STRUCTURAL AND FUNCTIONAL HETEROGENEITY OF THE SURFACE OF RAT LEUKEMIA CELLS

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ABSTRACT

Rat leukemia cells IRC 741 in suspension culture form single cytoplasmic protrusions by which the cells preferentially adhere to one another. The induction and/or maintenance of these protrusions is sensitive to changes in intercellular contact, pH, temperature, and nutritional conditions. The protrusions are stable, rigid structures which take part in intercellular adhesion but not in adhesion to substrata. Movement on substrata occurs by means of ruffling membranes formed on the main cell body. This asymmetry in cellular form and function is associated with specialized cell surface regions. Ultrastructurally, the cell surface over the protrusions lacks microvilli, and is covered with a 3,000–4,000-Å thick cell coat consisting of 200–500-Å electron-dense particles in an amorphous matrix. In contrast, the surface over the main cell body has microvilli and a 200-Å wide cell coat which lacks particles. The extracellular particles overlying the protrusions have electron-lucent cores, are protease- and pepsin-resistant, and do not stain with colloidal iron, while the matrix in which they are embedded is sensitive to proteolytic enzymes and contains acidic moieties. The negative surface charge density over the protrusions is higher than that over the main cell body, as shown by the orientation of the cells in an electric field. The unexpected observation that a region of higher charge density is one of increased intercellular adhesiveness might be explained, in part, by the rigidity of the protrusions and by the very small radius of curvature of the overlying extracellular particles. The protrusions permit the observation of discrete regions, differing in charge density, on the surface of living leukemia cells.

One of the difficulties in relating specific aspects of the behavior of cells to their surface characteristics lies in the structural and functional heterogeneity of the cell surface. Topological differences in chemical composition, charge density, or conformation of the plasma membrane, and the related distribution of sites of adhesive, locomotor, or metabolic activities at the cell periphery are fre-

quently difficult to identify. In solid tissues or monolayer cultures where major structurally and functionally distinct cell surface regions exist, difficulties arise because the cells have to be dissociated before study and thereby their surface properties are altered. On the other hand, cells which grow singly in suspension, including leukemic and leukocytic cultures, (6, 7), are charac-

teristically symmetrical in shape and surface properties, and here the dispersed nature of specialized sites on the cell surface is one of the main problems encountered in attempts to correlate different functions with specific cell surface regions.

We found that some of these difficulties could be overcome by using, as a model, suspension cultures of the leukemic cell line IRC 741 (10). This line seemed of particular interest because while most IRC cells in suspension assumed a round shape, a significant percentage of them had a stable cytoplasmic protrusion by which the cells preferentially adhered to one another. This unusual asymmetry in shape and adhesiveness of the IRC cells was indicative of extensive specialized cell surface regions and suggested that their surface might also be asymmetric in chemical composition, ultrastructure, or surface charges. An attempt was therefore made to correlate specific functional characteristics of the cells with specific areas on their surface and to characterize these areas chemically and morphologically. As IRC cells in suspension culture grow singly or as loosely coherent cell clumps, it was possible to study their surfaces without prior enzymatic or chemical dissociation.

The investigation of the morphology and properties of the IRC cells reported here shows that the proportion of cells with cytoplasmic protrusions is influenced by several environmental factors, including nutritional conditions, temperature, and intercellular contact. Furthermore, the protrusion appears to be the preferred region for intercellular adhesion but not for cell-substratum adhesion, and it is characterized by an unusually thick and complex cell coat. Finally, it was found that the cell surface overlying the protrusion appears to have a higher negative charge than does the remainder of the cell body.

MATERIALS AND METHODS

Cell Culture

Line IRC 741 is derived from a transplantable, acute leukemia which arose spontaneously in an inbred line of Fischer rats (10) and has been maintained in our laboratory as a tissue culture line for several years. The IRC cells were grown as stationary suspension cultures in serum bottles at 37°C in Fischer's medium supplemented with 10% horse serum, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. Medium was changed twice weekly. The cells were routinely subcultured every 3 days at an initial density of 2×10^4 /ml. Their doubling time was approximately 12 h.

Effects of Environmental Factors on the Incidence of Asymmetric (Pear-Shaped) Cells

In all experiments dealing with environmental effects on the incidence of cells with cytoplasmic protrusions, IRC cells were grown at an initial density of 1×10^4 cells/ml of medium, and the proportions of viable pear-shaped cells and of dead cells were determined at intervals by cell counts on glutaraldehyde-fixed, alcian blue-stained samples (38). Approximately 1,000 cells per sample were counted from each culture.

CULTURE AGE

Cells were grown in series of six culture bottles, without renewal of medium, for 5 days. Samples from each bottle were fixed daily for cell counts.

