The Electrophoretic Properties and Aggregation of Mouse Lymphoma Cells, Chinese-Hamster Fibroblasts and a Somatic-Cell Hybrid

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1. The electrophoretic mobilities of a mouse lymphoma cell, a Chinese-hamster fibroblast and a somatic-cell hybrid (also fibroblastic), produced by fusion of the hamster cell and a mouse lymphoma cell, were measured at 25°C over a range of pH, concentration of Ca^{2+} ions and concentration of La^{3+} ions. 2. All the cells have pI at pH 3.5. 3. Ca^{2+} ions decrease the mobilities and zeta potentials of the cells to zero in the range 1–100 mM. 4. La^{3+} ions lower the mobilities and zeta potentials in the range 10 μM –1 mM, and the cells become positively charged above 1 mM. 5. The data are consistent with specific adsorption of La^{3+} ions on approx. 2×10^{14} sites/m² of cell surface with a free energy of approx. –37 kJ/mol. 6. The effects of Ca^{2+} , La^{3+} and ionic strength on the extent of aggregation of the cells and of neuraminidase-treated cells were studied. 7. Ca^{2+} ions do not markedly increase aggregation, whereas La^{3+} ions gave rise to extensive aggregation in the range 10 μM –1 mM, corresponding to the region of La^{3+} adsorption. 8. Both fibroblastic cell lines are aggregated at high ionic strength. 9. The fibroblastic cells have larger amounts of trypsin-sensitive carbohydrate than does the lymphoma cell; the possible role of this material in cellular aggregation is discussed.

The interaction between cells is of importance in morphogenesis and in maintaining the integrity of tissues. Cell–cell interaction in tissues has been approached from biological, biochemical and biophysical aspects (see, e.g., Weiss, 1967; Curtis, 1967; Trinkaus, 1969), but at present there is no unified theory of cellular cohesion. The biochemical mechanisms proposed include short-range specific interactions of the enzyme–substrate type (Roseman, 1970), the existence of oscillating receptors mediated by membrane-bound contractile proteins which change the conformation of membrane glycoproteins (Jones, 1966; Jones *et al.*, 1970*a,b*), and specific aggregation factors (Hausman & Moscona, 1973; McClay & Moscona, 1974; Kudo *et al.*, 1974; Balsamo & Lilien, 1975) or inhibitors (Merrell *et al.*, 1975).

The biophysical approach to the problem has been dominated by attempts to explain cellular cohesion in terms of the classical theory for the interaction between lyophobic colloidal particles formulated by Derjaguin, Landau, Verwey and Overbeek (Verwey & Overbeek, 1948; Pethica, 1961; Curtis, 1967; Weiss & Harlos, 1972). More recently, attention has been drawn to the possible importance of the glycolipids and glycoproteins at the cell surface, which could give rise to both a steric potential-energy barrier to cell aggregation (Maroudas, 1975*a,b*; Greig & Jones, 1976) and an attractive force if hydrogen-bonding occurred between monosaccharide chain segments (Jones, 1976).

The study of the conditions which bring about the aggregation of cells *in vitro* can yield information about factors which might be involved in aggregation *in vivo*. Whether a given cell is stable in a dispersion or aggregates must depend to a large degree on the interactions between the plasma membranes of adjacent cells in close contact. The surface characteristics of the factors which affect the interactions between plasma membranes are thus of importance.

We have investigated the electrophoretic properties and ion-induced aggregation of three tissue-cell lines, a mouse lymphoma cell (P388-F36) (Fisher & Sartorelli, 1964), a Chinese-hamster ovary cell (CH23) (Tijo & Puck, 1958) and a hybrid cell line produced by fusion of the mouse and hamster cells by u.v.-inactivated Sendai virus (PCM1) (Ayad & Delinassios, 1974). The mouse lymphoma cells grow in dispersion, whereas the other two cells are fibroblastic and grow as monolayers. The objective of the investigation was to attempt to relate the different modes of growth of genetically linked cells with their surface characteristics and susceptibility to aggregation.

Experimental

Cell culture

Mouse lymphoma cells (P388) and Chinese-hamster fibroblasts (CH23) were cloned in this Department (Ayad & Delinassios, 1974). The clone (P388-F36)

was characterized by the presence of 36 chromosomes (range 34–46, polyploidy 27%), and the clone (CH23) was characterized by the presence of 23 chromosomes (range 21–26, polyploidy 8.0%). The somatic-cell hybrid (PCM1) was isolated between CH23 and P388-F36 cells by using a non-selective pressure. Chromosome stability was reached at a stage when two to four mouse chromosomes coexisted with not less than 19 hamster chromosomes.

