INITIAL ATTACHMENT OF BABY HAMSTER KIDNEY CELLS TO AN EPOXY SUBSTRATUM

Ultrastructural Analysis

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Cells that have been attached to a substratum for only a short period of time appear to be attached around the entire cell periphery (2, 8, 10, 21, 22, 27). At later times the adhesion sites occur as discrete loci predominantly at the leading and trailing margins of cells and, to a lesser extent, beneath cells (1, 3, 5, 13–16, 22, 23). Sites of adhesion may be characterized by a close (10 nm) approach of the cell plasma membrane to the substratum and by the presence of bundles of microfilaments (plaques) in the adjacent cytoplasm (1, 3, 11, 16, 19). Also, in serum-containing medium, cells are thought to be attached to a serum layer, which may be covered by cellular microexudate, and not directly to the substratum (3, 9, 19, 20, 23, 28).

We have developed a new technique that permits clear visualization of the relationship between the cell, the substratum, and any intervening materials. Using this technique, we have studied the ultrastructural details of cell adhesion and spreading during *initial* cell interaction with substratum. In order to analyze whether changes in the organization of the cell surface occur during initial adhesion and spreading, we have carried out studies on the distribution and mobility of anionic cell surface sites during this time period, and compared the results of these studies with previous, similar experiments utilizing cells in suspension (12). The technique reported in this manuscript has been presented in abstract form (26).

METHODS AND MATERIALS

Epoxy substrata for cell attachment were prepared by polymerizing an Epon mixture (18) in small (approx. 10 × 4 × 3 mm) flat embedding molds (Polysciences, Inc., Warrington, Pa.) for 24 h at 60°C. Excess unpolymerized epoxy was frozen and used later for embedding. Before use for cell attachment, the epoxy substrata were washed for 1 h at room temperature in adhesion salts (0.8 mM $MgSO_4 - 7H_2O$; 116 mM NaCl; 5.4 mM KCl; 10.6 mM Na_2HPO_4; 5.6 mM D-glucose; and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES); final pH adjusted to 7.0) to remove surface impurities.

BHK-21-13s cells were grown in suspension culture as described previously (12). Suspension cultured cells in the logarithmic growth phase were collected by centrifugation at 500 *g* for 2 min, resuspended in 4.0 ml of adhesion salts containing fetal calf serum as indicated in the experiments, and placed in Falcon 10 × 35-mm petri dishes (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.), each dish usually holding four epoxy substrata. 1–2 × 10^6 cells were used in each experiment. The petri dishes were incubated at 37°C for the time periods indicated in the experiments; at the end of the incubations, nonattached cells were removed by immersing the epoxy substrata in a beaker containing Dulbecco's phosphate-buffered saline (PBS).

In experiments requiring labeling of cells with polycationic ferritin, the epoxy substrata, with attached cells, were placed in Falcon 30 × 10-mm petri dishes containing 4 ml of polycationic ferritin (0.32 mg/ml) in PBS. The incubations were carried out at room temperature for the time periods indicated and then stopped by washing the cells in PBS.

At the conclusion of all experiments, attached cells were prepared for electron microscopy as follows. The epoxy substrata with attached cells were placed in tissue vials and fixed with 3% glutaraldehyde in 0.1 M Na-phosphate (pH 7.4) or with a solution of 1% tannic acid, 2% glutaraldehyde, 0.1 M Na-phosphate (pH 7.4) (23). Fixation was carried out for 15 min at room temperature

followed by 2 h at 4°C. Subsequently, the attached cells were washed with 0.2 M sucrose in 0.1 M Na-phosphate (pH 7.4) at 4°C and postfixed in 2% osmium tetroxide in 0.1 M Na-phosphate (pH 7.4) for 30 min at 4°C. After dehydration, the epoxy substrata were placed longitudinally in Beem capsules (Better Equipment for Electron Microscopy, Inc., Bronx, N. Y.) and embedded in the original Epon 812 mixture. Gray-to-silver sections of the embedded attached cells were stained for 10 min in saturated uranyl acetate in 50% ethanol followed by 10 min in lead citrate (24). Electron micrographs were taken on a JEOL 100B electron microscope at 60 kV using a 20-μm objective aperture.

Baby hamster kidney cells which were suspension culture adapted (BHK-21-13s) were the gift of Dr. Adrian Chappel, Communicable Disease Center, Atlanta, Ga. Eagle's minimal essential medium (MEM) ("spinner modified"), MEM amino acids, MEM vitamins, and fetal calf serum were obtained from Grand Island Biological Co., Grand Island, N. Y. Polycationic ferritin was obtained from Miles Laboratories, Kankakee, Ill. Other reagent grade chemicals were purchased from Fisher Scientific Co., Houston, Texas.