RENEWAL OF GROWTH MEDIUM

Cells were divided among two groups of culture tubes and incubated on a roller drum (4 rpm). Every 24 h, over 5 days, four tubes from each group were fixed for cell counts while the remaining tubes were centrifuged at low speed. The medium in one group of tubes was renewed daily for 4 days while the cells in the control group were resuspended in their original medium.

INTERCELLULAR CONTACT

Cells were grown, in addition to the standard stationary cultures, in spinner culture flasks to reduce intercellular contact and in 50-ml Erlenmeyer flasks on a gyratory shaker at 70–80 rpm to increase the amount of contact between cells over that in stationary cultures (18). Samples were fixed daily over 3 days for cell counts. During this period the medium was not changed to avoid disturbing intercellular relationships. Cultures grown on the gyratory shaker showed a consistent increase in the proportion of pear-shaped cells over the stationary suspension cultures, and therefore cells maintained for 48 h in the shaker system were used for the subsequent experiments on temperature and pH effects.

TEMPERATURE

Cells grown at 37°C in gyratory shaker cultures were dispersed, transferred to spinner culture flasks (to minimize effects of intercellular contact), incubated at either 4°C, 25°C, or 37°C for up to 5 h, and then restored to 37°C. Samples for counts were fixed every hour during the cooling and reheating period.

EFFECT OF PH

Cells from gyratory shaker cultures were suspended in Hanks' balanced salt solution (BSS) which had been adjusted to pH 6.5, 7.4, or 8.0, and incubated at 37°C for periods up to 3 h, and samples were counted every hour.

CELL ELECTROPHORESIS

IRC cells, washed with and suspended in serum-free medium, were transferred to a cylindrical chamber equipped with Ag/AgCl electrodes (28) at 25°C. The movements and the orientation of the protrusion and the cell body of the pear-shaped cells in an electric field at 50–60 V were observed with the built-in microscope.

TIME-LAPSE CINEMATOGRAPHY

IRC cells were transferred to Sykes-Moore chambers (25). Pictures were taken through an inverted Wilde microscope inside a Prior incubator at 37°C, with Kodak double-X negative black and white 16-mm film (Eastman Kodak Co., Rochester, N.Y.) in a Bolex movie camera (Kern-Paillard, La Chaux-de-Fonds, Switzerland) at speeds varying from 1 to 10 frames per minute.

Ultrastructure and Cytochemistry

ELECTRON MICROSCOPY

Cells were fixed by adding 0.1% glutaraldehyde in phosphate buffer to an equal volume of cell suspension in culture medium at pH 7.3 at room temperature. After 5 min, the cells were centrifuged and the resulting cell pellet was covered with 2.5% glutaraldehyde for another 30 min, washed in buffer, postfixed for 15 min with 1% OsO₄, and washed again. The fixed cells were then covered with 5% agar (12), dehydrated through graded alcohols and propylene oxide, and embedded in Epon 812.

Epon sections about 0.5- μ m thick were stained with 1% toluidine blue in 1% borax for light microscopy. For electron microscopy, silver-gold sections were mounted on carbon-coated grids, stained with uranyl acetate (31) and lead citrate (29), and examined with a Hitachi HS-7S electron microscope.

ACID MUCOPOLYSACCHARIDES

IRC cells were fixed as for electron microscopy, stained overnight with colloidal iron (21) at pH 1.9, washed with 25% acetic acid at pH 2.2, and then processed and embedded as for electron microscopy (37). For light microscopy, 0.5–1.0- μ m thick-sections were treated with 10% potassium ferrocyanide in 10% HCl for 60 min (26). For the ultrastructural demonstration of acid mucopolysaccharides, ultrathin sections, fixed, stained with colloidal iron, and embedded as above, were examined electron microscopically without further staining.

EFFECT OF PROTEOLYTIC ENZYMES

TRYPSIN: Living IRC cells were treated with 0.12% crystalline trypsin (1:250, Difco Laboratories, Detroit, Mich.) in Hanks' BSS for 30 min at pH 7.2 at 37°C, fixed and processed for electron microscopy as above, and examined after staining with either uranyl acetate

and lead citrate, or with colloidal iron for acid mucopolysaccharides.

