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MECHANISMS OF CELL ADHESION: EARLY-FORMING JUNCTIONS BETWEEN AGGREGATING FIBROBLASTS

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SUMMARY

When cultured fibroblasts (16C) are mildly dissociated with EGTA or trypsin/EDTA, they aggregate rapidly. The formation of aggregates has been found to involve junctions of the gap and adhaerens types which are seen by electron microscopy within minutes of allowing cells to come together. The process of adhesion between freshly dissociated, transformed 16C fibroblasts is therefore organized and establishes its usefulness as a model for studying cellular interactions in relation to supracellular organization.

INTRODUCTION

When 2 cells collide, re-organization probably occurs in both the intra- as well as the inter-cellular regions for that contact to result in a stable adhesion – at least, that is the point we explore in this paper. Adhesion can be generated experimentally by the technique of cell aggregation, used originally for bringing together dissociated embryonic tissue cells in suspension and subsequently as a device for quantitating the adhesiveness of cells on a much shorter time-scale. In this second application, non-embryonic material is often used such as that from cultured cell lines, when the aim is to study the mechanism of adhesion rather than the outcome of exchanging partners in mixed cell aggregates.

One major problem of interpretation with isotypic cultured cells is that there is rarely any evidence that aggregation proceeds beyond passive flocculation such as can be induced with inert particles or droplets, and therefore there is no guarantee that the behaviour can illuminate biological questions. Again the cells may be damaged in the dissociation step that is used to bring them into suspension so that it is not clear what relation, if any, the measurements have to natural interactions between native cell surfaces.

If an adequate justification could be advanced for the use of cultured cells to study mechanisms of adhesion, many powerful advantages would follow. For example, subconfluent monolayers can be separated gently from the substratum using brief and mild conditions to yield suspensions composed mainly of single cells, of a single defined and reproducible type, which may even be synchronized with respect to the cell cycle if desired. The object of this paper is therefore to explore the organization of the adhesions that form in suspension of aggregating rat dermal fibroblasts

(the 16C line). We report that these adhesions involve structural re-organization that is rapid and extensive and that it can be taken as evidence for the involvement of authentic processes of biological organization.

MATERIALS AND METHODS

Cell cultures and their dissociation

The cells used in this study are an established line of rat dermal fibroblasts (16C, Colworth strain). They are grown in 230-ml glass bottles in Dulbecco's medium (Gibco-Biocult, special formulation) containing 10 % (v/v) foetal calf serum, fungizone and penicillin/streptomycin in a gas phase of 10 % (v/v) CO_2 in air. Cultures were routinely passaged at a 1:3 split ratio and taken for aggregation assay after about 48 h.

Two methods for dissociating cells have been used. The first employs trypsin (0·05 %)/EDTA (0·01 %) (Gibco–Biocult) which is added (10 ml) to monolayers which have been washed with calcium- and magnesium-free Earle's balanced salts solution. After about 20–30 s at room temperature, the trypsin/EDTA is drained off and at 2 min the cells are removed by shaking with 10 ml of cold Dulbecco's phosphate-buffered saline (PBS) containing deoxyribonuclease (5 μg/ml). The cells are then washed 3 times by centrifugation in this buffer and suspended by gentle pipetting to a final concentration close to 1×10^6/ml – a density which provides optimal aggregation under the conditions we use. The second method is to dissociate washed monolayers with 0·05 % (w/v) EGTA in Tris-buffered calcium- and magnesium-free saline (10 ml). After 2 min at room temperature, the solution is decanted and at 4 min the cells are suspended and washed as for trypsin/EDTA-treated cells.

Cell suspensions (1 ml) at 1×10^6/ml are placed in siliconized scintillation vials and made to adhere by reciprocal shaking in a water-bath at 100 strokes/min at 37 °C. This was done in PBS for short-term experiments and in complete Dulbecco's medium, plus serum, buffered with HEPES, for longer-term experiments. Complete contents of a vial are sacrificed for sampling by gently diluting to 10 ml with cold PBS and then counting the cellular particles on a Coulter Counter (model ZB) electronic particle counter. At the settings we use, the machine counts all particles (i.e. cells plus aggregates) larger than subcellular debris which pass across the 140-μm diameter aperture of the sampling probe. In this way, the number of cellular particles, which declines as single cells go into making aggregates, gives a quantitative measure of adhesion since no cells adhere to the siliconized flasks and vital staining shows that viability remains high over the experimental period (Lloyd & Cook, 1974).

Scanning electron microscopy

Aggregating cells were prepared for scanning electron microscopy by adding 3 % (v/v) glutaraldehyde in PBS (5 ml) to the suspension at the temperature of the experiment. After 30 min, the fixative was carefully removed and the aggregates washed twice by allowing them to settle in changes of PBS. They were then postfixed in 1 % (w/v) osmium tetroxide in PBS for 30 min and stored, if necessary, at 2 °C in distilled water before dehydrating with graded changes of acetone. With this fixation procedure, we have found that the morphology of delicate projections, such as lamellipodia and filopodia on substrate-attached fibroblasts, is preserved and compares well with the unfixed state observed under phase and interference contrast optics. Fixed, suspended aggregates were dried by the critical point method of Anderson (1951) in specially designed containers. These were BEEM (00) (Polaron Equipment Ltd.) capsules with the conical end cut off and replaced by a second cap. The caps at either end – freely perforated by a dissecting needle – were placed over Millipore solvent-resistant membranes (Solvinert). Using this apparatus, excellent preservation of cell shape was obtained after critical-point drying, far superior to results obtained by air-drying from acetone. Dried aggregates were attached to specimen stubs by double-sided adhesive tape and coated with a layer (~ 20 nm) of gold using an SEM coating unit (E5000, Polaron Equipment Ltd).