The method of cell fusion and hybrid isolation, together with details of the respective cell karyotypes, have been described in full previously (Ayad & Delinassios, 1974). Biochemical studies using this genetic system have been reported (Ayad & Foster, 1974). The three cell lines were cultured as a routine in Eagle's minimum essential medium with Hanks salts (Flow Laboratories Ltd., Irvine, Scotland) supplemented with 10% heat-inactivated newborn-calf serum, 2.0 mM-glutamine, 100 units of penicillin/ml, 100 µg of streptomycin/ml and 1.32 mM-NaHCO₃, and were gassed with air+CO₂ (95:5). The P388-F36 cells, which have a generation time of 14 h (initial seeding density 5×10^4 cells/ml), were harvested at confluence, when the cell density was 1.8×10^6 cells/ml. The monolayer cells were seeded into Roux bottles at an initial density of approx. 4×10^4 cells/ml; when the monolayers were confluent (approx. 10^5 cells/cm²) they were washed free of medium with a standard Difco trypsin buffer solution (0.05%, w/v; activity 225 α-N-benzoyl-L-arginine ethyl ester hydrochloride units/mg), pH 7.6 (Paul, 1970a), and incubated for 5 min with the trypsin solution and finally washed twice with phosphate-buffered saline, pH 7.5 (4.8 mM-Na₂HPO₄+0.96 mM-NaH₂PO₄), containing 10.8% (w/v) sucrose, $I = 0.0172$ mol/litre, before suspension in the required medium.

Microelectrophoresis

Electrophoretic mobilities were measured at $25 \pm 0.1^\circ\text{C}$ in a horizontal flat cell equipped with grey-platinum electrodes. The apparatus was obtained from Rank Bros., Bottisham, Cambridge, U.K., and has been previously described by Bangham *et al.* (1958a,b). The rate of migration of the cells was determined by timing their transit over a known distance such that the time was of the order of 10 s at an applied potential gradient of 500–1000 V/m. The velocity of at least 25 individual cells was measured in both directions. The mobilities [u (m²·s⁻¹·V⁻¹)] were calculated from eqn. (1):

$$u = \frac{dL_e}{Vt} \quad (1)$$

where d is distance travelled, L_e the effective length between the electrodes, V the applied voltage and t the time. The mean mobility and standard deviation was calculated from at least 50 velocity measurements

under a given set of conditions. The final alignment of the apparatus and the position of the 'stationary levels' was checked by determining the mobility of human erythrocytes as a function of pH at $I = 0.0172$ mol/litre. The experimentally located stationary levels were found to be in agreement with those theoretically calculated. The mobility of human erythrocytes at pH 7.0 at $I = 0.172$ mol/litre was $-1.05 \times 10^{-8} \pm 0.07 \times 10^{-8} \text{ m}^2 \cdot \text{s}^{-1} \cdot \text{V}^{-1}$. This value, which has been corrected to the viscosity of water at 25°C , are in agreement with literature values (Furchgott & Ponder, 1941; Hunter, 1960; Zerial & Wilkins, 1974).

Buffered saline/sucrose solutions were used for determining the pH-mobility profiles. The buffer solutions to cover the pH ranges required were made up from stock solutions as follows: pH 2.0–4.0 (0.1 M-glycine / 0.1 M-NaCl / 0.2 M-HCl); pH 4.0–6.0 (0.2 M-sodium acetate/0.85 M-acetic acid); pH 6.0–8.0 (0.05 M-Na₂HPO₄ / 0.4 M-NaH₂PO₄); pH 8.0–9.5 (0.2 M-HCl / 0.05 M-sodium veronal); pH 9.5–11.0 (0.1 M-glycine/0.1 M-NaCl/0.2 M-NaOH). In addition, all the buffers contained 0.0152 M-NaCl and 10.8% (w/v) sucrose. The final ionic strength of all the buffers was constant at $I = 0.0172$ mol/litre.

The cells were prepared for electrophoresis by washing twice with 100 times their packed volume of phosphate-buffered saline, pH 7.5, containing 10.8% (w/v) sucrose ($I = 0.0172$ mol/litre) and harvested by centrifugation at 500g for 5 min. Further washes did not alter the mobility. The cells (approx. 10^6) were suspended in the required buffer, placed in the electrophoretic cell and mobility measurements usually completed within 15 min, during which time the cells remained at least 90% viable, as judged by the Trypan Blue-exclusion test (Paul, 1970b).

