STUDIES ON THE FUNCTION OF CELL SURFACE GLYCOPROTEINS

II. Possible Role of Surface Glycoproteins in the Control of Cytoskeletal Organization and Surface Morphology

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ABSTRACT

Immunoglobulin from goat antiserum directed against purified surface membranes from transformed BHK\textsubscript{21}/C\textsubscript{13} cells (anti-M) has been shown to cause both control and transformed hamster cells to round and detach from the substrate (see accompanying paper). This paper documents the effects of the antiserum on the cytoskeletal organization and cell surface morphology of control BHK\textsubscript{21}/C\textsubscript{13} cells examined by scanning and transmission electron microscopy. As a result of antiserum-induced rounding, the normally smooth cell surface becomes covered with filopodia and blebs, and the organization of all three components of the filamentous cytoskeleton is altered. In terms of cell surface morphology and cytoskeletal organization, the cells resemble rounded, postmitotic or trypsinized BHK cells rather than cells treated with either anticytoskeletal drugs or lectins. Immunocytochemical and radioimmune assay experiments support the suggestion that the rounding reaction induced by anti-M serum results from the specific interaction of antibodies with molecules on the cell surface. It is suggested that anti-M serum induces alterations in cytoskeletal organization via a transmembrane signal and that cytoskeletal reorganization is a fundamental part of the rounding and detachment process.

KEY WORDS  surface membrane  ·  cytoskeleton  ·  adhesion  ·  glycoproteins  ·  ultrastructure  ·  immunoelectron microscopy

In recent years, evidence has accumulated which suggests that subsurface cytoskeletal proteins can interact with the surface membrane and that this interaction is important to cellular morphology and behavior (4, 9, 21, 22). A subsurface cytoskeletal network has been implicated in substrate adhesion (1, 5, 11, 12, 14, 20, 26), and its organization has been shown to be affected by exposure of cells to agents such as immobilized trypsin (25), specific immunoglobulin (30), and lectins (3, 17, 33), all of which bind to or react with components on the cell surface but are thought not to penetrate the cell. It is also apparent that alterations in the organization of cytoskeletal proteins below the cell surface have an effect on the distribution of cell surface components and on cell surface morphology (24, 34). These findings suggest that a transmembrane signaling system, responsive to changes on either side of the membrane, might exist between the cytoskeleton...
and components facing the external environment (see references 7, 11, 16, 23, and 28 for reviews of this subject). To document the existence of such a signal, it is necessary to have a system in which a known membrane component on one side of the membrane can be manipulated to produce a detectable alteration in the behavior of the cell and a reorganization of components on the other side of the cell surface.

In the accompanying paper (32), we described the effects of antisera against surface membrane-derived components on adhesiveness and gross morphology of hamster fibroblasts. Treatment of these cells with antiserum to purified surface membranes (anti-M serum) caused them to round, detach from the substrate, and grow as clumps suspended in culture medium. Data from immunoprecipitation studies implicated the binding by the antiserum of one or more surface membrane glycoproteins in this process. In this paper, we document the ultrastructural changes which accompany the antiserum-induced alterations in adhesion and gross morphology. Our results show that dramatic alterations in the organization of the cytoskeleton and cell surface morphology take place as a result of antiserum treatment.

MATERIALS AND METHODS

Cell Culture System

BHK21/C13 and C13/B4 cells were maintained as described in the previous paper (32). Cells to be fixed for electron microscopy were grown in six-well Costar dishes (Costar, Data Packaging, Cambridge, Mass.), 300 mm diam/well. Cells were treated with immune serum or preimmune serum at concentrations of 25 mg serum protein/ml of complete medium. When purified immunoglobulin (IgG) was used, the protein concentration was 10 mg IgG/ml complete medium. Cells were exposed to antiserum or preimmune serum for 3 or 24 h before fixation.

Production of Anti-M and Anti-LIS Sera

Anti-M serum was produced in goats injected with a purified surface membrane preparation from C13/B4 cells. Anti-LIS serum was produced in goats injected with a partially purified glycoprotein of 120,000 daltons obtained from a lithium diidososalicylate (LIS)-hot phenol extract of C13/B4 membranes. Details of the preparation of antigens and the evaluation of antiserum and immunoglobulin preparations are given in reference 32. IgG from immune and preimmune sera were purified by sodium sulfate precipitation as described in reference 32.