Transmission electron microscopy

For TEM, the aggregates were allowed to sediment and most of the buffer was removed. The material was fixed for 30 min at either 2° or 37 °C in 3 % (v/v) cacodylate-buffered glutaraldehyde at pH 7·4 containing 3 mM calcium chloride (5 ml). Aggregates were washed twice in sucrose/cacodylate, postfixed for 30 min in 1 % Palade's buffered osmium tetroxide, pH 7·4, and stained with 1 % (w/v) aqueous uranyl acetate. They were then dehydrated through a graded series of ethanol and embedded in Spurr's low viscosity resin. Thin sections were stained with lead citrate.

RESULTS

Whether they are dispersed by EGTA or trypsin/EDTA, 16C fibroblasts aggregate extensively and rapidly, although EGTA-dissociated cells are somewhat less aggregable (see Fig. 1). With both reagents, aggregation is accompanied by the formation of specialized organizations at points of contact; these are of 2 types, recognizable as gap junctions and adhaerens-type junctions (for a review, see McNutt & Weinstein, 1973).

Fig. 2 shows such a gap junction formed between fibroblasts aggregated for

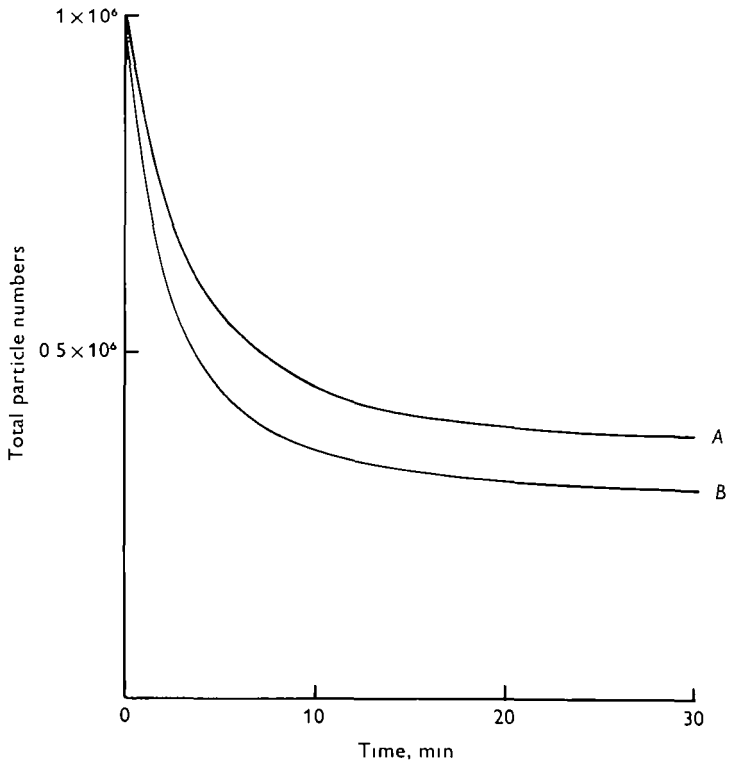
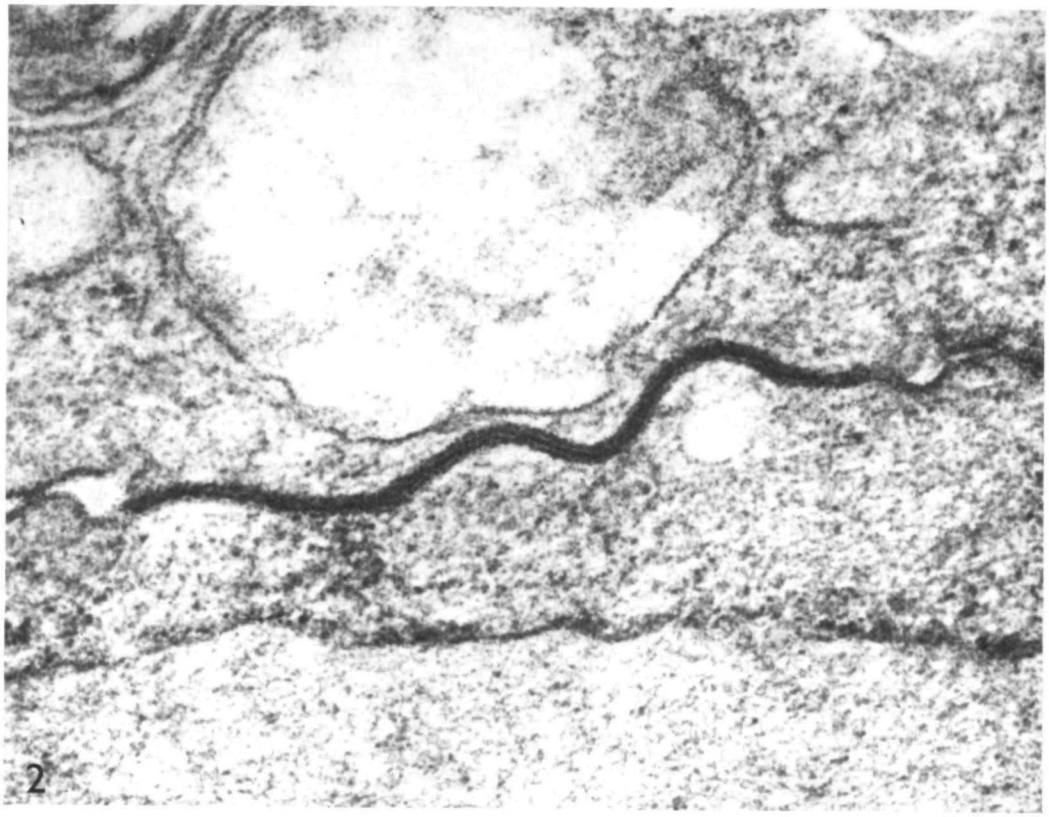


Fig. 1. Trypsin/EDTA-dissociated 16C fibroblasts (curve *B*) aggregate more rapidly and extensively than EGTA dissociated cells (curve *A*). This is shown by the speedier decrease in particle number as the cells adhere to form fewer total cellular particles.