PROTEASE AND PEPSIN: Uncoated gold grids (300 mesh) bearing silver ultrathin sections of Epon-embedded IRC cells were immersed in 3–4 ml of 5% H₂O₂ at 37°C for 10–20 min (1). After the oxidation, the grids were thoroughly washed in distilled water and then were immersed in 1–2 ml of one of the following solutions at 37°C for 1–4 h: (a) protease (bovine pancreatic, partially purified, Nutritional Biochemicals Corp., Cleveland, Ohio), 0.03% solution in phosphate buffer at pH 6.8 (9). Control: in same buffer at pH 6.8. (b) pepsin (three times crystallized, Nutritional Biochemicals), 0.2 and 0.5% in 0.1 N HCl. Control: 0.1 N HCl (1).

RESULTS

Morphology of Living Cells

All cultures of IRC leukemia cells contained a fraction of asymmetric pear-shaped cells, i.e., of cells with a single cytoplasmic protrusion. The protrusions varied in size from about one-third to one-eighth of the diameter of the main cell body. Cytoplasmic material was asymmetrically distributed, with most particulate organelles at the side of the protrusions (Fig. 1). The cells tended to adhere to one another even at very low densities, at which stage small aggregates predominated, consisting of two to four cells characteristically joined together by their protrusions (Fig. 2). As the cell density increased, larger cell clumps of no definable organization appeared in increasing numbers (Fig. 3).

Although a varying proportion of pear-shaped cells was found in every IRC culture, these asymmetric cells never comprised all or none of any one

FIGURE 1 Aggregate of IRC 741 cells. Organelles are concentrated near the cytoplasmic protrusions (P). Arrows indicate microspikes on the main cell body. Living cells, phase microscopy. $\times 1,800$.

population. As the average cell density increased after subculture, from 1×10^4 /ml on day no. 1, to $5-6 \times 10^5$ /ml on day no. 3, the proportion of pear-shaped cells rose concomitantly from about 10% to about 40%. If the medium in the cultures was not renewed, then after three days both the density of live cells and the proportion of pear-shaped cells decreased as the cultures aged. Dead cells (by dye exclusion) were always round rather than pear-shaped, and appeared in increasing numbers when the total cell density exceeded $5-6 \times 10^4$ (Fig. 4).

Effects of Environmental Factors

(All data presented in this section are corrected for cell death, i.e., they refer to live cells only).

RENEWAL OF GROWTH MEDIUM: As shown in Fig. 5, there appeared to be little difference in the proportion of pear-shaped cells between cultures grown with or without daily renewal of culture medium, until the cell density reached about 5×10^5 /ml. Beyond this point, the absolute number as well as the proportion of pear-shaped cells in cultures with daily renewal of growth medium greatly exceeded those of cultures maintained for 4 days without medium renewal.

pH: Upon incubation in Hanks' BSS for 3 h at different levels of pH, there was a significant

reduction in the proportion of pear-shaped cells when the pH was raised from 7.4 to 8.0. If the pH was lowered from 7.4 to 6.5, the proportion of pear-shaped cells remained unchanged or increased slightly (Fig. 6).

INTERCELLULAR CONTACT: Differences in the extent of intercellular contact, induced by

FIGURE 4 Change in cell number and in the proportion of pear-shaped IRC 741 cells with culture age, in the absence of medium renewal. Density of living (O—O) and dead (●—●) cells (means of six cultures) and proportion of pear-shaped cells (X—X) (means $\pm$ SE of six cultures).

FIGURE 2 Low-density culture of IRC 741 cells. Single cells with protrusions (arrows), some forming small aggregates. Living cells, standard optics. $\times 100$.

FIGURE 3 High-density culture of IRC 741 cells. Formation of large cell clumps (see Fig. 14 for ultrastructure). Living cells, standard optics. $\times 100$.

FIGURE 5 Changes in the number of round IRC 741 cells (open areas) and pear-shaped IRC 741 cells (shaded areas) with daily changes of medium (c) and in unchanged medium (u). (Data presented in Figs. 5-8 are corrected for cell death, i.e., proportions, cell densities, and cell numbers refer to live cells only).

modifications of the culture system, also affected the proportion of cells with cytoplasmic protrusions. At comparable cell densities, the proportion of pear-shaped cells was consistently highest in gyratory shaker cultures, i.e., under conditions where cell contact was enhanced, lowest in spinner flasks where cell contact was most limited, and intermediate in stationary cultures with intermediate degrees of cell contact (Fig. 7). Spinner cultures contained the highest number of dead cells.

TEMPERATURE: In response to a temperature reduction from 37°C to either 25°C or 4°C, the proportion of asymmetric cells dropped rapidly over 1 h, when it leveled off and maintained a steady level for up to 5 h. If at any point during this period of time the temperature was restored to 37°C, then the proportion of pear-shaped cells rose again, sometimes approaching the original level within 1 h (Fig. 8). Contrary to possible expectations, there was no difference in the magnitude or in the reversibility of this change between samples cooled to 25°C and samples cooled to 4°C.