Zeta potentials (ξ) were calculated from the mobilities (u) by using the Smoluchowski equation (Henry, 1931):

$$\xi = \frac{u\eta}{\epsilon_0 \epsilon_r} \quad (2)$$

where η is the viscosity of the medium of relative permittivity ϵ_r , taken as 78.5, and ϵ_0 is the permittivity of free space, $8.854 \times 10^{-12} \text{ J}^{-1} \cdot \text{C}^2 \cdot \text{m}^{-1}$. For the mobility measurements made in 10.8% (w/v) sucrose solutions (i.e. pH profiles) $\eta = 12.6 \times 10^{-4} \text{ N} \cdot \text{s} \cdot \text{m}^{-2}$, and in 9.4% (w/v) sucrose solutions (containing La³⁺ and Ca²⁺ ions) $\eta = 12.0 \times 10^{-4} \text{ N} \cdot \text{s} \cdot \text{m}^{-2}$. The mean standard deviation of the zeta potentials, based on all the mobility data, was 1.9 mV.

Aggregation experiments

Cell aggregation was assessed by measuring the total number of particles, n_t (single cells plus aggregates), as a function of time after the addition of electrolyte solution to a dispersion of cells, initial density approx. 2×10^6 cells/ml, in an unbuffered

medium containing 9.4% (w/v) sucrose plus 17.2 mM-NaCl at room temperature ($\sim 20^{\circ}\text{C}$). During the period of the measurements the dispersions were positioned at 45° to the horizontal and turned end-over-end at a frequency of approx. 72 inversions/min. The pH of the dispersions was constant during the course of the experiments but varied according to the ion concentration used. For the solutions containing Ca^{2+} the pH was in the range 6.5–5.6; for the solutions containing La^{3+} the pH range was 5.9–4.9. Over the latter pH range, hydrolysis of the trivalent La^{3+} ion would be negligible (Biedermann & Ciavatta, 1961). The measurements of n_t were made on portions of the cell suspension with a model B Coulter counter. In general, n_t reached a limiting value (n_{30}) within 30 min after the addition of electrolyte, and a percentage aggregation was calculated from the equation:

$$\text{Aggregation (\%)} = \left(1 - \frac{n_{30}}{n_0}\right) \times 100 \quad (3)$$

where n_0 is the initial number of particles in the cell suspension. For each set of conditions the percentage aggregation was measured on at least five batches of cells. The deviations in percentage aggregation from one cell batch to another were within $\pm 10\%$.

The Coulter counter was used in conjunction with a model J particle-size analyser to determine cell volumes in iso-osmotic NaCl (0.152 M). The volumes obtained were: P388-F36, $5.24 \times 10^{-16} \text{ m}^3$; CH23, $21.5 \times 10^{-18} \text{ m}^3$; PCM1, $14.4 \times 10^{-18} \text{ m}^3$. The corresponding equivalent spherical radii are $5 \times 10^{-6} \text{ m}$, $8 \times 10^{-6} \text{ m}$ and $7 \times 10^{-6} \text{ m}$ respectively.

Sialic acids

These were liberated from the cell surface by digestion with 50 units (defined below) of neuraminidase per 10^8 cells at pH 5.5 for 60 min at 37°C . The supernatant was deproteinized with trichloroacetic acid (final concn. 5%, w/v) and the precipitate removed by centrifugation. The resulting fluid was concentrated by evaporation under decreased pressure and the sialic acids assayed as *N*-acetylneuraminic acid by the method of Warren (1959). Controls of cells without neuraminidase, and neuraminidase without cells, were also included.

Carbohydrate

Cell-surface carbohydrate was liberated from approx. 10^8 cells by incubation with trypsin [type III; Sigma (London) Chemical Co., London S.W.6, U.K.] at 37°C , pH 7.6, activity of 1 α -*N*-benzoyl-L-arginine ethyl ester hydrochloride unit/ml for 60 min for the P388-F36 cells and 55 α -*N*-benzoyl-L-arginine ethyl ester hydrochloride units/ml for 15 min for the CH23 and PCM1 cells. Longer incubation times did

not change the amount of carbohydrate released. The supernatant from the trypsin-treated cells was deproteinized with trichloroacetic acid (final concentration 5%, v/v) and the precipitate removed by centrifugation (500g, 10 min). The resulting solution was concentrated under decreased pressure and carbohydrate was assayed by the anthrone method (Herbert *et al.*, 1971). Controls of cells without trypsin treatment, and trypsin without cells, were also included. Total cellular carbohydrate was measured in a similar manner on cell homogenates produced by sonication by using a Kerry's ultrasonicator (Kerry's Ltd., Basildon, Essex, U.K.) at full power for two intervals of 10 s duration.

