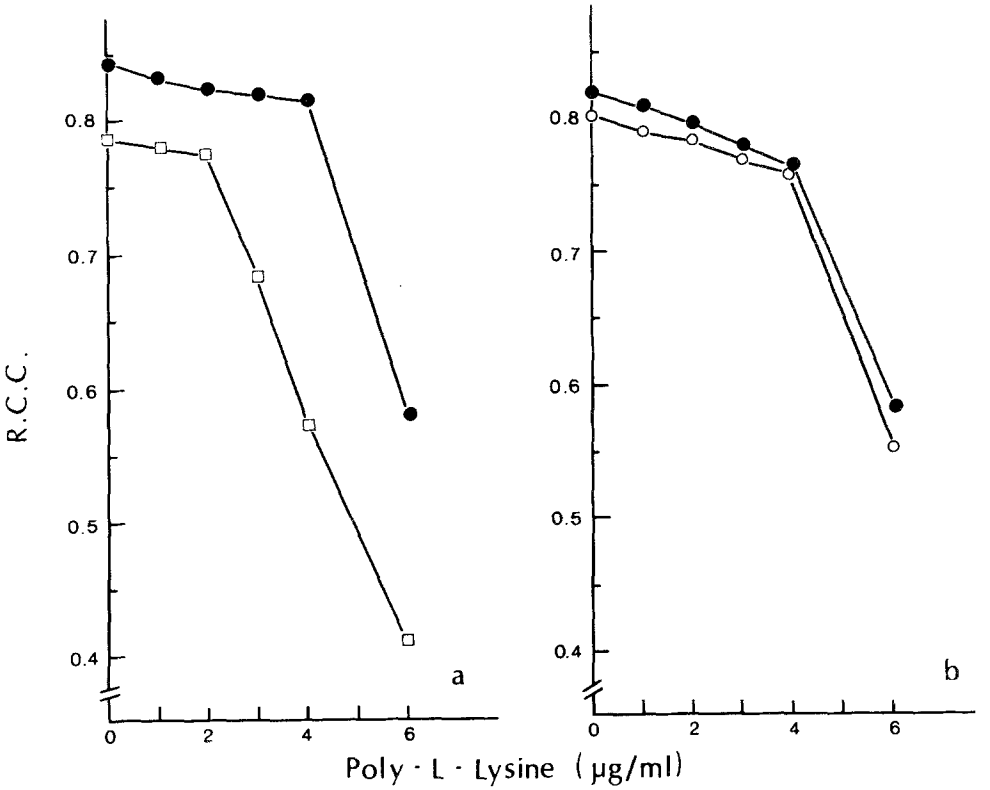

Fig. 2. Cells from density-inhibited cultures. Reversible alteration in repulsive force following incubation in a 0.5% NaCl solution. (*a*) Harvest at 2.43×10^6 cells/ml (mean density of two suspensions), □—□ : incubation in NaCl 0.5%, final mean cell diam. 15.5 ± 2.2 µm; ●—●: incubation in NaCl 0.5% followed by incubation in NaCl 0.9%, final mean cell diam. 12.9 ± 1.7 µm. (*b*) Harvest at 2.69×10^6 cells/ml (mean density of two suspensions), ○—○ : incubation in NaCl 0.9%, mean cell diam. 14.3 ± 1.7 µm; ●—● : incubation in NaCl 0.5% (15.2 ± 1.8 µm) followed by incubation in NaCl 0.9%, final mean cell diam. 13.0 ± 1.6 µm

In order to investigate the reversibility of the hypotonic effect, HeLa cells harvested from the same DI culture were divided into two portions. Cells from one portion were incubated in NaCl 0.5% and then were agglutinated with polylysine. The cells from the other portion were first treated with NaCl 0.5%; the solution was discarded after centrifugation, after which the cells were reincubated in NaCl 0.9%. Fig. 2*a* shows that the smaller cells, i.e. those agglutinated in NaCl 0.9%, show a stronger repulsion effect than the larger cells in hypotonic medium. Fig. 2*b* shows the results of a next experiment. Previous incubation in NaCl 0.5% followed by centrifugation and incubation in NaCl 0.9% gave an agglutination curve very similar to that of cells which immediately were incubated in NaCl 0.9%.