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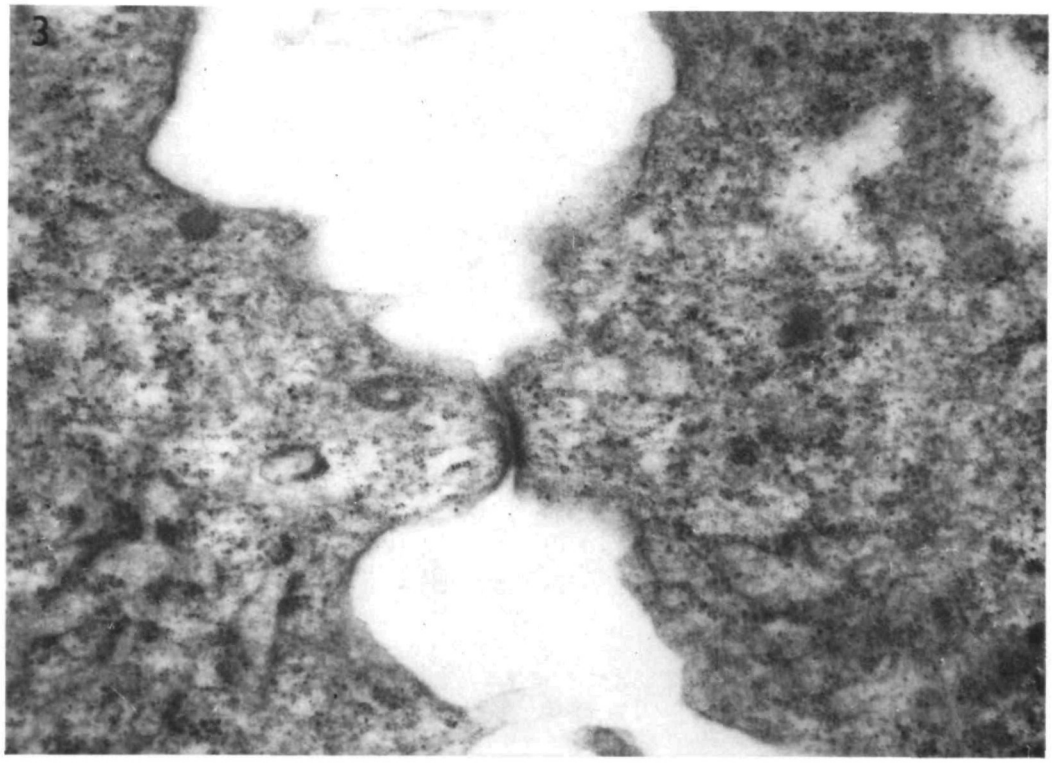

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24 h at 2 °C. In places, the characteristic septilaminar structure becomes more apparent as the gap between the 2 membranes is revealed where the structure is incompletely stained. Pentalaminar structures seen in our photographs are not tight junctions – the primary sealing union of epithelial cells. This is shown not only by the occasional separation of faces but also by an overall width which is greater than the sum of the contributing membranes. For true tight junctions, membrane 'unification' would lead to a dimension which is less than this sum. The structures we show exhibit the characteristic staining pattern found in junctions treated with uranyl acetate/lead citrate as McNutt (1970) has demonstrated with heart tissue. Further evidence of the identification of these structures as gap junctions, is the periodicity in the mid-region found in oblique sections (not shown) where electron-lucent spaces break up the line which probably reflects the intervals between globular subunits of the junction. Revel (1974) has recently suggested that gap junctions between fibroblasts have often in the past been mis-identified as tight junctions.

Our adhaerens junctions, especially in the early stages of formation, do not easily fit into Farquhar & Palade's (1963) classification of adhaerens junctions in epithelial tissue. In shape, the junction is initially focal (Fig. 3) and this closely resembles the 'spot weld' of the macula adhaerens (desmosome) but unlike the desmosome it does not bear tonofilaments and does not have a dense central stratum in the interspace (Fig. 4). In this respect it is similar to the zonula adhaerens (intermediate junction) but does not share its overall morphology. The most evocative description is given by the informal term of 'primitive desmosome'. Hay (1968) has called these 'maculae adhaerentes diminutae'.

In 24-h aggregates, structures are occasionally found where cytoplasmic micro-filaments run along either side of opposed cell membranes in parallel alignment. Belt-like adhesions would most often be sampled as 'focal' transverse sections so it is conceivable that such views represent less-common longitudinal sections of adhaerens junctions in the zonular form. However, the dense cytoplasmic mat is absent from these structures and for this reason they seem to represent instead a specialization of the cytoskeleton between areas of close cellular apposition in much the same way that the filamentous system stretches between the focal adhesion plaques of cells attached to a substrate (unpublished).

These observations on well established aggregates (24-h) therefore show that the cells are capable or forming 2 species of intercellular junction. After shorter periods of aggregation (as early as 1 min), junctions can be clearly identified and distinguished even though they may be less well developed. It is unlikely that such early unions had formed between the times of cell dissociation and assay because cells fixed at