FIGURE 6 Changes in the proportion of pear-shaped cells after incubation of IRC 741 cells at 37°C for 3 h in Hanks' BSS adjusted to pH 6.5, 7.4, and 8.0. Star, proportion of pear-shaped cells in the culture before incubation in the three test solutions. Means $\pm$ SE of six cultures.

FIGURE 7 Effect of cell contact on the proportion of pear-shaped cells. Groups of five cultures, from same original cell population, grown for 3 days in stationary suspension culture (x), gyratory shaker culture (●), or spinner-flask culture (○). Lines represent changes of mean values for each group of cultures over 3 days.

Cell Electrophoresis

The electrophoretic movement of the cells is illustrated in Fig. 9. When there was no electric potential applied through the medium, the asymmetric cells sank slowly in response to gravity, with the protrusion on the top. When a voltage gradient of 50–60 V was applied, the cells were attracted to the anode in a direction which was the

FIGURE 8 Change in the proportion of pear-shaped cells with changes in environmental temperature. $\blacktriangle$, control cultures at 37°C, $\bullet$, at 25°C for 4 h, then restored to 37°C. Each curve represents a spinner culture.

FIGURE 9 The change in orientation of the protrusion of IRC cells with the change of the electrical polarity in cell electrophoresis. (A), no electrical current, (B), (C), and (D) illustrate changes of polarity. Large broken arrow, gravitational force; large solid arrows, electrical attractive force; small arrows, resultant direction of cell movement.

resultant of the gravitational and the electrical attractive forces. However, in addition to this standard response, the cells underwent another unusual series of movements: as the whole cell was moving, the protrusion of the IRC cells gradually turned further towards the anode than the cell body. Thus the protrusion would turn left or right in response to changes in the polarity of the current. The degree of turning, i.e., the angle of deflection from the vertical, seemed to vary with the size and shape of the protrusion, and the time for assuming the maximum angle, although also variable, was approximately 1 min.

This peculiar behaviour of the protrusion was recognized in over 90% (34/37) of the observed pear-shaped cells on two separate occasions and indicated that the electrophoretic mobility of the protrusions exceeded that of the opposite side of the cells, i.e., the main cell body.

Cinematography

The characteristic type of locomotion of IRC cells was gliding motion (2). This movement was associated with the formation of a ruffled membrane at the leading edge of the cytoplasmic mass while the remainder of the cell followed in a rather rigid fashion. Microspikes appeared and disappeared rapidly on the main cell body. In contrast, the protrusion of pear-shaped cells remained in the same location, although its size fluctuated and although it occasionally seemed to disappear momentarily and then reform at the same site. There were no microspikes on the surface of this part of the cells. On single migrating cells, the protrusions were usually at the back, but they were also seen on the side, front, or top of moving cells. Protrusions did not appear to take part in adhesion to the substratum, and they frequently appeared as rigid structures carried by cells in a position raised above the substratum. They disappeared before the onset of mitosis and were not observed on daughter cells immediately after cell division.

Collisions between single cells, occurring during random movement, were almost invariably followed by intercellular associations of some duration rather than by immediate cell separation. When pear-shaped cells collided, they remained attached to one another by their protrusions if these were the sites of initial contact. If the protrusion of one cell first made contact with the main body of another pear-shaped cell, an adhesion also resulted but appeared less stable, as

rearrangements in the positions of the two cells relative to one another usually continued until they were again joined by their protrusions (Fig. 10). This type of contact seemed quite stable and was maintained, sometimes for hours, in spite of the vigorous locomotion of the adhering cells. Aggregates of two to four cells thus formed moved on the substratum as units while adhering by their protrusions. Small aggregates in turn collided and formed larger cell clumps so that with time, the cell populations consisted of actively moving cell aggregates of increasing size. It appeared thus, on the basis of cinematographic observations, that the cells interacted with the substratum only by the surface overlying the main cell body, and that the rigid cytoplasmic protrusions were the preferred sites of relatively long-lasting and stable intercellular adhesion.

Ultrastructure

On sections through the pear-shaped cells, most mitochondria, microtubules, and microfilaments were accumulated in or near the protrusion while the remainder of the cytoplasm was occupied by polyribosomes and by a large nucleus with a single nucleolus (Fig. 11). The cells contained many mitochondria, some short sections of rough endoplasmic reticulum, and only occasional lysosomes and Golgi bodies. Microtubules tended to be oriented along the long axis of the protrusion (Fig. 12), while no particular organization was noted among microfilaments. The cell surface overlying the protrusions lacked microvilli, but in most cells was covered by aggregations of 200–500-Å electron-dense extracellular particles (ECP) which were embedded in a thick cell coat and were separated from the plasma membrane by a 100-Å electron-lucent space (Fig. 12). In contrast, the cell surface over the main cell body formed numerous microvilli but lacked overlying ECP (Fig. 13). Secondary protrusions were occasionally seen (Fig. 13), but they lacked the adjacent accumulation of organelles or the ECP on the surface, and did not seem to participate in cell adhesion.