Protein

This was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Materials

The sources and grades of the materials used were as follows: bovine serum albumin, Sigma, fraction V, essentially fatty acid-free; β -lactoglobulin, Sigma, crystallized and freeze-dried from milk; dextran, Sigma, average mol. wt. 82 000 (range 60 000–90 000); trypsin, Sigma, type III, twice-crystallized from bovine pancreas, dialysed, freeze-dried, essentially salt-free, 5000 units/mg; trypsin, Difco Laboratories, Detroit, MI, U.S.A., used for treating P388-F36 cells before pH profile, activity 225 units/mg; neuraminidase, Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K. (according to the suppliers the enzyme contained trace amounts of proteinase, peptidase and α - and β -glycosidases), activity 500 units/ml (1 unit liberates 1 μg of *N*-acetylneuraminic acid from a glycoprotein substrate in 15 min at 37°C , pH 5.5); mixed glycosidase, Miles Laboratories Ltd., Stoke Poges, Slough, Bucks., U.K., extracted from *Charonia lampus*; *N*-acetylneuraminic acid, Koch-Light, synthetic, pure, used as standard for sialic acid assay. All the inorganic salts and sucrose were of analytical grade. The water used was double-distilled from an all-glass still.

Results

The zeta potentials of the three cell lines were measured as a function of pH. The results at pH 7.0 are given in Table 2. All three curves of zeta potential plotted against pH showed isoelectric points at approx. pH 3.5 and approximately constant zeta potentials in the range pH 6–9. The zeta potentials of the P388-F36 cells were unaffected by treatment with trypsin at an activity sufficient to remove the monolayer cells from a glass surface. The cells behaved reversibly in that after exposure to a medium of

Fig. 1. Zeta potentials of cells in the presence of Ca^{2+} ions at 25°C

The cells were suspended in a background medium of 17.2 mM-NaCl plus 9.4% (w/v) sucrose. —, Debye length; ●, P388-F36 cells; △, CH23 cells; ○, PCM1 cells.

Fig. 2. Zeta potentials of cells in the presence of La^{3+} ions at 25°C

The cells were suspended in a background medium of 17.2 mM-NaCl plus 9.4% (w/v) sucrose. —, Debye length; ●, P388-F36 cells; △, CH23 cells; ○, PCM1 cells.

pH 2.8 the zeta potential at pH 7.0 was within experimental error of the original value. Treatment of the P388-F36 cells with neuraminidase resulted in a decrease in zeta potential by 26% at pH 7.0. Neuraminid-

ase had no significant effect on the zeta potentials of the fibroblastic cells.

The zeta potentials of the cells were also measured as a function of the concentration of Ca^{2+} and La^{3+}

ions, as shown in Figs. 1 and 2, which also show the Debye length ($1/\kappa$), a measure of the electrical double-layer thickness, calculated from the equation:

$$\kappa^2 = \frac{2e^2 N \times 10^3}{\epsilon_0 \epsilon_r kT} \cdot I \quad (4)$$

where e , N , k and T are the electronic charge, Avogadro's number, the Boltzmann constant and the absolute temperature respectively and I is the ionic strength of the solution. The decrease in the double-layer thickness at higher Ca^{2+} concentrations gives rise to a decrease in the zeta potentials of the cells. By contrast, La^{3+} ions, which compress the double layer in a manner similar to Ca^{2+} ions, have a large effect on the zeta potentials at low concentrations and the cells show a charge reversal (negative to positive) above about 1 mM. The effects of these cations on the zeta potentials of the cells are reversible; after exposure to high cation concentrations, if the cells are washed in aqueous NaCl solution the zeta potentials return to their initial values.

Fig. 3 shows the percentage aggregation of the P388-F36 and CH23 cells as a function of Ca^{2+} concentration. In the absence of Ca^{2+} the cells usually had a basal aggregation of approx. 10–20%. Ca^{2+} ions do not significantly affect the aggregation of P388-F36 or CH23 cells over the $[\text{Ca}^{2+}]$ range of from 0.1 M–1 μM , nor do they effect neuraminidase-treated P388-F36 cells. For the CH23 cells there is a small increase in aggregation on neuraminidase treatment. The PCM1 cells behave in a way very similar to that of the CH23 cells. By contrast, La^{3+} ions dramatically increase aggregation at concentrations between 10 and 100 μM , as illustrated in Fig. 4. Neur-

aminidase treatment decreases the aggregation of P388-F36 cells at $[\text{La}^{3+}] = 10 \text{ mM}$ and enhances the aggregation of CH23 cells in the range 100 μM –10 mM. Fig. 5 shows the effect of ionic strength and Debye length on aggregation for cells suspended in unbuffered NaCl maintained iso-osmotic by addition of sucrose. For both fibroblastic cell lines, aggregation increases with increasing ionic strength and compression of the thickness of the electrical double layer. Aggregation of P388-F36 cells was not affected by ionic strength.