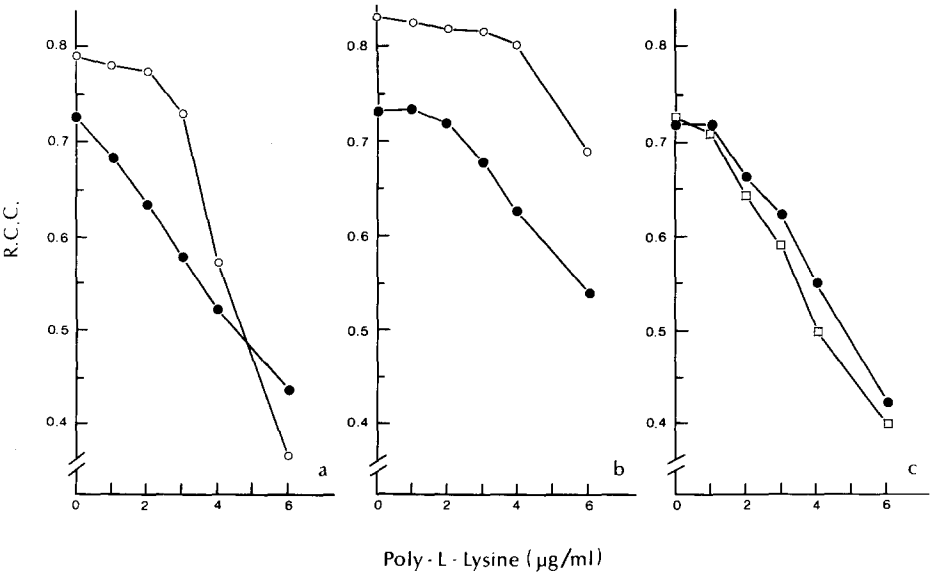

Fig. 3. Fast growing cells from low density cultures after 24 hr of growth. Reversible induction of repulsive forces by incubation in a 1.5% NaCl solution. (*a*) Harvest at 1.10×10^6 cells/ml (mean density of four suspensions); ●—●: incubation in NaCl 0.9%, mean cell diam. 16.1 ± 2.1 μm; ○—○: incubation in NaCl 1.5%, mean cell diam. 14.4 ± 1.9 μm. (*b*) Harvest at 1.47×10^6 cells/ml (mean density of four suspensions); ●—●: incubation in NaCl 0.9%, mean cell diam. 14.9 ± 1.7 μm. ○—○: incubation in NaCl 1.5%, mean cell diam. 13.9 ± 1.8 μm. (*c*) Harvest at 1.00×10^6 cells/ml (mean density of four suspensions); ●—●: incubation in NaCl 0.9%, mean cell diam. 15.5 ± 1.8 μm: □—□: incubation in NaCl 1.5% (13.3 ± 1.5 μm) followed by incubation in NaCl 0.9%, final mean cell diam. 15.1 ± 1.7 μm

Fast Growing Cells

The observation that weak LRER forces on DI cells after hypotonic swelling, could be restored to their original strength by reincubation of the cells in isotonic medium, led us to investigate whether repulsion forces could be induced on FG cells by means of hypertonic shrinkage. Previous experiments (*see* Introduction) indicated that LRER forces were absent on cells harvested from exponentially growing cultures.

HeLa cells were harvested from four fast-growing suspensions, 24 hr after start of the cultures at a density of ca. 0.5×10^6 cells per ml. The combined suspensions were divided into two portions. The cells from one portion were incubated in NaCl 0.9%. Agglutination of these cells with polylysine, showed absence of LRER forces. The cells from the other portion were incubated in NaCl 1.5% due to which the mean size was reduced. Such treatment resulted in the appearance of LRER forces (Fig. 3*a*).