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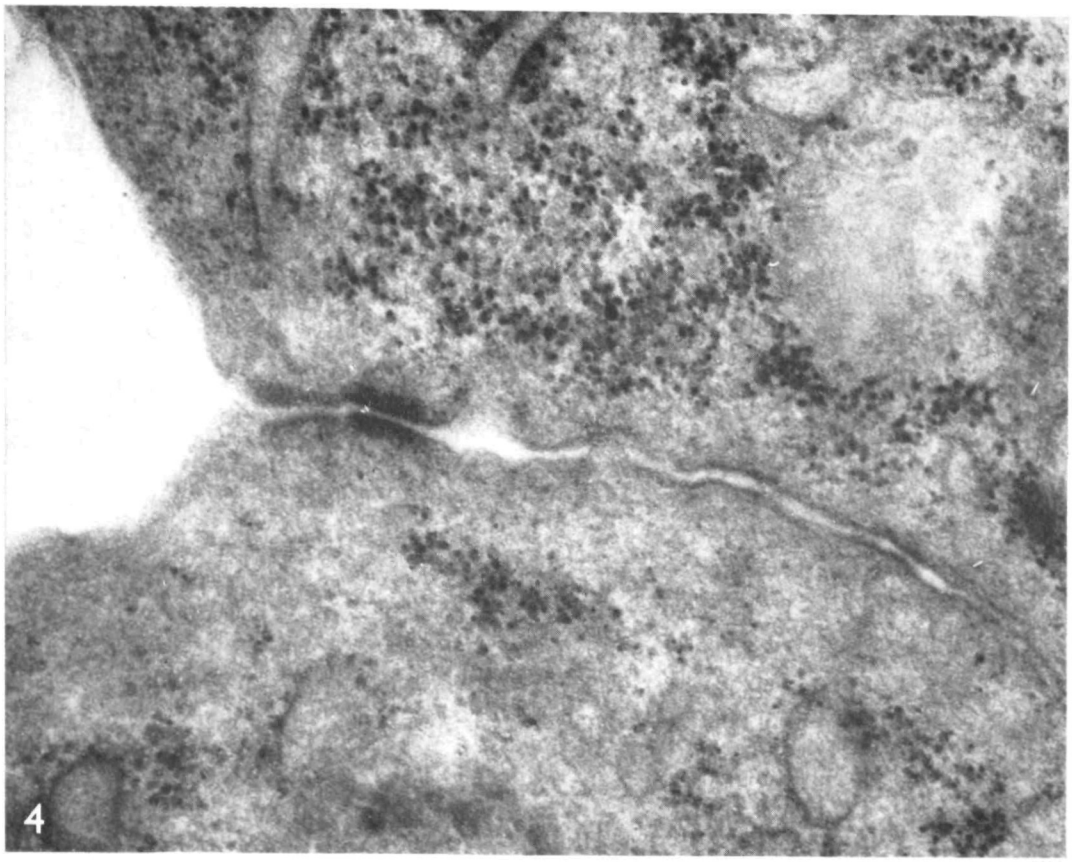
Fig. 2. A well developed gap junction, seen in thin section, between 16C cells aggregated at 2 °C for 24 h. The heavily stained mid-line is characteristic of osmium/uranium lead stained preparations. × 105000.

Fig. 3. An adhaerens-type junction formed in a suspension of trypsin/EDTA-dispersed cells which had been aggregated at 37 °C for 4 min. × 50000.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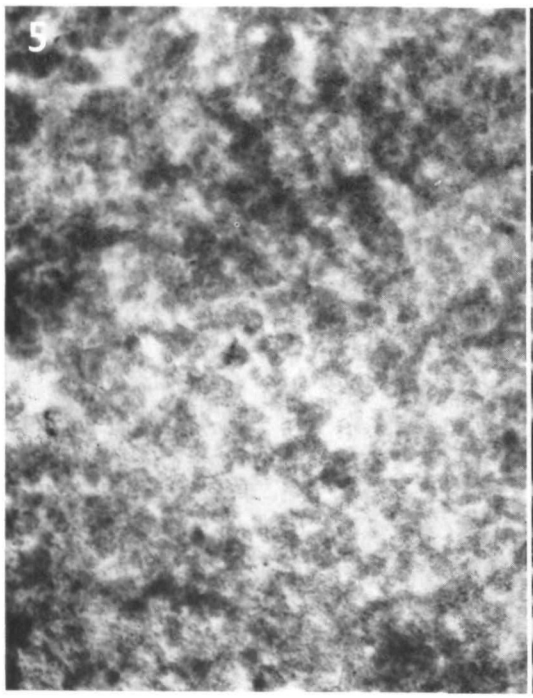
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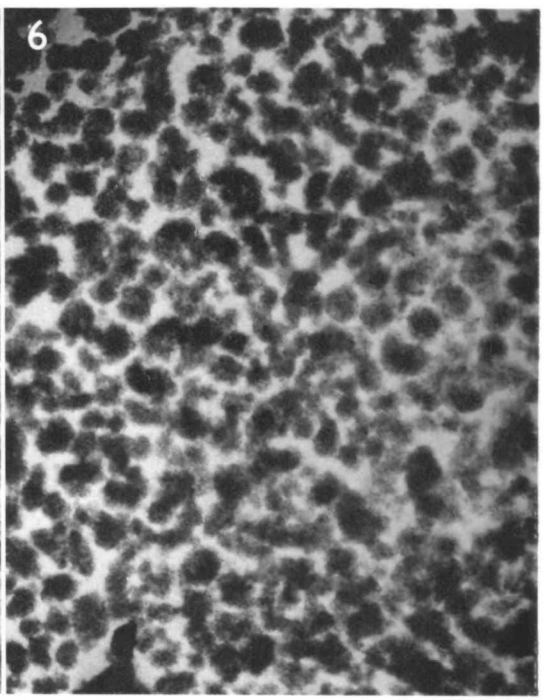
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zero time (the instant before the shaking water-bath was started) showed few junctions compared with 1-min aggregates; the very few junctions that were observed are indeed likely to be derived from the residual 5% or so of cell particles which Coulter counting and haemocytometry indicate to have been undissociated by our dispersal procedures. Analysis of the numbers of junctions in thin sections taken from representative areas of blocks confirms this. At zero time, 3% of the cells examined show junctions in the plane of the section; at 1 min, 13% have junctions and at 8 min (the time by which the rapid aggregation rate begins to decline), 25% have junctions. We are aware of the problems of sampling large numbers of cells by thin sections but even as a semiquantitative index, this indicates the speedy formation of defined junctions.