Experiments were performed to investigate the effects of trypsin and other macromolecules on La^{3+} -induced aggregation of the P388-F36 cells. These were carried out by preincubation of the cells with La^{3+} ions over a range of concentration under static conditions for a period of 5 min. The percentage aggregation was then measured with and without the addition of trypsin. The measurements were made approx. 5 min after the addition of the trypsin. The results of these experiments are shown in Fig. 6; the percentage aggregation was less under static conditions than when the cell suspensions were gyrated, as can be seen by comparing the curve for the cells without trypsin in Fig. 6 with that of Fig. 4. However, addition of trypsin resulted in instantaneous aggregation, to an extent comparable with that observed under gyration. That this effect was not specific to trypsin was established by a series of experiments in which batches of cells were incubated for 5 min under static conditions with a fixed La^{3+} concentration (100 μM), followed by the addition of β -lactoglobulin, dextran and bovine albumin. These three macromolecules induced aggregation to a

Fig. 3. Percentage aggregation of cells as a function of Ca^{2+} concentration

The cells were suspended in a background medium of 17.2 mM-NaCl plus 9.4% (w/v) sucrose. ----, Ionic strength; ○, P388-F36 cells; ●, P388-F36 cells treated with neuraminidase (50 units/ 10^8 cells) at pH 5.5 for 60 min at 37°C; △, CH23 cells; ▲, CH23 cells treated with neuraminidase (50 units/ 10^8 cells) at pH 5.5 for 60 min at 37°C.

Fig. 4. Percentage aggregation of cells as a function of La^{3+} concentration

The cells were suspended in a background medium of 17.2mM-NaCl plus 9.4% (w/v) sucrose. ----, Ionic strength; ○, P388-F36 cells; ●, P388-F36 cells treated with neuraminidase (50 units/ 10^8 cells) at pH 5.5 for 60 min at 37°C; △, CH23 cells; ▲, CH23 cells treated with neuraminidase (50 units/ 10^8 cells) at pH 5.5 for 60 min at 37°C; □, PCMI cells; ■, PCMI cells treated with neuraminidase (50 units/ 10^8 cells) at pH 5.5 for 60 min at 37°C.

similar extent as did trypsin. This macromolecule-mediated instantaneous-aggregation process is specific to the presence of La^{3+} ions, since no aggregation is induced when the cells are similarly incubated with Ca^{2+} ions, followed by addition of trypsin. Attempts to modify the surface of P388-F36 cells by treatment with glycosidases and a range of other enzymes neither promoted aggregation in the presence of Ca^{2+} ions nor affected La^{3+} -induced aggregation. We were unable to detect any surface carbohydrate released by the action of the mixed glycosidase preparation on the cells or on cells which had surface sialic acid removed by prior treatment with neuraminidase. The latter experiment eliminated the possibility that the presence of terminal sialic acid residues of membrane-bound glycoproteins was inhibiting the action of the glycosidases. It was found, however, that treatment of the cells with 1M-urea at pH 7.2 resulted in 28% instantaneous aggregation with retention of viability. Table 1 shows the results of the assays for sialic acids, carbohydrate and protein for the three cell lines.

Discussion

Zeta potentials

The zeta potential-pH curves (and corresponding mobility-pH curve) found for the cells are broadly similar to the curves that have been reported for a wide variety of other cells (Bangham *et al.*, 1958a,b;

Fig. 5. Percentage aggregation of cells as a function of Debye length in NaCl

The cells were suspended in iso-osmotic solutions containing various amounts of NaCl plus sucrose. ●, P388-F36 cells; ▲, CH23 cells; ○, PCMI cells.