Electron Microscopy of Cells

Cell cultures treated with anti-M, preimmune serum, or IgG for 3 or 24 h were fixed in situ with 1.5% glutaraldehyde (Polysciences, Inc., Warrington, Pa.) in 0.1 M phosphate buffer, pH 7.2, containing 5 mM MgCl₂ for 30 min at room temperature. Cells were postfixed in 1% osmium tetroxide in the same buffer for 15 min. Cells were washed with distilled water three times over a 20-min period in the cold and stained in the cold with saturated aqueous uranyl acetate for 30 min. Cultures were dehydrated in ethanol, removed in sheets from the plastic dish in propylene oxide, and embedded in Epon. Sections were cut on an MT2 microtome (DuPont Instruments-Sorvall, DuPont Co., Wilmington, Del.), stained with lead citrate, and examined on a Hitachi HU II or Zeiss 10 electron microscope. The volume density of microtubules and lysosomes in control and anti-M-treated preparations was determined by the method of Weibel and Bolender (31). A multi-purpose test grid containing 250 points was laid over micrographs prints. The numbers of points lying over the whole cell and over the profiles of the organelles being quantitated were tabulated. Volume density of the organelles was calculated and expressed as a percentage. Micrographs of ×30,000 or above were used, and >50 micrographs of randomly sectioned cells were scored from each group.

For immunocytochemical experiments designed to detect the binding and distribution of antiserum on the cells, cells were incubated with immune or preimmune IgG (10 mg/ml) for 3 h, washed four times over a 30-min period with phosphate-buffered saline, fixed 15 min with 1% glutaraldehyde in 0.1 phosphate buffer (pH 7.2), washed four times over a 30-min period with 0.1 M phosphate buffer (pH 7.2) containing 10% sucrose, and finally incubated with rabbit anti-goat IgG conjugated to peroxidase (Cappel Laboratory, Inc., Downingtown, Pa.) for 1 h. The cultures were washed four times over a 1-h period with 0.1 M phosphate buffer, pH 7.2, and then incubated for 30 min with 0.3 mg/ml diaminobenzidine-HCl (Sigma Chemical Co., St. Louis, Mo.) in 0.05 M Tris-HCl, pH 7, containing 0.01% hydrogen peroxide (13). After a thorough washing with 0.05 M Tris buffer, the cells were postfixed for 45 min in 0.1 M phosphate buffer containing 1% osmium tetroxide and processed for electron microscopy as described above. Controls were prepared in the same way, except that one of the following was omitted: (a) goat anti-M IgG, (b) peroxidase-conjugated second antibody (rabbit anti-goat IgG), or (c) diaminobenzidine-H₂O₂.

The amount of immunoglobulin from anti-M and anti-LIS preparations bound by cells was tested by indirect radioimmunoassay (29) by using horse anti-goat immunoglobulin labeled with 125I by the chloramine-T method (18). Threefold serial dilutions of anti-LIS, anti-M, or preimmune IgG in tissue culture medium were applied to cells in microtiter plates (2 x 10⁴ cells/well) which had been fixed with 0.15% glutaraldehyde, washed with
Scanning electron micrographs (SEM) of control and anti-M serum-treated BHK/C cells. Bar in Figs. 1-3 is equivalent to 10 μm.

**FIGURE 1** SEM of a culture of BHK/C cells. Most cells are smooth surfaced and flat. There is extensive overlapping of cellular processes. × 1,800.

**FIGURE 2** SEM of a culture of BHK/C cells treated with anti-M serum for 1 h. Cells have rounded and formed clumps but have not yet detached. Surface convolutions are apparent. Retraction fibers connecting cells to the substrate and to other cells (arrows) are abundant. Single cells in the culture appear to round up completely before detaching. × 900.

**FIGURE 3** Higher magnification of rounded detached cells. The free surfaces of cells are entirely covered with blebs and filopodia. × 5,200.
buffer containing 0.15 M glycine, and processed by the procedure of Segal and Klinman (29). Some wells were incubated with labeled horse anti-goat IgG only, to obtain background levels for nonspecific binding of this reagent.

RESULTS

Ultrastructural Alterations in the Surface and Subsurface Morphology Induced by Anti-M Immunoglobulin

Anti-M-induced changes in surface morphology and cytoskeletal organization were examined both by scanning and transmission electron microscopy. Fig. 1 shows a scanning electron micrograph of a group of adherent control BHK21/C15 cells. The cells are well spread and the elongated processes of neighboring cells overlap extensively. The surfaces of most of the cells in the culture are smooth except for the presence of variable numbers of microvilli. Very few blebs are present. The two rounded cells in this field are covered with surface blebs and are most likely in some stage of mitosis (9). This micrograph shows that there is significant natural variation in the surface morphology of a presumably homogeneous cell population. This variation is thought to represent different stages in the cell cycle (9).