Sections cut through small cell aggregates showed cells adhering only by their protrusions with an interposed layer of ECP (Fig. 13), while sections cut through large aggregates showed three types of cell contacts: (a) protrusion to protrusion, with interposed ECP; (b) protrusion to main cell body, also with interposed ECP; and (c) adhesion between the main cell bodies of two cells with

FIGURE 10 The interaction of a single pear-shaped cell with an aggregate of three cells is illustrated here by composite diagrams made by tracing the images from a time-lapse movie on the screen of a film editor. The numbers indicate the positions of the approaching cell in different frames. The time interval between consecutive numbers is 6 s. In (A), a single cell advances with its protrusion in front at various speeds (1–3, 0.5 $\mu\text{m/s}$; 3–4, 1.2 $\mu\text{m/s}$; 4–5, 1.7 $\mu\text{m/s}$). Then the protrusion turns towards the adhering protrusions until it joins them (B). But the adhesion is not yet stable. The newcomer separates and rejoins the group several times (C)–(E) until finally a stable association is accomplished which lasts about 1 min (not shown). (F) depicts the same cell moving away from the group. The speed was approximately 0.1 $\mu\text{m/s}$.

interdigitating microvilli but no ECP (Fig. 14). These observations suggest that intercellular adhesion took place preferentially over surface regions covered with ECP, but once these were occupied by adjoining cells, further aggregation into larger cell clumps occurred through contact via interdigitating microvilli.

ACID MUCOPOLYSACCHARIDES: Light-microscope sections containing colloidal iron-stained cells treated with ferrocyanide showed the presence of mucopolysaccharides over the whole cell surface but stained particularly heavily at the area surrounding the protrusions (Fig. 15). Ultrastructurally, the cell coat over the main cell body showed a rather sparse layer of colloidal iron particles, which resembled in amount and distribution the iron staining of the cell coat of the normal

macrophage (4). Within the cell coat overlying the protrusion, however, there was a heavier deposition of iron particles in the matrix between the ECP, although the particles themselves did not stain (Fig. 16). On the surface of microvilli and of protrusions, filamentous structures were observed which were not visible after staining with lead citrate and uranyl acetate only (Fig. 17).

EFFECTS OF PROTEOLYTIC ENZYMES: Treatment of live cells with 0.12% trypsin before fixation and colloidal iron staining did not abolish the asymmetry of pear-shaped cells, but did remove the ECP and the surrounding matrix from the surface of the protrusions. A sparse layer of iron particles remained in this region, resembling the staining over the remainder of the cell surface which did not seem to be altered by trypsin treatment (Fig. 18). Treatment of ultrathin sections at 37°C for 1 h with either protease in phosphate buffer at pH 6.8, 0.1 N HCl, or pepsin in 0.1 N HCl at pH 1.2–1.5 removed the amorphous matrix from among the extracellular particles (Fig. 19). This change was not observed after treatment with the buffer alone. The particles themselves became more irregularly shaped with definite electron-lucent round areas in the center (Figs. 20, 21). No further changes in the morphology of the particles were observed over 4 h of treatment, although by the end of that period the mitochondrial matrix and some of the nonparticulate cytoplasm were removed. No difference was observed between the results of the three types of treatment. Thus the extracellular particles differed both in colloidal iron reactivity and in sensitivity to proteolytic enzymes from the surrounding matrix, which appeared to be mucoprotein in nature.

DISCUSSION

The cytoplasmic protrusions formed by IRC 741 leukemia cells are responsible for the characteristic pear shape of the cells and represent sites of differential adhesiveness. This cellular asymmetry does not appear to be a temporary *in vitro* modification, because microscopically and ultrastructurally similar cytoplasmic protrusions were

found in IRC 741 cells maintained in this laboratory as ascites tumors in rats, and in cells frozen several years ago. In addition, a photograph of the original leukemia cells, i.e., the *in vivo* ancestor of line IRC 741, shows asymmetric cells similar to those found in current cultures (10).