RESULTS

Attachment of Baby Hamster Kidney (BHK) Cells to an Epoxy Substratum

In order to study the ultrastructure of initial cell attachment, we chose to use a substratum prepared from the same epoxy mixture (18) routinely used in our laboratory for embedding cells fixed in suspension. The substratum was sectioned readily and the presence of a layer of penetration of osmium into the substratum, which occurred during fixation, identified the substratum surface precisely. Thus, clear visualization of the ultrastructural relationships between the cells and the substratum was made possible. Using light microscopy, we determined that the time-course and overall morphology of BHK cell attachment to the epoxy substratum was similar to that observed with Falcon polystyrene as a substratum.

Initial attachment of the cells in serum-containing medium occurred within 15 min of incubation (Fig. 1). The cells remained rounded, and a number of surface microextensions could be observed. The area of apparent cell-to-substratum attachment was usually limited to a small portion of the cell body, with a few cell microextensions encountering the substratum. Within 30 min of incubation (Fig. 2), partial spreading of the cells onto the substratum resulted in a much larger area of contact between the cell surface and the substratum. Cell microvilli were diminished; however, blebs increased. Within 60 min the cells were observed to be completely spread, and there was a marked diminution of all cell microextensions (Fig. 3). When attached cells were studied at high magnification, an electron-opaque layer approx. 3–5 nm thick and about the same distance away from the substratum could be resolved in the space between the cells and the substratum (Fig. 4). This layer was observed only in the presence of serum in the medium, irrespective of the presence or absence of cells in the incubation.

The space between the undersurface of attached cells and the serum protein-coated substratum varied markedly, often exceeding 100 nm. However, many discrete regions of close apposition were observed between the plasma membrane and the serum layer and were characterized by a space of approx. 9 nm. Such spacing was determined by tilting thin sections of the attached cells in a goniometer holder. During the course of initial cell attachment and spreading, we did not detect any cytoplasmic specializations, such as bundles of microfilaments (plaques), adjacent to regions of close apposition; however, the cytoplasm of spread cells often showed a marked alignment of microtubules parallel to the surface of the substratum.

Presence of Cellular Substances between the Cell and Substratum

Experiments were carried out using a tannic acid-glutaraldehyde fixation/staining method (25) to determine if there were any protein-containing substances in the 9-nm space between the plasma membrane on the undersurface of attached cells and the adsorbed serum layer on the substratum. The presence of a diffuse substance was visualized in this space (Fig. 5), and material with similar staining characteristics was also observed on the upper surfaces of the attached cells, as well as on the surfaces of cells free in suspension. The tannic acid technique also made it possible to visualize the trilaminar appearance of the plasma membrane with much greater clarity than observed with our conventional fixation procedure (cf. Fig. 5 with Fig. 4).

Anionic Sites on the Surfaces of Attached BHK Cells

In a previous report, we described the distribution and mobility of anionic sites on the surfaces of