Collins, 1966a,b; Kiremidjian & Stotzky, 1975; Wilkins *et al.*, 1962a,b). Many cells show an isoelectric point in the region pH2–4. The rapid change in zeta potential in the pH range 2–5 is usually attributed to the ionization of carboxylic groups on the cell surface. The decrease of 26% in zeta potential at pH7 on treatment of the P388-F36 cells with neuraminidase is very similar to the decrease observed by Cook & Jacobson (1968) for normal mouse lymphoma cells (27%), but less than the decrease they observed for leukaemic lymph-node cells (49%). The amount of sialic acid released by neuraminidase is within experi-

mental error of that found by Cook & Jacobson (1968) for leukaemic cells.

The decrease in zeta potentials brought about by the addition of Ca^{2+} ions accompanies the decrease in the Debye length ($1/\kappa$) and can be attributed to a non-specific effect on the electrical double layer at the cell surface. It has been shown by Hunter (1960) that for values of $\kappa a > 10^3$, where a is the cell radius, the zeta potential can be related to the Debye length by the equation:

$$\zeta = \frac{\sigma}{\epsilon_0 \epsilon_r} \left(\frac{1}{\kappa} + a_1 \right) \quad (5)$$

where σ is the surface charge density and a_1 the average radius of the counterions. Fig. 7 shows the plots of ζ against $1/\kappa$ for the cells in the presence of Ca^{2+} . The linearity of the plots implies that the cells do not specifically interact with Ca^{2+} ions. The slope of the plot for the P388-F36 cells gives a surface charge density of $6.1 \times 10^{-3} \text{ C} \cdot \text{m}^{-2}$. For the fibroblastic cells the corresponding value is $7.8 \times 10^{-3} \text{ C} \cdot \text{m}^{-2}$. An interesting feature of Fig. 7 is the intersection of the lines at $\zeta = 0$ at a positive value of $1/\kappa$. This means that a_1 is negative. The physical significance of this observation is unclear, but it may reflect the roughness of the cell surfaces. At physiological ionic strength, $1/\kappa = 8 \times 10^{-10} \text{ m}$, the zeta potentials for the three cells in the presence of Ca^{2+} ions are all approx. -3 mV . Taking the zeta potentials at $I = 0.0172 \text{ mol/litre}$ in NaCl and $a_1 = 0.27 \text{ nm}$ (Hunter, 1960), the zeta potentials in the presence of Na^+ ions at physiological ionic strength would be -7 mV (P388-F36), -9 mV (CH23) and -8 mV (PCM1).

The effect of La^{3+} ions on the zeta potentials is distinctly different from the effect of Ca^{2+} ions in that there is a large decrease over a range of almost constant Debye length followed by a charge reversal. In this case there is no linear relationship between ζ and $1/\kappa$, and we conclude that the La^{3+} ions are interacting specifically with sites on the cell surface. From these data the number of La^{3+} -binding sites can be estimated. Specific binding will give rise to a change

Fig. 6. Effect of trypsin on the percentage aggregation of mouse lymphoma cells as a function of La^{3+} concentration under static conditions

The cells were suspended in a background medium of 17.2 mM-NaCl plus 9.4% (w/v) sucrose. ●, P388-F36 cells plus $\text{La}(\text{NO}_3)_3$ (15 min incubation); ○, P388-F36 cells plus $\text{La}(\text{NO}_3)_3$ (15 min incubation), followed by the addition of $0.0323 \mu\text{g}$ of trypsin (Sigma)/ml; the percentage aggregation was measured 5 min after the addition of the trypsin.

Table 1. Biochemical characterization of cell lines

'Carbohydrate' denotes anthrone-sensitive material liberated by trypsin treatment, expressed as μg of glucose equivalents. The errors given are the S.E.M.; where no error is given the value refers to a single determination. The numbers of determinations are given in parentheses.

Cell line ...	Content		
	P388-F36	CH23	PCM1
Surface sialic acid ($\mu\text{mol}/10^8$ cells)	0.006 ± 0.002 (4)	0.021 ± 0.002 (3)	0.031 ± 0.002 (3)
Surface carbohydrate ($\mu\text{g}/10^8$ cells)	5 ± 2 (3)	50	105
Total carbohydrate ($\mu\text{g}/10^8$ cells)	26	237	345
Total protein ($\text{mg}/10^8$ cells)	10 ± 1.5 (4)	15 ± 2.4 (4)	16 ± 2.5 (4)

Fig. 7. Variation of zeta potential (ξ) with Debye length ($1/\kappa$) for cells

The conditions were as described for Fig. 4. ●, P388-F36 cells; △, CH23 cells; ○, PCM1 cells; △, denotes overlapping points for CH23 and PCM1 cells. The points at 0.5 nm coincide for the three cell lines.