The protrusions on the IRC cells represent a type of localized peripheral differentiation which is particularly unusual for cells growing in suspension culture. It is of interest in this regard, however, that embryonic neural retinal cells dissociated *in vitro* retain a somewhat similar type of cellular polarity and reaggregate into multicellular structures whose intercellular organization resembles that of IRC cell aggregates (23). Morphologically and functionally, the cytoplasmic protrusion in IRC cells resembles the "tail" or "uropod" of lymphocytes (17, 24), although in the latter there is no cell coat specialization similar to that of IRC cells. Instead, uropods form numerous long microvilli which are usually in contact with cellular debris, the surface of the culture vessel, and other cells in the environment. Thus it is apparent that although both the protrusion of IRC cells and the uropod of lymphocytes are structures serving for intercellular adhesion, the underlying mechanisms are probably different in these two systems. Some of the unusual surface characteristics of the leukemic IRC cells might represent normal tissue-specific properties retained from a normal leukocytic precursor cell. This possibility is suggested by the presence of somewhat similar cell coat specializations found on adherent cells in aggregates of normal macrophages (3).

The fact that the proportion of pear-shaped IRC cells in otherwise round cell populations first increased and subsequently dropped with the age of the cultures suggested a connection between the presence of the protrusion and at least three environmental factors, i.e., pH, cell density, and nutritional supply. Experiments designed to investigate these variables separately tended to rule out the lower pH of old, crowded cultures as a contributing factor; however, they indicated that the increase in the incidence of pear-shaped cells,

FIGURE 11 Cross section through a pear-shaped cell. Asymmetric distribution of organelles, with the particulate organelles accumulated on the side of the cytoplasmic protrusion. Microvilli (*mv*) are prominent over the main cell body. *f*, microfilament bundle; *m*, mitochondria. $\times 10,000$.

FIGURE 12 Centre of small cell aggregate. Adhesion by protrusions which are covered with extracellular particles (ECP). These are separated from the plasma membrane by an electron-lucent space (arrows). *mt*, microtubules. $\times 13,000$. *Insert*: higher magnification of cell periphery showing ECP, electron-lucent space, and plasma membrane. $\times 30,000$.

FIGURE 15 Plastic section through cells stained with colloidal iron before embedding. Treatment of sections with 10% KCN-10% HCl demonstrates a thin layer of mucopolysaccharides over the whole cell surface and a thick, filamentous, colloidal iron-positive cell coat surrounding the cytoplasmic protrusions (arrow). $\times 1,000$.

occurring in proportion to cell density, was the result of increasing degrees of intercellular contact and this relationship continued as long as there was an adequate supply of nutrients. It is not possible to state on the basis of the available data whether intercellular contact augmented the proportion of pear-shaped cells by initiating the formation of new cytoplasmic protrusions, or by stabilizing preexisting protrusions.

Temperature reduction also decreased the proportion of asymmetric cells. In view of its rapid reversibility, it is unlikely that this temperature effect was due to diminished synthetic activity alone. The pattern observed would be more in keeping with temperature-dependent conformational changes, either at the cell periphery or within the protrusion. A similar mechanism has been suggested as the possible basis for the temperature-dependent changes in surface charge density,

FIGURE 16 Electron micrograph of a cell stained with colloidal iron. The protrusion is covered with a cell coat, 3,000-4,000-Å thick (I-bar). Unstained extracellular particles (p) are embedded in an iron-positive matrix. The surface of the remaining part of the cell (arrow) is covered with a sparse layer of iron particles. $\times 2,400$.

FIGURE 17 Same preparation as Fig. 16. Filamentous structures are present on the surface of the protrusion (arrows). $\times 40,000$.

FIGURE 13 Two cell aggregate, adhesion by protrusions. Extracellular particles (ECP) are localized over the protrusions and absent over the remaining cell surface. (SP), secondary protrusion. $\times 8,000$.

FIGURE 14 Three types of adhesion in a large cell clump: protrusion to protrusion (PP), protrusion to body (PB), and body to body (BB). The latter (BB) lacks interposed extracellular particles and involves interdigitating microvilli. $\times 12,000$.

FIGURE 18 Trypsinized IRC cell stained with colloidal iron. Most of the extracellular coat overlying the protrusion is removed (arrow points to a remnant of it). A sparse layer of colloidal iron-positive material remains over the protrusion (P) and over the main cell body. $\times 18,000$.

FIGURE 19 Epon-embedded thin section of a cytoplasmic protrusion. The section was immersed for 1 h in 0.5% pepsin/0.1 N HCl, and poststained with lead citrate and uranyl acetate. Extracellular particles overlying the protrusion persist. $\times 12,000$.

FIGURE 20 Epon-embedded thin section of a cytoplasmic protrusion. The section was immersed for 1 h in 0.03% protease-phosphate buffer and poststained with lead citrate and uranyl acetate. The amorphous matrix of the cell coat has been removed by protease treatment, but the extracellular particles persist and show electron-lucent centers (arrows). $\times 32,000$.