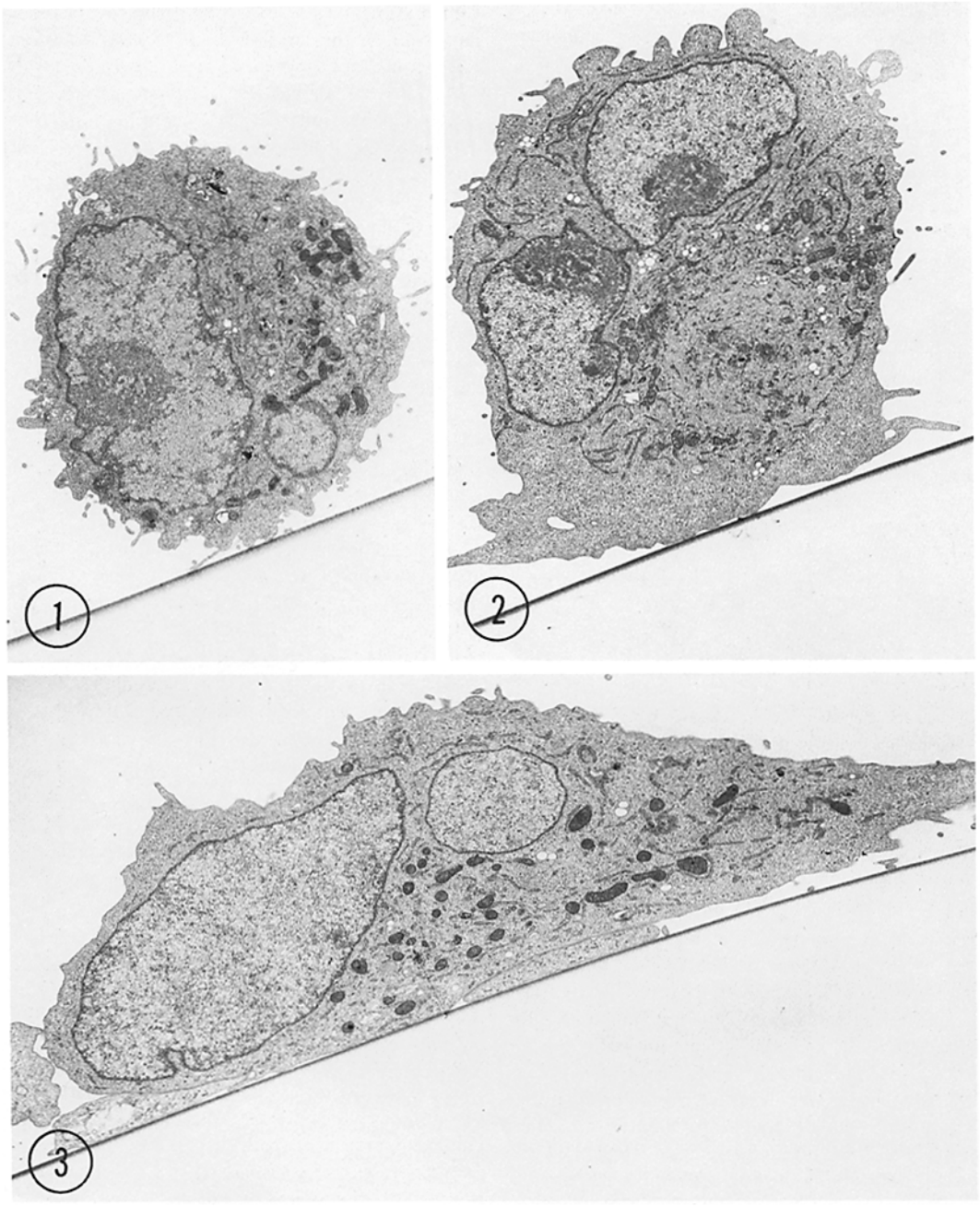


FIGURE 1 BHK cell attached to the epoxy substratum for 15 min in attachment incubation medium containing 5% fetal calf serum. The cell is rounded; many microvilli are apparent. × 6,000.

FIGURE 2 BHK cell attached to the epoxy substratum for 30 min in attachment incubation medium containing 5% fetal calf serum. Cell flattening has begun; microvilli are decreased; numerous blebs are apparent. × 6,000.

FIGURE 3 BHK cell attached to the substratum for 1 h in attachment incubation medium containing 5% fetal calf serum. Cell flattening is essentially complete; few microvilli remain. × 6,000.