Comparison of aggregates formed in the cold and in the warm reveals significant differences. At 2 °C, the aggregates (Fig. 5) are smaller and looser than the corresponding solid, spheroidal aggregates (Figs. 6–8) in the warm. Thin sections show that fewer junctions are formed in the cold and that they are mostly of the gap type. We take this as evidence that adhaerens-type junctions are formed by a movement of membrane components that is prevented in the cold. Filamentous structures that could be involved in such movement, can be seen beneath these junctions (Fig. 9). In addition, some early adhaerens junctions (which could equally be forming or separating, see Fig. 10) exhibit radially oriented material in the interspace with a condensation in a mid-line which resembles the more well developed central stratum of the desmosome proper and which, although not common in other adhaerens junctions, has previously been observed in the fascia adhaerens of pigeon heart (McNutt, 1970). This structure (Fig. 10) gives a strong impression that lateral associations are formed on the outer face to consolidate this type of junction.

When 16C fibroblasts are brought into suspension, they round up and form numerous microvilli over their surface (Fig. 11). The first contacts between suspended cells are seen to occur at or in the neighbourhood of these actual cell protrusions (Fig. 3). There is no evidence from our work to show whether such sites are already specialized for adhesion, or whether contacts tend to form between them merely because of their positional advantage.

DISCUSSION

Although the rapid formation of junctions has not as far as we know been reported previously in thin sections of cells aggregated in suspension, our findings may be compared with those of Heaysman & Pegrum (1973) who found intermediate-type junctions forming between the leading edges of substrate-attached chick heart

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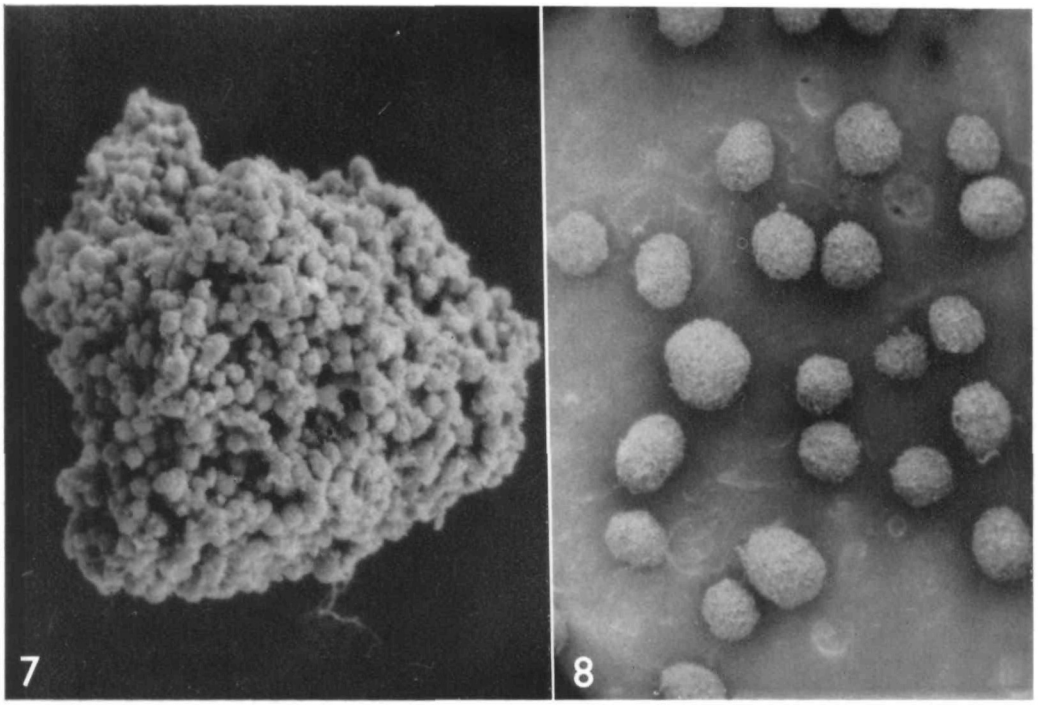
Fig. 4. An adhaerens junction after 30 min aggregation. Note the cell flattening compared with Fig. 3. × 100000.

Figs. 5, 6. Low-power (× 30) light micrographs of cells aggregated for 2·5 h at 2 and 37 °C, respectively. The warm aggregates are more dense and compact than the loose aggregates formed in the cold.