FIGURE 21 Control to Fig. 20. This section was immersed for 1 h in phosphate buffer and poststained with lead citrate and uranyl acetate. The extracellular particles remain embedded in an amorphous matrix and their electron-lucent centers are indistinct. $\times 32,000$.

observed in some types of cultured cells (33). Among specific structures involved in the temperature effect on IRC cells, microtubules should be considered, because they are important in the maintenance of asymmetric cell shapes (14), are known to be cold-sensitive (22), and occurred as parallel bundles along the long axis of the protrusions of IRC cells. The mechanism underlying the reappearance of protrusions after restoration of temperature to 37°C seems to require little if any intercellular contact, since it takes place in spinner cultures. It is possible that the specialized cell coat overlying protrusions before cooling persists when the cells become round at reduced temperatures, and that this localized surface modification initiates the reversion to the asymmetric shape upon return to 37°C.

The most striking ultrastructural observation was the presence of a complex cell coat overlying the protrusion and consisting of 200–500-Å electron-dense particles embedded in a thick (3,000–7,000-Å) matrix. By means of colloidal iron staining, acid mucopolysaccharides were shown to be present over the whole surface of the IRC cells as well as in the matrix over the protrusion. The staining over the cell body was similar in amount and distribution to that of normal macrophages (4), but the region localized over the protrusion stained more intensively. The removal by trypsin treatment of the thick coat covering the protrusion of live cells, and the digestion in plastic sections of the cell coat matrix by protease, HCl, and pepsin, indicate the presence of a proteinaceous moiety in this region. After trypsin treatment, there still remained a thin layer of acid mucopolysaccharides over the whole cell surface, a finding consistent with results obtained by others (4, 11). In contrast to the matrix, the electron-dense particles were unstained with colloidal iron, and their persisting ring-shaped structure after extensive enzyme digestion suggests a protease- and pepsin-resistant framework. While the precise chemical nature of the extracellular particles remains unresolved, their staining characteristics, lack of penetration by colloidal iron, resistance to enzymatic treatment, and ring-shaped appearance would all be in keeping with a membranous structure and suggest the possibility that the extracellular particles might represent minute vesicles derived from the plasma membrane by blebbing.

When the movement of pear-shaped cells was observed in an electrophoretic field, it was found that the cytoplasmic protrusion turned further

towards the anode than did the remaining cell body. This unusual movement indicates that the protrusion has a higher electrophoretic mobility, which in turn suggests that the average surface-charge density is higher in this particular surface area (34). However, the electrophoretic mobility of a particle is also influenced by its shape and size. To rule out the possibility that the protrusion moved more rapidly towards the anode because of its smaller size, some theoretical considerations are necessary.

For an electrically charged sphere of radius a , the density of charge σ can be related to η , the viscosity of the suspending medium; μ , the electrophoretic mobility in $\mu/s/v/cm$; and κ , where $1/\kappa$ is the Debye-Huckel parameter which indicates the "effective" thickness of the diffuse electrical double layer surrounding the cell surface, by the following equation (27):

$$\sigma = (\eta\mu/a) (1 + \kappa a) \quad (1)$$

When κa is greater than 100, the above equation can be reduced to

$$\sigma = \eta\mu\kappa \quad (2)$$

The parameter $1/\kappa$ depends on the ionic strength of the suspending medium. Under physiological conditions, when the ionic strength is about 0.145, $1/\kappa$ equals 8–10 Å and κ approximates 10^7 cm^{-1} (27, 34).

From equations (1) and (2), it is obvious that if the radius of the particle is large enough to make κa greater than 100, the parameter of size and shape of the particle can be eliminated from the calculations. Consequently, if the ionic strength and the viscosity of the suspending medium are kept constant, the charge density will be directly proportional to the electrophoretic mobility of the particle, without considering its size and shape.

In the present case, the radii of the cell body and the protrusion of IRC cells are about $5 \mu\text{m}$ ($5 \times 10^{-4} \text{ cm}$) and $1 \mu\text{m}$ ($1 \times 10^{-4} \text{ cm}$), respectively. Accordingly, the products κa for them are

$$\kappa a = 10^7 \text{ cm}^{-1} \times 5 \times 10^{-4} \text{ cm} = 5 \times 10^3$$

and

$$\kappa a = 10^7 \text{ cm}^{-1} \times 10^{-4} \text{ cm} = 1 \times 10^3$$

i.e., both are greater than 100. Therefore equation (2) is valid and the difference in electrophoretic mobility between these two entities, as demon-

strated by the present experiments, is *attributable to the difference in charge density over their surface*.