($\Delta\sigma$) in the surface charge density which over the region of approximately constant ionic strength ($[\text{La}^{3+}] = 1 \mu\text{M} - 1 \text{ mM}$) can be related to the number of binding sites, N_1 , and the electrochemical free energy of adsorption, $\Delta\bar{G}$, by the equation (Ottewill *et al.*, 1960):

$$\Delta\sigma = \frac{k_1 c}{1 + k_2 c} = \frac{2\sqrt{2} \cdot kT \epsilon_0 \epsilon_r \kappa}{eV} \left(\sinh \frac{ve\xi^0}{2kT} - \sinh \frac{ve\xi}{2kT} \right) \quad (6)$$

where $k_1 = veN_1k_2$ and $k_2 = \exp(-\Delta\bar{G}/RT)/5.56 \times 10^4$.

In eqn. (6) v is the valency of the counterions (here 3) and c is their concentration. ξ^0 is the initial zeta potential of the cell and ξ is the potential at concentration c . It should be pointed out that in the formulation of eqn. (6) the measured zeta potentials are assumed to be approximately equal to the surface potentials. However, this is a good approximation for the low zeta potentials, considered here. Taking the zeta potentials at $[\text{La}^{3+}] = 1 \mu\text{M}$ and $100 \mu\text{M}$ and at $[\text{La}^{3+}] = 1 \mu\text{M}$ and 1 mM , the two simultaneous equations in k_1 and k_2 can be solved and the number (N_1) and free energy ($\Delta\bar{G}$) of the La^{3+} -binding sites calculated. The calculations give the following values for N_1 (sites/ m^2): $1.8 \pm 0.1 \times 10^{14}$ (P388-F36), $2.5 \pm 0.1 \times 10^{14}$ (CH23) and $2.2 \pm 0.1 \times 10^{14}$ (PCM1). The corresponding values of $\Delta\bar{G}$ (kJ/mol) are -38 ± 4 , -37 ± 4 and -36 ± 4 respectively. The errors given are based on a mean standard deviation of $\pm 1.9 \text{ mV}$ in the zeta potentials.

Cellular aggregation

The failure of Ca^{2+} ions to increase appreciably aggregation of the cells (Fig. 3), even though the ions compress the electrical double layer and decrease the zeta potential, strongly indicates that stability of these cell dispersions is not predominantly controlled by non-specific electrostatic forces. This conclusion is further substantiated by the observation that decrease in the net surface charge by neuraminidase has no effect on the P388-F36 and PCM1 cells and only a small effect on the aggregation of the CH23 cells.

The La^{3+} -induced aggregation (Fig. 4) occurs at a La^{3+} concentration which coincides with the large decrease in zeta potential. With the exception of the CH23 cells, the extent of aggregation is not significantly affected on treatment with neuraminidase, implying that the specific adsorption of La^{3+} ions by the cells probably does not occur at peripheral sialic acid sites. In contrast with the PCM1 and P388-F36 cells, the aggregation of CH23 cells by La^{3+} ions is enhanced by neuraminidase treatment. Enhancement of aggregation after neuraminidase treatment has been previously observed (Lloyd & Cook, 1974). This observation, together with the fact that the untreated cells only aggregate to the extent of 45%, implies that the surface sialic acid groups probably play some part in decreasing the extent of aggregation.

Several molecular mechanisms by which the cells may be aggregated by La^{3+} ions can be envisaged. (1) The metal ions may bind at sites near neighbouring carboxyl groups on the cell surface, forming a bridge between them (changing the local net charge from -2 to $+1$) and facilitating cell-cell interaction; (2) binding at such sites may induce conformation changes in membrane-bound proteins or glycoproteins, perhaps exposing hydrophobic groups which facilitate cell-cell interaction (Chothia & Jamin, 1975); (3) direct bridging could occur between two carboxyl groups on different cells by the La^{3+} ions. Apart from the above possibilities it is known that La^{3+} ions bind strongly to some phospholipid liposomes, in particular those formed from phosphatidylserine and phosphatidylcholine. The binding site is believed to be the phosphodiester group (Hauser *et al.*, 1975).