Fig. 2 shows cells that have been exposed to a 1:10 dilution of anti-M serum in whole tissue culture medium for 60 min. The cells have rounded and formed clumps. Their surfaces appear highly convoluted even at low magnification. Retraction fibers still connecting rounded cells to the substratum are abundant. Preliminary time lapse cinematography of anti-M-treated cells shows that extensive membrane ruffling and blebbing begin within 15 min of exposure to antibody and precede other changes in overall cell morphology at the light microscope level. Blebbing and ruffling continue throughout the rounding process (Damsky and Buck, unpublished observations). When cells were incubated with the same dilution of antiserum for 2–3 h, all of the cells detached completely from the substratum. Fig. 3 shows clearly that the surfaces of these detached cells are covered with blebs and filopodia and that the cells resemble the rounded cells in untreated cultures (Fig. 1) at least with respect to their surface contours. The alterations in surface morphology as well as changes in the overall arrangement of intracellular components are apparent in the transmission EM (Figs. 4 and 5).

Antiserum-treated cells were examined by transmission EM at higher magnification with special attention to the organization of cytoskeletal components. All three major components—microfilaments, microtubules, and 10-nm filaments—were present in treated cells but were changed in their organization and distribution. The bundles of 6 to 8-nm microfilaments found just beneath the cell surface of adherent well-spread control BHK21 cells (Fig. 6) were not found in antiserum-treated cells (Fig. 7). Small organized bundles of microfilaments were found in association with surface microvilli and filopodia, but most of the subsurface cytoplasm of treated cells was characterized by amorphous fibrillar material. Similar material found in the subsurface cytoplasm of round postmitotic BHK21 cells has been shown to contain actin (11).

Microtubule profiles are present in thin sections in all areas of antiserum-treated cells (Fig. 7). In fortuitous sections of the cell which include the centriole (Fig. 8), it is clear that the microtubules are still capable of forming complex patterns. A rough comparison of the amount of polymerized tubulin in control and antiserum-treated cells was obtained by morphometric analysis (31). The results (Table I) showed that the volume densities of microtubules in control and anti-M-treated cells were ~1.5 and 1%, respectively. Because of the

Figure 4  Control BHK21/C15 fibroblast sectioned perpendicular to the substrate. The process of a second cell that underlaps the first is visible. Both cells adhere to the substrate by dense plaques (arrows) and have smooth contours over most of their free and attached surfaces. Some surface convolutions are evident at the leading edge of the cell. × 7,000. §, substrate; N, nucleus. Bar, 1 μm.

Figure 5  Thin section through a clump consisting of several rounded BHK cells exposed to anti-M for 3 h. Surface membranes of all cells are highly convoluted except in regions where cells in the clump contact one another (arrowheads). The perinuclear region (arrows) is discrete and excludes most large organelles. At higher magnification (see Fig. 7) it is shown to be packed with 10-nm filaments. × 7,300. Bar, 1 μm.
Figure 6  Higher magnification of control BHK cells cut slightly oblique to the substrate, showing the arrangement of cytoskeletal elements in elongated cellular processes of adherent cells. Bundles of microfilaments, microtubule profiles, and 10-nm filaments all run parallel to the long axis of the process. MFB, microfilament bundles; MT, microtubules; IF, intermediate (10-nm) filaments. × 45,000. Bar, 1 μm.
Figure 7. Higher magnification of a cell portion exposed to anti-M serum for 24 h. In contrast to the control cell, the cell surface is highly convoluted. Discrete microfilaments (MF) are distinguishable only in microvilli and other sharp cellular processes. The subsurface cytoplasm in other regions contains amorphous fibrous material which excludes organelles. Microtubule profiles (MT) are present in many different orientations. 10-nm filaments (IF) are confined to a discrete area of the cell. L, lysosome. × 45,000. Bar, 1 μm.
The centriolar region of an anti-M-treated cell with radiating microtubules. This demonstrates the ability of antiseraum-treated cells to organize their microtubules into complex patterns. C, centriole. × 26,000. Bar, 1 μm.

Table I

| Morphometric Analysis of the Volume Density of Microtubules and Lysosomes in Control and Anti-M-Treated BHK21/C13 Cells |
|-------------------------------------------------|
|                                        | Anti-M treated |
|                                        | Control | 3 h | 24 h |
| Microtubules                           |         |     |      |
| %                                      | 1.5 ± 0.6 | 0.95 ± 0.5 | - |
| Lysosomes                              | 0.8 ± 0.2 | 1.4 ± 0.5 | 2.0 ± 0.6 |

Micrograph prints of control and antiseraum-treated cells were analyzed for the volume density of lysosomes and microtubules as described in Materials and Methods (31).