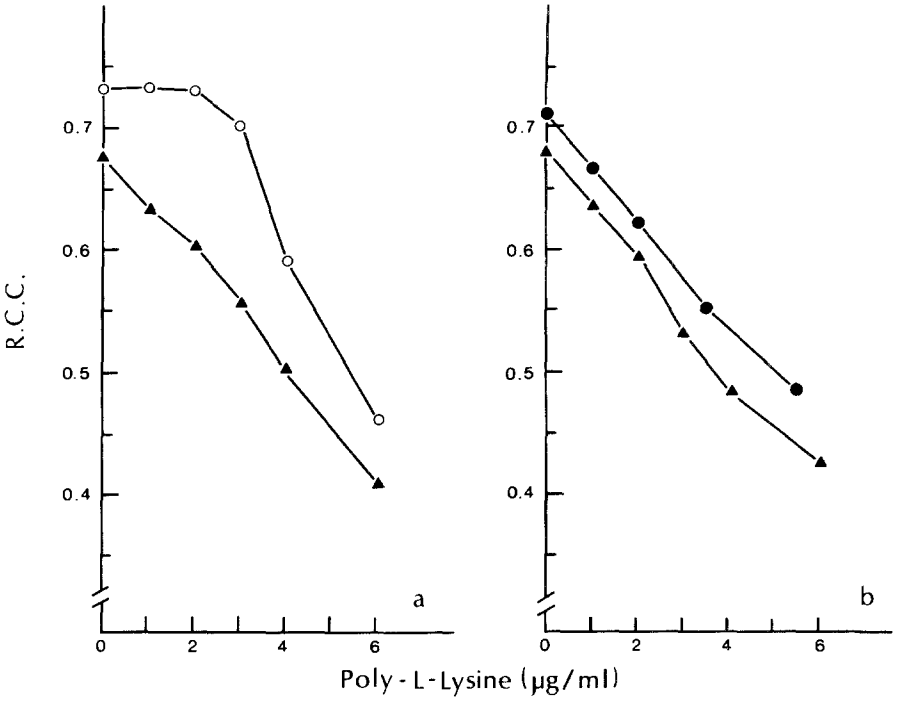

Fig. 4. Fast growing cells from low density cultures after 24 hr of growth. Previous neuraminidase treatment prevents the induction of repulsive forces following incubation in NaCl 1.5%. (*a*) Harvest at 1.00×10^6 cells/ml (mean density of four suspensions, mean cell diam. 16.5 ± 2.1 µm. ○—○ : Incubation in NaCl 1.5%, final mean cell diam. 15.0 ± 1.8 µm; ▲—▲ : treatment with neuraminidase followed by incubation in NaCl 1.5%, final mean cell diam. 14.3 ± 1.7 µm. (*b*) Harvest at 0.90×10^6 cells/ml (mean density of four suspensions); ●—● : treatment with neuraminidase buffer followed by incubation in NaCl 0.9%, mean cell diam. 16.2 ± 1.9 µm; ▲—▲ : treatment with neuraminidase followed by incubation in NaCl 1.5%, final mean cell diam. 14.2 ± 1.7 µm

Basically, the same observation was made in another experiment (Fig. 3*b*) in which the cells were harvested after 24 hr at a somewhat higher density than that of the previous experiment. The cell density at start of these cultures was ca. 0.75×10^6 cells per ml. A small repulsion effect was detectable in NaCl 0.9%. The effect became more pronounced after shrinkage of the cells in NaCl 1.5%.

Fig. 3*c* demonstrates that the effect is reversed, and LRER forces are weakened again, when shrunken cells are replaced in isotonic NaCl solution.

Previous neuraminidase treatment of the cells from FG cultures prevents the generation of repulsion forces by hypertonic treatment. This is shown in Fig. 4*a*, where it is observed that hypertonic size-reduction

of desialylated cells does not produce repulsion forces, whereas intact cells from the same stock become repulsive when incubated in the same medium. The agglutination curve in 1.5% NaCl solution, obtained with neuraminidase-treated cells, is very similar to that of intact cells in an isotonic NaCl solution (Fig. 4*b*).

In a last experiment, not represented here, it was found that the reverse treatment was ineffective: hypotonic swelling of FG cells did not give rise to the appearance of LRER forces, nor did treatment with neuraminidase whether or not followed by hypotonic incubation.

We wish to draw attention to the fact that the values of the relative cell concentrations in the absence of polylysine, are inversely related to mutual cell adhesiveness. From Figs. 1–4, it is apparent that a diminution in repulsive force is accompanied by an increase in adhesiveness.

Discussion

The results contain additional evidence to support the conclusion that the strength of LRER forces between cells can be evaluated by agglutinative titration with short-chain polylysines. There is an inverse relationship between the lengths of the low-slope phases and the strengths of mutual cell adhesiveness. Anisotonicity produces appreciable changes in adhesiveness only under those conditions in which the shape of the agglutination curve is significantly altered. The increase in adhesiveness of the smaller, DI cells following hypotonic swelling, described in the companion paper (Deman *et al.*, 1976), can be ascribed with reasonable certainty to the reduction of LRER forces on these cells. Conversely, the decrease in adhesiveness of the larger, FG cells in hypertonic media, observed in the present study, can be explained by the appearance of repulsion forces. The latter effect is absent after neuraminidase treatment of the FG cells, which confirms the essential role of surface sialic acids for the generation of the LRER forces.