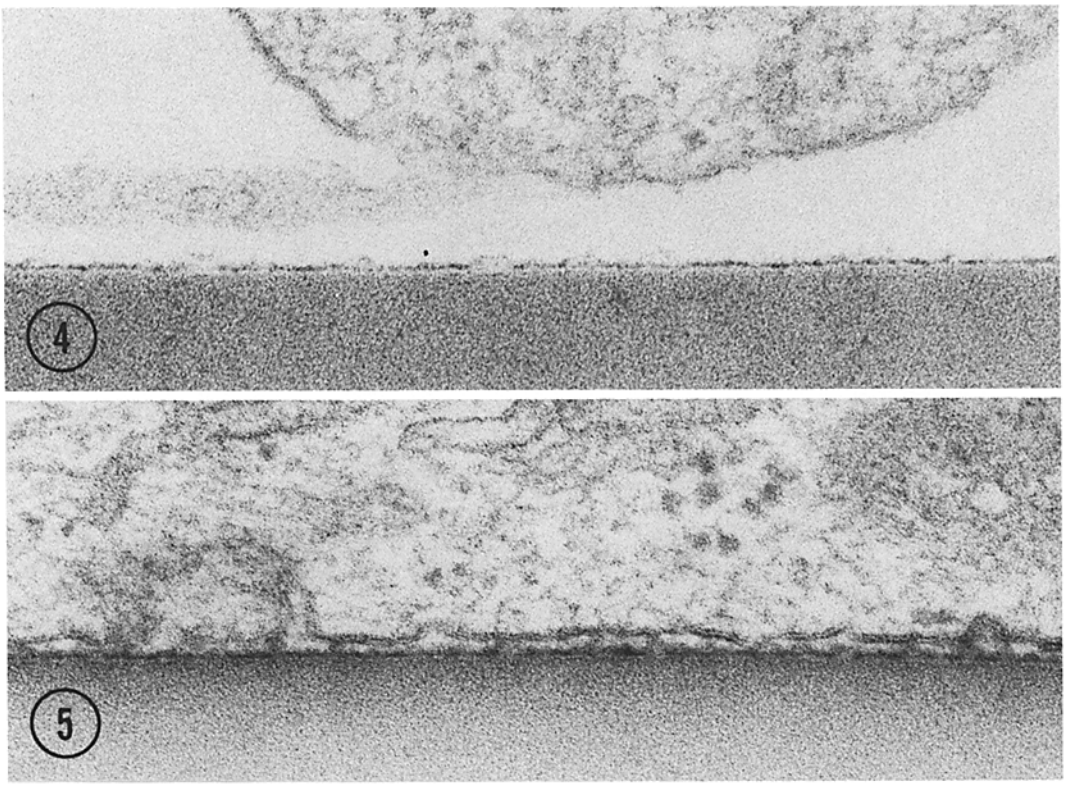
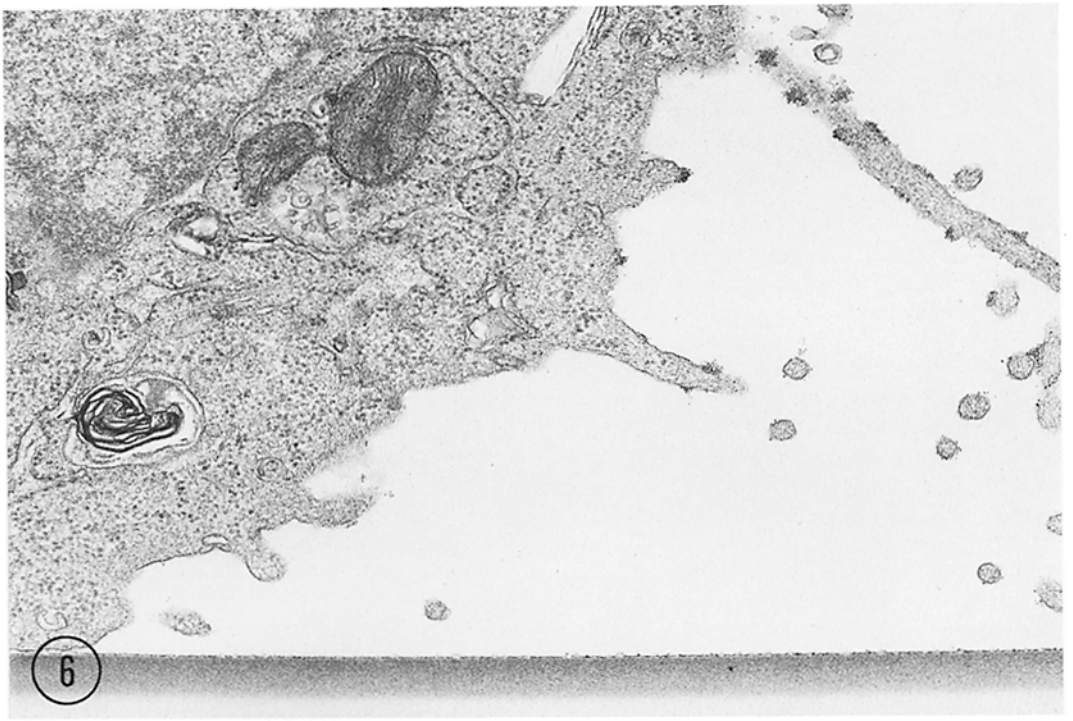


FIGURE 4 BHK cell attached to the substratum for 15 min in attachment medium containing 5% fetal calf serum. High magnification demonstrating the serum layer on the substratum. × 157,000.

FIGURE 5 BHK cell attached to the substratum for 60 min in medium containing 5% fetal calf serum. Fixation was carried out with tannic acid-glutaraldehyde, and the unit membrane appearance is obvious. Diffuse staining of extracellular material is observed between the plasma membrane and the serum layer on the substratum. × 157,000.