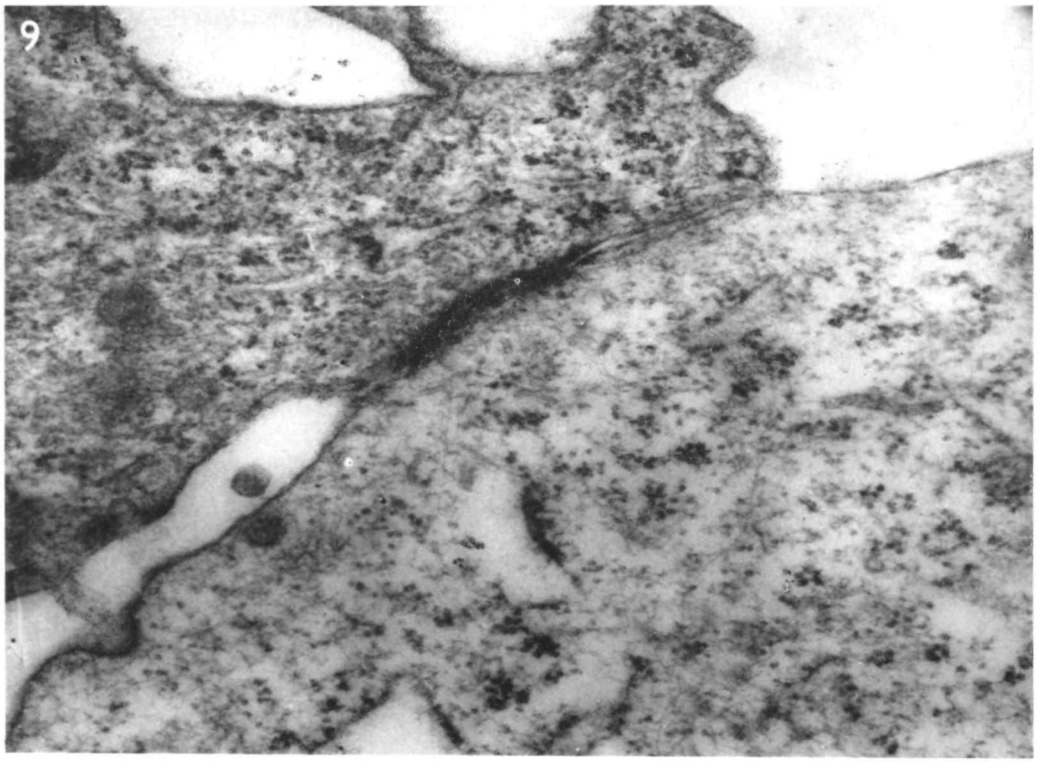
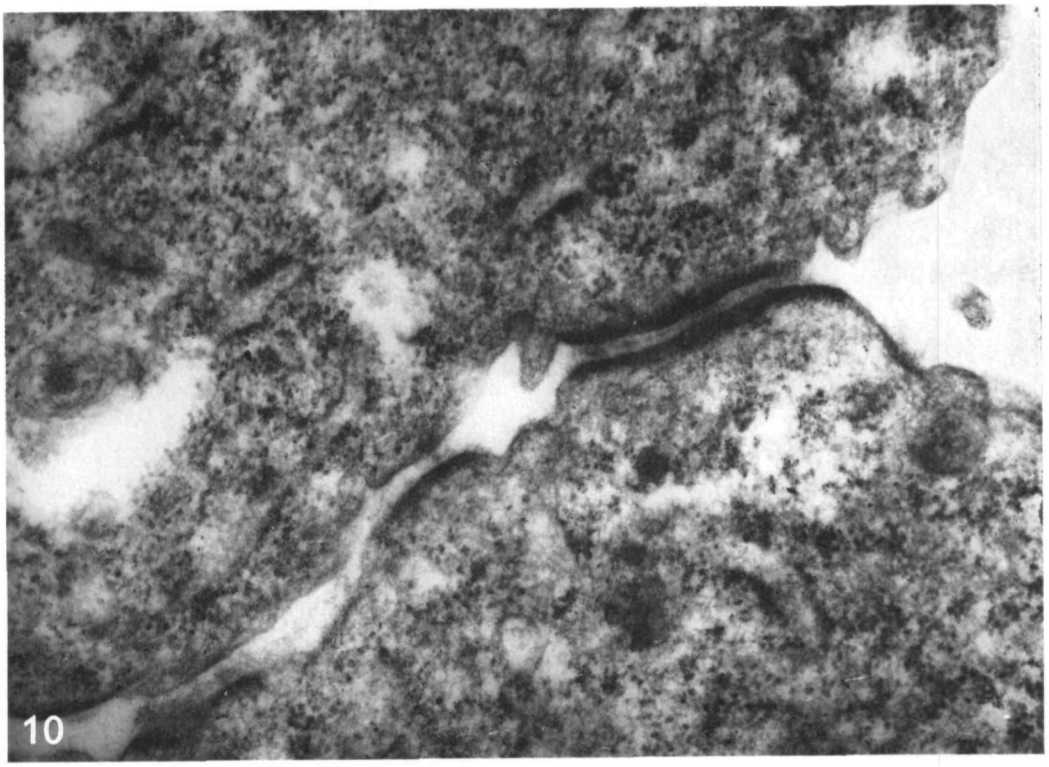


Fig. 7. Aggregate formed after 30 min at 37 °C (scanning EM, × 270).

Fig. 8. After 24 h at 37 °C aggregates of fibroblasts are very dense, compact and rounded (scanning EM, × 27).

Fig. 9. Fibroblasts after 8 min aggregation at 37 °C. The initial focal adhesion can be seen to be broadened, bringing more membrane into parallel alignment. The cytoskeletal meshwork can be seen in the vicinity of the adhaerensjunction. × 50000.



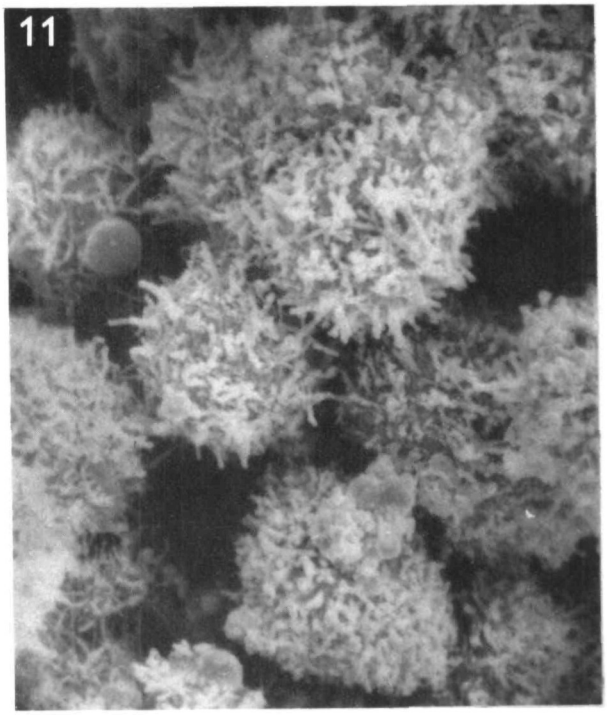


Fig. 10. In the act of either separating or forming, this adhaerens junction from a 1-min aggregate gives possible insight into its organization. Note condensation in the interspace. × 52000.