The effects observed are contrary to the supposition that Na^+ ions should affect the strength of repulsion through neutralization of the negative cell surface charge, because in that case, a decrease in NaCl concentration should be expected to enhance the repulsive forces on DI cells, which is contrary to observation. The same applies, mutatis mutandis, for the effect of hypertonic NaCl solutions on FG cells. The fact that the effects of hypotonicity and hypertonicity are reversible indicates that modulation of the repulsive forces by anisotonicity cannot

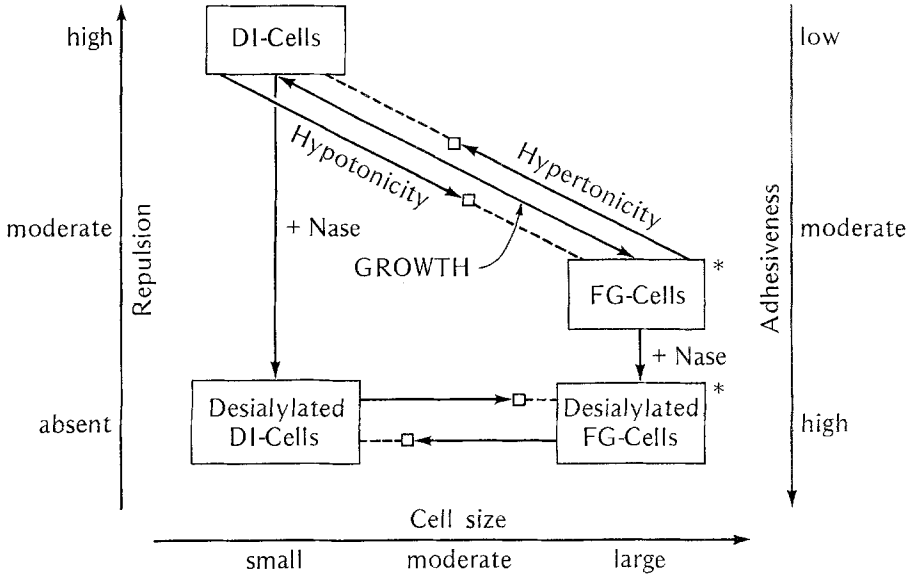

Fig. 5. Long-range electrostatic repulsion and intercellular adhesiveness are affected in opposite directions by alterations in mean cell size. The Figure shows the analogy between the effects of size changes arising during growth in suspension, and those induced by anisotonic treatment of harvested cells. Boxes marked with an asterisk may coincide when growth rate is maximal (exponential)

be ascribed to loss or addition of essential cell surface material involved in repulsion or adhesion. This, taken together with the improbability of a direct effect of the ionic medium on repulsion, favors an interpretation in which modulation of the LRER forces is due directly to the change in cell size itself.

HeLa cells during asynchronous growth in suspension culture undergo size alterations, the magnitude of which is comparable to that of the size alterations induced by anisotonicity (Deman *et al.*, 1976). The changes in adhesiveness and repulsive strength which accompany the growth-induced size alterations, are exactly analogous to those occurring as a result of anisotonicity. In both cases, removal of cell surface sialic acids has no, or only a slight, influence on the adhesiveness of the larger cells, whereas the same treatment increases the adhesiveness of the smaller cells by annihilation of the LRER forces. The analogy is schematically represented in Fig. 5.

The above considerations suggest that changes in bulk adhesiveness of the cells during growth, are brought about mainly by cell size alterations, the effect of which is exerted by modulation of the repulsive

forces generated by the sialic acid residues. We cannot exclude the possibility that differences in intercellular attractive forces might also exist. Differences in surface glycoprotein composition between cells in different stages of cell growth have been reported (Buck, Glick & Warren, 1971; Glick & Buck, 1972; Muramatsu, Atkinson, Nathenson & Ceccarini, 1973). Further research must show whether LRER forces are characteristic for GO or G1 cells.

In recent years it has become increasingly clear that in many circumstances, the cell membrane may be sufficiently fluid to allow for floating and rearrangements of certain of its components, including sialic acids (Nicolson, 1973; Nicolson & Painter, 1973; Weiss, 1974). The finding (Deman & Bruyneel, 1975) that FG and DI HeLa cells have approximately equal sialic acid contents leaves little doubt that differences in the topographical distribution of these negative residues, are responsible for the differences in LRER forces. This altered distribution may be accounted for either by the presence of different cell surface sialoglycoproteins or to a modified positioning of the sialoglycopeptide residues of identical glycoproteins. The possibility of induction of LRER forces on FG cells by hypertonicity, and conversely, the weakening of these forces on DI cells by hypotonicity, clearly is in favor of the latter possibility.