BHK cells in suspension utilizing polycationic ferritin as a visual probe for such sites (12). Since there is a marked change in the overall topography of cells during attachment and spreading, it was of interest to know if changes in membrane organization at the molecular level occurred during this process. Thus, we attempted to determine the distribution and mobility of anionic sites on the surfaces of attached and spreading BHK cells using the polycationic ferritin technique. BHK cells which were attached to the epoxy substratum for 30 min were treated with polycationic ferritin for 10 s, 1, 5, and 20 min at room temperature. Labeling of the cells was predominantly in small clusters on the cell surface after 1 min (Fig. 6). At the end of 20 min, larger patches replaced the smaller clusters, and in general more ferritin appeared to be bound (Fig. 7). Large patches of ferritin also occurred between folded-over cell surface microextensions and the cell body, while endocytosis was quite common. In addition, there was some labeling between the margin of the cells and the substratum, but for the most part the polycationic ferritin did not penetrate beneath the cells to any great extent.

The overall pattern of labeling of attached cells by polycationic ferritin was very similar to that observed previously with suspended BHK cells (12); however, shorter incubation periods were found to result in markedly less binding of polycationic ferritin on the surfaces of attached cells than was previously observed for cells in suspension. Also, less binding of polycationic ferritin to the substratum immediately adjacent to the cells was noted at the shorter time periods (cf. Fig. 6 and 7).



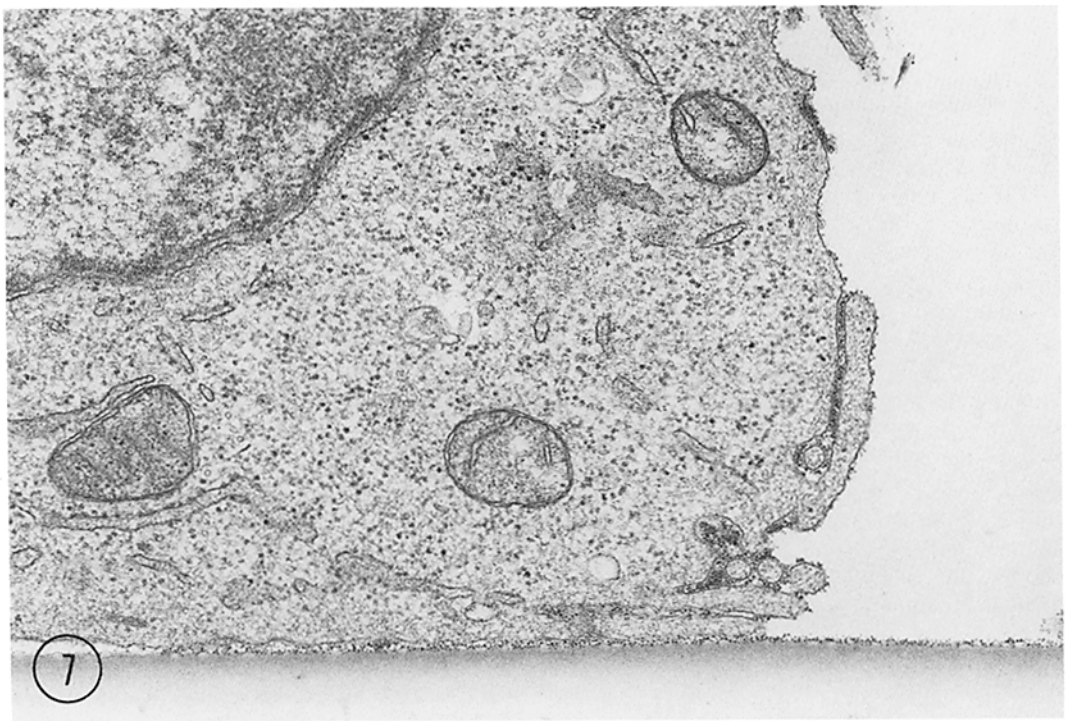


FIGURE 6 BHK cell attached to the substratum for 30 min in medium containing 5% fetal calf serum, then treated with 0.32 mg/ml polycationic ferritin for 1 min. Polycationic ferritin occurs predominantly in small clusters on the cell body and microextensions. Little polycationic ferritin is observed on the substratum; however, the serum layer is apparent. × 39,000.