Fig. 11. Scanning electron micrograph of a region of an aggregate at 30 min. The numerous microvilli on the free outer surfaces are an obvious feature which disappears between contacting surfaces. × 3000.

fibroblasts as early as 20 s of contact, and of Johnson, Hammer, Sheridan & Revel (1974) who reported the formation of gap junctions between suspended Novikoff hepatoma cells as early as 5 min when examined by freeze-fracture analysis. The initial aggregation of our cultured fibroblasts seems to differ from that of dissociated embryonic tissue cells in at least 2 respects. First the cells aggregate rapidly without a detectable lag. This behaviour of cultured cells is not confined to the present system, for it has been recorded by others (Edwards & Campbell, 1971; Cassiman & Bernfield, 1975; Lackie, 1974) for other cell types which have been gently suspended. On the other hand, an initial lag of 30–40 min after trypsinization, has been reported by Steinberg, Armstrong & Granger (1973) using 6- or 7-d chick embryo neural retinal cells. Secondly specialized junctions begin to form quickly whereas, as McNutt & Weinstein (1973) have stated, 'During the initial phases of reaggregation of dissociated (embryonic) cells, there is an impressive lack of specialized cell junctions that are recognizable in standard thin sections'. We suggest that the use of drastic conditions for the dissociation of cells can impair their subsequent ability for organized aggregation until relevant surface structures have been regenerated. That the speed of aggregation should differ according to the rigour of dissociation is hardly surprising since trypsin is known to degrade or detach the cell surface components such as glycoproteins, proteoglycans, and proteins, that are concentrated in adhesive junctions. But by paying increased attention to the mild dissociation of embryonic chick cells, McGuire (1976) has recently shown that these cells can aggregate rapidly, showing selective adhesion without a time lag and, importantly, that this behaviour shows a strong inverse correlation with the amount of trypsin used for dissociation. For cultured cells too, extensive trypsin treatment affects the speed of aggregation adversely as Edwards, Campbell, Robson & Vicker (1975) have found for BHK21 cells.

Paradoxically, although higher levels of trypsin progressively impair the ability of cells to adhere, very mild trypsinization actually gives enhanced adhesiveness relative to EGTA-treated cells. A similar effect for embryonic chick cells has been attributed (McGuire, 1976) to an adverse effect of chelators on adhesive components. This is unlikely to be the explanation of our results because EGTA or EDTA was used in both our dissociation methods. We suggest that low levels of trypsin loosen the peripheral structure of the cell much in the way that this enzyme increases the agglutinability by lectins of a variety of cells. This increased lateral mobility of membrane components (Nicolson, 1971) could allow adhesive molecules to redistribute themselves more readily to form new adhesions. In support of this, our unpublished studies (Thom, Powell, Lloyd & Rees) show that even the mild trypsinization conditions we use, remove some labelled sialic acid residues from the cell surface whereas EGTA does not.

Although a relatively undamaged surface structure may be necessary for speedy cell aggregation, it may not always be sufficient because not all cultured cells aggregate well. O'Neill & Burnett (1974), for instance, have established that transformed and sparse normal BALB/c 3T3 fibroblasts aggregate but that normal cells derived from dense cultures hardly aggregate at all. This points to an importance for normal cell

of the growth status. The cells used in the present study aggregate well no matter what the density at which they are taken, which is in keeping with the findings of O'Neill & Burnett for spontaneously transformed fibroblasts. However, not all cultured cells aggregate as rapidly as 16C. Our preliminary studies show that hamster fibroblasts and their virally transformed derivatives (NIL8 and NIL8HSV) form aggregates consisting of considerably fewer cells after several hours of aggregation and only if dissociated with EGTA.

Our findings that gap junctions can form readily in the cold, can perhaps be compared with lectin-induced patching of surface receptors in lymphocytes (Raff, de Petris & Lawson, 1974) and fibroblasts which can also occur in the cold, and which is believed to involve passive regrouping of membrane components rather than active movement of them by the cytoskeleton. In contrast, the formation of adhaerens-type junctions is more sensitive to cold and like the capping of lectin receptors, is also probably controlled by a submembranous contractile system. Consistent with this would be the accumulation of filamentous material at adhaerens junctions as seen in our electron micrographs (Fig. 9). We conclude that although gap junctions alone are capable of maintaining the cohesion of aggregates, an energy-dependent process in which cytoskeletal elements become inserted upon or organized at localized regions of the plasma membrane is necessary for compact, shapely aggregates.

As aggregation proceeds, it is apparent from our electron micrographs that the entangled, tortuous interfaces between contacting cells become smooth as adhering cell faces flatten against each other. This is also seen in SEM where the free outer surfaces of cells in aggregates bear numerous microvilli but, as can be seen through gaps, cells in the next layer inwards bear few. An explanation of how this could occur is provided by the structure of the microvilli. We have found that, as in other systems (Mooseker & Tilney, 1975; Buckley, 1975), microvilli in 16C cells possess a core of microfilaments which could have a contractile function to pull cell surfaces together upon contact. Whether or not the cell surface is pre-specialized for adhesion at these villus tips is not clear, although histochemical evidence does show that polyanionic surface components are relatively concentrated at such points in some other cells (Weiss & Subjeck, 1974; Grinnell, Tobleman & Hackenbrock, 1975). All this suggests that the villi could be adhesive probes with contractile ability. Certainly when adhesions occur at the tips of 16C microvilli they are almost always of the filamentous adhaerens type.