As a hypothesis, we propose that cell size increase caused by hypotonicity or occurring following the release from DI of growth, causes a change in the distribution of the sialic acids in the cell coat. The ensuing diminution in LRER force expresses itself as an enhancement of intercellular adhesiveness. A decrease in cell size has an opposite effect and diminishes intercellular adhesiveness (Table 1).

Table 1. Causal sequence between cell surface alterations during growth

Low cell density		High cell density
Large	Cell size	Small
	↓	
Disorganized	Distribution of sialic acids	Local increases in charge density
	↓	
Absent	Long-range electrostatic repulsion forces	Present
	↓	
Strong	Intercellular adhesiveness	Weak
Growth		Inhibition of growth

It has been pointed out elsewhere that there are reasons to believe that the changes in sialic acid distribution under discussion must be situated below the electrokinetic shear plane, i.e. in the deeper regions of the cell coat (Deman & Bruyneel, 1975). It is proposed that the redistribution of the sialic acids which accompanies cell size reduction results in the appearance of a large increase in local negative charge density. Under this condition, mobile positive counterions are no longer able to interdigitate between individual sialic acid changes, but have to 'neutralize them at distance', giving rise to an electrical double layer structure and a zeta-potential which expresses itself as a long-range repulsive force.

The correlation of a difference in agglutinative action of polylysine with a change in charge distribution in DI and FG cells, is in good analogy with the conclusion reached by Marikovsky, Inbar, Danon and Sachs (1974), for the difference between normal and transformed cells. The authors demonstrated that polylysine agglutinated normal cells at a higher rate than transformed cells. Labeling with cationized ferritin resulted in a more regular distribution on normal cells than on transformed cells.

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References

Brooks, D.E., Seaman, G.V.F. 1972. Electroviscous effect in dextrane-erythrocyte suspensions. *Nature, New Biol.* **238**:251

Buck, C.A., Glick, M.C., Warren, L. 1971. Effect of growth on the glycoproteins from the surface of control and Rous sarcoma virus transformed hamster cells. *Biochemistry* **10**:2176

Curtis, A.S.G. 1973. Cell adhesion. *Prog. Biophys. Mol. Biol.* **27**:316

Deman, J.J., Bruyneel, E.A. 1974. Evidence for long-range electrostatic repulsion between HeLa cells. *Exp. Cell Res.* **89**:206

Deman, J.J., Bruyneel, E.A. 1975. Intercellular adhesiveness and neuraminidase effect following release from density inhibition of cell growth. *Biochem. Biophys. Res. Commun.* **62**:895

Deman, J.J., Vakaet, L.C., Bruyneel, E.A. 1976. Cell size and mutual cell adhesion. I. Increase in mutual adhesiveness of HeLa cells from density-inhibited suspension cultures by hypotonic treatment. *J. Membrane Biol.* **26**:189

Glick, M.C., Buck, C.A. 1972. Glycoproteins from the surface of metaphase cells. *Biochemistry* **12**:85

Jan, K.M., Chien, S. 1973. Role of surface electric charge in red blood cell interactions. *J. Gen. Physiol.* **61**:638

Jones, G.E. 1974. Intercellular adhesion: Modification by dielectric properties of the medium. *J. Membrane Biol.* **16**:297

Marikovsky, Y., Inbar, M., Danon, D., Sachs, L. 1974. Distribution of surface charge and concanavalin-A binding sites on normal and transformed cells. *Exp. Cell Res.* **89**:359

Muramatsu, T., Atkinson, P.H., Nathenson, S.G., Ceccarini, C. 1973. Cell-surface glycopeptides: Growth-dependent changes in the carbohydrate-peptide linkage region. *J. Mol. Biol.* **80**:781

Nicolson, G.L. 1973. Anionic sites on human erythrocyte membranes. I. Effects of trypsin, phospholipase C, and pH on the topography of bound positively charged colloidal particles. *J. Cell Biol.* **57**:373

Nicolson, G.L., Painter, R.G. 1973. Anionic sites on human erythrocyte membranes. II. Antispectrin-induced transmembrane aggregation of the binding sites for positively charged colloidal particles. *J. Cell Biol.* **59**:395

Pethica, B.A. 1961. The physical chemistry of cell adhesion. *Exp. Cell Res.* **8**:123

Weiss, L. 1974. The topography of cell surface sialic acids and their possible relationship to specific cell interactions. *Behring Inst. Mitt.* **55**:185