FIGURE 7 BHK cell attached to the substratum for 30 min in medium containing 5% fetal calf serum, then treated with 0.32 mg/ml polycationic ferritin for 20 min. Polycationic ferritin occurs predominantly in large patches on the cell body and between the cell body and folded-over cell microextensions. Endocytosis is evident. Polycationic ferritin has only penetrated beneath the margin of the cell. × 39,000.

DISCUSSION

This report presents the ultrastructural aspects of initial adhesion and spreading of BHK cells onto an epoxy substratum. Cells attached and spread onto a thin (3–5 nm) electron-opaque layer which coated the substratum surface. Since this layer could be visualized on substrata treated with serum in the absence of cells, but was not present on substrata to which cells were previously attached in the absence of serum, we believe that it is a serum protein layer adsorbed onto the substratum. Previous studies reporting the use of a technique in which fixed, embedded cells were peeled away from the substratum revealed a layer of osmiophilic material to which cells appeared attached and identified the layer as a serum layer (3, 19, 23). Although the osmiophilic layer previously reported is probably the same material that we observed in the present study, our technique has the distinct advantage of preserving the physical relationships between the attached cells, serum layer, and substratum.

The closest approach of the plasma membrane to the serum layer on the substratum was observed to be about 9 nm. This finding was determined by tilting the sectioned cells in a goniometer holder. In the absence of serum in the medium, the closest distance we observed between the cell plasma membrane and uncoated substratum was still about 10 nm. Thus, we cannot confirm a recent finding that close molecular contacts, i.e., <0.5 nm, occur between the cell and substratum in serum-free medium (19). However, that study utilized the peel-off technique, and the substratum was not directly visualized.

The existence of a definitive space between the cell plasma membrane and the substratum in the regions of close contact has been reported by numerous laboratories. However, previous ultrastructural studies of cells grown on substrata have demonstrated the presence of unidentified substances within this space (9, 20). Our present observations support these findings. We find a diffusely stained material (using the tannic acid-glutaraldehyde fixation/staining technique) located between the plasma membrane and the serum layer. This material may contain cell surface receptors involved in adhesion.

Several investigators have reported the presence of plaques (bundles of microfilaments) associated with regions of cell-substratum close contact (1, 3, 16), even soon after initial cell attachment (11, 19). However, a recent systematic study of the formation of plaques during initial adhesion and spreading indicated that formation of these structures is a late event (2). We concur in this finding and were unable to observe plaques after initial adhesion. Thus, although plaques may be insertion sites of cytoplasmic filaments and important in cell movements (1, 5, 11, 13, 17), we believe their role in initial cell adhesion to be doubtful.

Studies were carried out on the distribution and possible mobility of anionic sites on the surfaces of attached and spreading BHK cells analogous to our previous studies on BHK cells free in suspension (12). The distribution and mobility of such sites on attached cells, as determined by the distribution of polycationic ferritin label after incubations ranging from 10 s to 20 min, was similar to that previously observed with suspended cells (12). However, the absolute amount of polycationic binding after the shorter incubations was markedly less than that previously observed using suspended cells under similar labeling conditions. Decreased binding might result from fewer anionic sites on the cell surface, secretion of cellular products that mask the sites, or the presence of a diffusion barrier around attached cells. Whatever the final explanation, this observation may relate to the loss of adhesiveness of the upper surface of attached cells reported by others (6, 7).

SUMMARY

In the presence of serum-containing medium, BHK cells attached and spread during a 1-h period onto a 3–5-nm thick serum layer adsorbed on the substratum surface. The closest approach of the plasma membrane to the serum layer was observed to be about 9 nm, which was determined by tilting the sectioned cells in a goniometer holder. Bundles of microfilaments or other cytoplasmic specializations were not observed in association with the regions of close contact. However, in the space between the plasma membrane and the adsorbed serum layer, a diffusely stained material could be visualized after fixation/staining by the tannic acid-glutaraldehyde technique. This technique also permitted increased clarity of visualization of the trilaminar appearance of the plasma membrane. The distribution and mobility of anionic sites on the surfaces of attached and spreading cells was determined by labeling with polycationic ferritin. We observed movement of polycati-

onic ferritin into large clusters on the cell surface, collapse of cell surface microextensions, and endocytosis, all of which were similar to our previous findings utilizing cells in suspension. However, the absolute amount of ferritin bound to the upper cell surface was less than that previously observed when suspended cells were put under similar labeling conditions. Also, polycationic ferritin did not appear to penetrate between the lower cell surface and the substratum.

This research was supported by grants CA14609 and GM21698 from the National Institutes of Health.

Received for publication 17 February 1976, and in revised form 17 May 1976.

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