The filaments we observe in adhaerens junctions are present in an amorphous mat closely applied to the cytoplasmic face, and we have not yet identified the molecular species present in them; among the possibilities are actin, myosin, α-actinin and spectrin (McNutt, 1970; Mooseker & Tilney, 1975; Maltoltsy, 1975; Painter, Sheetz & Singer, 1975). In contrast, in some normal cells, microfilament bundles, presumably of F actin, also insert this mat. Initial 16C junctions are clearly deficient in organized arrays of this second filamentous system – perhaps they are not yet oriented by stress – but unorganized meshwork can be found and isolated membranes of 16C do bear attached microfilaments (unpublished). This is consistent with

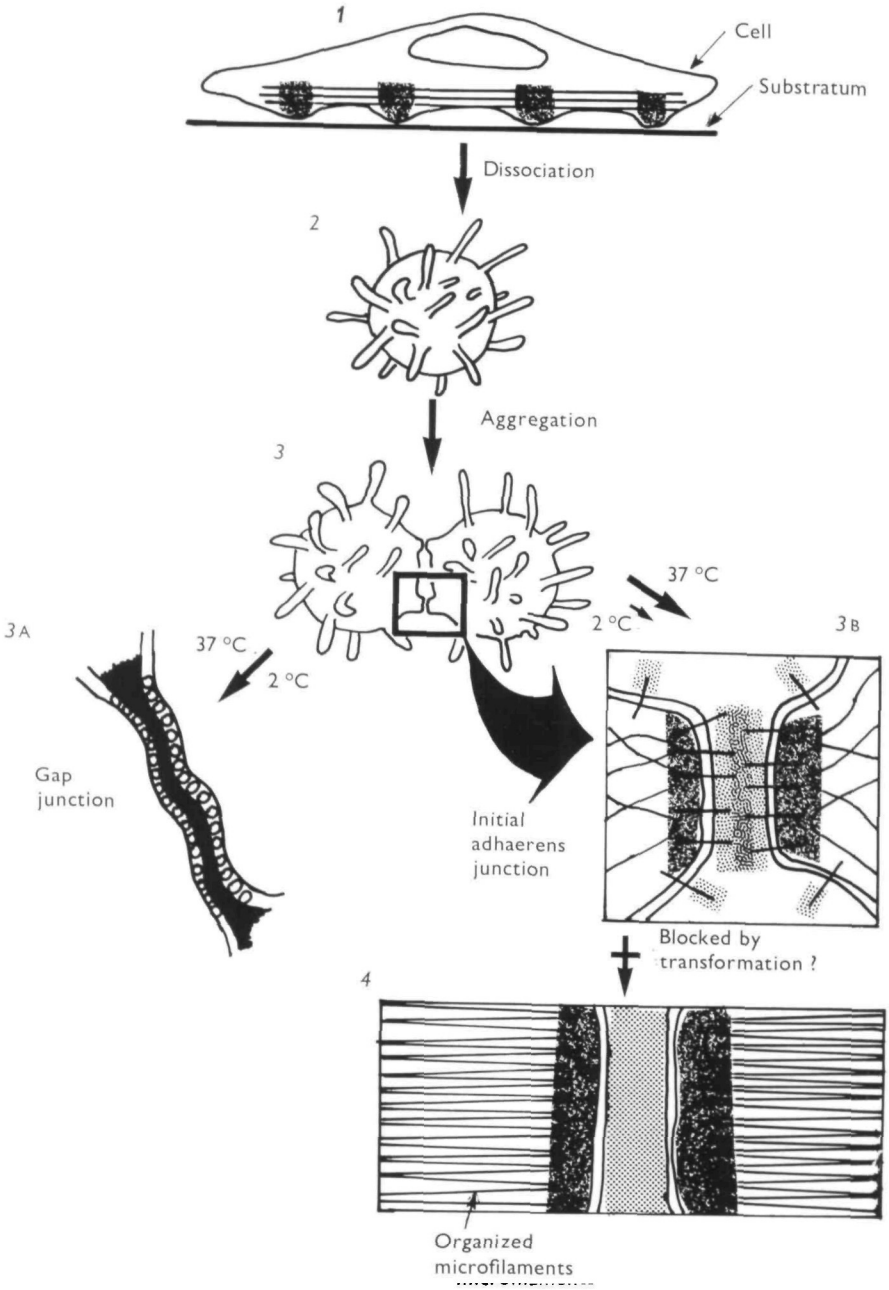


Fig. 12. Schematic representation of the processes involved in the aggregation of 16C fibroblasts. *1*. The fibroblast is attached to the substratum by discrete 'feet' which are associated with cytoplasmic microfilaments. *2*. After dissociation, the cell becomes rounded and the surface thrown into numerous microvilli. *3*. Initial cell contact generally involves microvilli. Adhesions formed are either: gap junctions (*3*A) which are found at 37 and at 2 °C, or (*3*B) adhaerens-type junctions which are encountered less frequently in the cold. For this latter junction, glycoproteins are suggested to be concentrated at the point of contact where they occupy the interspace in ordered array. It is suggested that trans-membrane glycoproteins are also immobilized by the cytoplasmic filamentous mat. *4*. The ability of actin microfilaments to organize themselves at such adhaerens junctions is suggested to be transformation-sensitive.

observations made by others that transformed cells are less able to form adhaerens junctions or, when they do so, the organization of associated microfilaments is impaired (McNutt, Culp & Black, 1973; Dermer, Lue & Neustein, 1974; Altenburg & Steiner, 1975). Analogous effects are well known in the influence of transformation on the organization of actin in substrate-attached cells (Pollack, Osborn & Weber, 1975; Revel & Wolken, 1973; McNutt, Culp & Black, 1971; Wickus, Gruenstein, Robbins & Rich, 1975).

A schematic representation of our general conclusions for the adhesion of 16C cells, is shown in Fig. 12.

The object of this investigation was to establish whether or not the aggregation of cells grown in culture could provide a suitable system for the study of mechanisms of cell adhesion. We have shown that these cells respond rapidly to contact with each other by re-organizing their surfaces to form structures that resemble typical elements of tissue organization, and thus we believe that the elucidation of the mechanisms involved will contribute to the understanding of other social processes of cells.

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