One possible explanation for the instantaneous aggregation induced by macromolecules in the presence of La^{3+} ions (Fig. 6) is the formation of macromolecular bridges between cells. This aggregation effect seems only to require the addition of a macromolecule to cells which have had their gross negative surface-charge density decreased by adsorption of La^{3+} ions. It does not occur when only the zeta potential is lowered at constant surface charge density, as the experiments for cells incubated with Ca^{2+} showed. Since under the conditions of these experiments (near neutral pH) trypsin is positively charged,

Table 2. *Comparison of cellular properties*

Property	Cell line	...	Mouse P388	Hamster CH23	Hybrid PCMI
Zeta potential (ξ) $I = 0.0172$ mol/litre			-17 mV	-22 mV	-20 mV
Isoelectric point (pI)			3.5	3.5	3.5
Effect of Ca^{2+} on ξ			All decrease below $[\text{Ca}^{2+}] = 1$ mM		
Effect of La^{3+} on ξ			All decrease below $[\text{La}^{3+}] = 10$ μM		
Aggregation at $[\text{Ca}^{2+}] = 10$ mM*			0	+10%	+10%
Aggregation at $[\text{Ca}^{2+}] = 10$ mM after neuraminidase treatment			0	+10%	+10%
Maximum aggregation by La^{3+}			+70%	+30%	+40%
Maximum aggregation by La^{3+} after neuraminidase treatment			+70%	+70%	+30%
Aggregation in NaCl ($I = 0.1$ mol/litre)			0	+20%	+20%
$10^{-8} \times$ No. of sialic acid molecules released per cell			0.37	1.3	1.9
$10^{-8} \times$ No. of glucose equivalent residues released per cell			1.7	16	35
$10^{12} \times$ Apparent cell area (m^2)			314	804	616
$10^{-17} \times$ Glucose equivalent residues per m^2			5.4	20	57
Karyotype (no. of chromosomes)†			36	23	2-4 mouse +19 hamster

* + denotes an increase in the extent of aggregation above the control value.

† Ayad & Delinassios (1974).

β -lactoglobulin and bovine serum albumin are negatively charged, and dextran is neutral, the electrostatic state of the macromolecule is not of major significance in the process.

Comparison of cellular properties

To clarify comparison between the three cell lines that we have studied, some of the most important results are summarized in Table 2. The electrophoretic behaviour of all the cells in the presence of Ca^{2+} ions adequately conforms to that expected for negatively charged particles surrounded by diffuse electrical double layers; the effects of La^{3+} ions are also consistent with such a model in which specific adsorption occurs. In general the two fibroblastic cells have a greater overall tendency to aggregate. The induction of aggregation by high-ionic-strength NaCl is to some extent consistent with electrical-double-layer effects. It is noteworthy that the hybrid cell resembles one parent (CH23) to the extent by which it is aggregated by La^{3+} ions, but resembles the other (P388-F36) in that La^{3+} -induced aggregation is not affected significantly by neuraminidase treatment. Comparison of the number of glucose equivalent residues per cell shows that the fibroblastic cells have more trypsin-sensitive surface carbohydrate than do the mouse lymphoma cells, both in absolute terms and in terms of the number of residues per unit area of cell surface. Hence it is tempting to attribute the greater tendency of the fibroblastic cells to aggregate and to grow as monolayers to the presence of surface carbohydrate, and to suggest that it is possibly involved in bridging between the cells. The bridging mechanism suggested

to explain the aggregation of erythrocytes by dextrans (Chien & Jan, 1972, 1973*a,b,c*), and the induction of aggregation of the P388-F36 cells by macromolecules would support such a hypothesis. However, there are reservations to this proposal. First, the values for surface-carbohydrate content only relate to trypsin-sensitive material (most of this must come from the cell surface, but there is always the possibility of cell lysis, resulting in the presence of intracellular carbohydrate), and, secondly, the true surface areas of the cells are not known. Scanning electron micrographs of cells in general show that their surfaces, even at a resolution considerably below the molecular level, are often ruffled and covered with microvilli (Shay *et al.*, 1974; Knutton *et al.*, 1975; Collard & Temmink, 1975). It follows that the true surface areas will be much greater than those calculated from the measured volumes on the assumption that the cells are spherical. Since both these factors have opposite effects on the calculation of the number of glucose equivalent residues per unit area of cell surface, it is difficult to assess how much reliance can be placed on the results.

The most dramatic effect that we have observed in these studies is the rapid induction of aggregation by La^{3+} ions. More recently the action of lanthanides in biological systems has received considerable attention (Haskar *et al.*, 1976; Darnall & Birnbaum, 1973; Abbot *et al.*, 1975; Gomez *et al.*, 1974). Our observations indicate that the effect of La^{3+} ions on cell aggregation may be a very general phenomenon, in contrast with the action of concanavalin A, where a diversity of results have been reported (Berman, 1975; Glimelius *et al.*, 1975; Duzgunes, 1975; Kao & Harris, 1975; Marikovsky *et al.*, 1974).

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