The results thus indicate that the protrusion of IRC cells not only has a thicker layer of acid mucopolysaccharides on its surface (3,000–7,000 Å) than elsewhere over the cell, but also that the density of the negative charges of the cell coat on the protrusion is higher than on the cell body. It should be noted here that electrophoretic mobility is not necessarily influenced by cell coat thickness, since it is determined only by charges within the superficial layer of the cell coat (8–10 Å).

On the basis of the cinematographic observations there is little doubt that the adhesion of IRC cells to the substratum was limited to the surface overlying the main cell body, while the protrusions were used exclusively and preferentially for intercellular adhesion. The question arising here would seem to concern the basis for this segregation of adhesive functions on different parts of the cells. Since all mammalian cells examined so far carry a net negative charge at their surface, it has been suggested that interactions between cells can be considered similar to the interactions of charged lyophobic particles (8, 30, 34). According to this theory, attractive forces largely of the London-Van der Waals type, tend to favour close contact between surfaces, but before cells can approach sufficiently for this short-range attraction to occur, the repulsive electrostatic forces which act over greater distances must be overcome, usually by locomotor energy. The repulsive forces encountered by approaching cells are proportional to the radius of curvature and to the charge density of the interacting cell surfaces, among other factors. This relationship is demonstrated, for example, by the importance of microvillus formation, i.e., a reduction of the radius of curvature, in the primary intercellular adhesion leading to cell fusion (19). Thus, if the above argument is applied to the IRC cells, it should be expected that the preferred site of adhesion would be the cell body with microvilli and a lower charge density, rather than the protrusion which lacks microvilli and has a higher surface charge density. Greater adhesiveness of the cell body of IRC cells was, in fact, observed in terms of interactions with the substratum. However, adhesion *between* cells occurred preferentially between protrusions. There are at least two factors that may be contributing to this apparent discrepancy. First, the high dynamic energy resulting from

locomotion of the highly motile IRC cells might have been sufficient to overcome the stronger electrostatic repulsive forces on the protrusions. Second, it is possible that the effective radii of curvature of two approaching cytoplasmic protrusions were those of the electron-dense extracellular particles embedded in the overlying cell coat. Under physiological conditions, two approaching cells will not experience significant electrostatic repulsive forces until they are about 50–60-Å apart (19, 34). Within this interaction distance, the surfaces contributing to the initial repulsive forces between IRC protrusions would be those of the overlying particles, with diameters of about 250 Å. Since the radius of curvature of these particles (0.01 μm) is much smaller than that of a microvillus (0.05–0.1 μm), the repulsive forces generated when two protrusions approach each other would be reduced manifold as compared to the electrostatic repulsion between microvilli under comparable conditions. Along the same line of reasoning, contact between the protrusions may have been facilitated further by the presence of numerous filamentous processes (radius 10–20 Å) on the specialized cell coat in this region. In contrast to its influence on interacting protrusions, the small radius of curvature of the 250-Å extracellular particles might not effectively reduce the repulsive forces between a protrusion and either the substratum or the body of another cell, because of the very large radius of curvature of the latter structures (34). In addition to this factor, the relative lack of adhesiveness between protrusions and either the substratum or cell bodies could be accounted for, at least in part, by the higher charge density over the protrusions and by their rigidity, which would interfere with the spreading and deformation required during the process of adhesion to substrata. This localized rigidity, probably the result of higher surface charge density (32) and greater cell coat thickness (19, 20), might also have contributed to the greater stability of established intercellular adhesions between protrusions, as compared to those involving the main cell body where there was considerable surface activity in the form of microspike formation and of undulating membranes (5).

The effect of the localized increase in cell coat thickness and charge density on the expression of malignancy by the IRC cells is unknown, but it may well influence their antigenicity, growth pattern, and interactions with other cell types. An

increase in cell coat thickness has been demonstrated to accompany malignant transformation by virus (16), has been linked to the ability of cancer cells to overcome the immune rejection by the host (13), and may also account for the lack of communication between tumor cells (15). A large body of information has also accumulated in attempts to correlate characteristics of cancer cells with their electrophoretic mobility (36). It has been pointed out (34) that these data are based on measurements of the *average* charge density of cells, and do not take into account the influence of the charge heterogeneity thought to exist over different parts of the cell surface. A localized difference in surface charge and cell coat composition that can be demonstrated electrophoretically and can be related to cell behavior by cinematography, as observed in the IRC cells, would appear to represent an unusual and useful system for the study of some of the interrelationships between cell surface properties and cell functions, that have been postulated to exist on the basis of histochemical data and less direct experimental evidence (34, 35).

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