Large standard deviation, however, it is difficult to assess the significance of this difference. Although these data tell us nothing about the possible role of microtubule redistribution, it is clear that large-scale depolymerization of microtubules, as is found after colchicine treatment, does not play a role in antiserum-induced rounding.

The alteration in the distribution of 10-nm filaments induced by antiseraum treatment was very striking. In control cells, these filaments are present both in the perinuclear region and in elongated cellular processes where they are aligned parallel to the long axis of the cell (Fig. 6). In antiseraum-treated cells, all of the 10-nm filaments appear to be sequestered into a dense mass near the nucleus (Figs. 5 and 7). This is very similar to their appearance in postmitotic or freshly trypsinized BHK cells (10).

In addition to changes in cytoskeletal organization, morphometric analysis (31) revealed an increase in the volume density of the cell occupied by lysosome-like structures in anti-M-treated cells (Table I). The increase may be indicative of the internalization of bound antibody by the cell. There were, however, no large endocytic vesicles and no formation of a large caplike structure at one end of the cell. A more extensive investigation of antibody internalization and its relevance to cell rounding will be undertaken when more specific antisera are obtained.
**Distribution of Antiserum on Cell Surfaces**

Anti-M serum may induce cell rounding and detachment by interacting specifically with cell surface molecules that control adhesion, or it may bind a large number of cell surface components and thus coat the cell sufficiently to interfere physically with the ability of components to maintain contact with the substrate. To determine which possibility is the more likely, the distribution of anti-M on the cell surface was compared with the distribution of another antiserum that interacts with the surface membrane but does not cause cell rounding. Antiserum raised against a water-soluble 120,000-dalton glycoprotein fraction derived from a crude membrane preparation of C12/B4 cells by the method of Marchesi and Andrews (19) was used for this purpose. This antiserum is designated anti-US serum (32).

Cells treated for at least 3 h with purified IgG (10 mg/ml of medium) from anti-M, anti-LIS, or preimmune sera (which does not produce complement-induced killing or rounding) were fixed lightly with glutaraldehyde and were then treated with rabbit anti-goat IgG conjugated to horseradish peroxidase. The peroxidase-conjugated immune complexes were rendered electron opaque by the method of Graham and Kamovsky (13). Fig. 9 shows an anti-M-treated cell that had rounded and detached from the surface before treatment with the second, peroxidase-conjugated antibody. The surface is highly convoluted. Antiserum is present over the entire surface of the cell, but there is no evidence that antiserum treatment has caused any damage to the integrity of the cell surface. Fig. 10 shows an anti-LIS-treated cell. Despite the presence of reaction product over its entire surface as well as on the substrate, this cell remains adherent. Fig. 11 shows the surfaces of several cells treated with preimmune serum. There is almost no reaction product present on their surfaces. There was also no reaction product present on preparations in which either the peroxidase-conjugated rabbit anti-goat immunoglobulin or the dianinobenzidine was omitted. Thus, the positive reactions observed in anti-M and anti-LIS treated preparations are not caused by insufficient removal of unbound material or by endogenous peroxidase activity.

Because immunoperoxidase is not very quantitative, it was important to demonstrate that at the concentrations of IgG used in the immunoperoxidase experiments, cells were able to bind similar amounts of anti-M and anti-LIS IgG. If cells bound much less anti-LIS than anti-M, one might still argue that anti-M-treated cells were detached because of a nonspecific protein coating of the cell surface. To quantitate the amount of bound IgG, cells were lightly fixed to microtiter plates with 0.15% glutaraldehyde (29). After extensive washing with phosphate-buffered saline (PBS) containing 0.15 M glycine, the cells were treated for 3 h at 4°C with anti-LIS, anti-M, or preimmune IgG over a concentration range of 0.1–20 mg/ml in tissue culture medium. Cells were washed free of unbound IgG and were treated with 100,000 cpm/well of 125I-labeled horse anti-goat IgG in PBS containing 0.15% fetal calf serum for 24 h at 4°C. After removal of the unbound labeled antibody, the individual wells of the microtiter plate were counted in a gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.). The results (Fig. 12) show conclusively that the binding of both IgG preparations is maximal at 10 mg/ml. At that concentration, cells bind as much IgG from the anti-LIS preparation as from the anti-M preparation. Binding by cells of preimmune IgG was slightly above background, but only at the highest protein concentrations.

**DISCUSSION**

The exposure of BHK21/C13 hamster fibroblasts to antiserum prepared against surface membranes from C13/B4 hamster cells results in reversible rounding and detachment of the cells from the substratum (32). At the ultrastructural level, changes are seen in both the cytoskeletal organization and surface morphology of such cells (Figs. 1–8). The rounding and detachment appear to be the result of the immunoglobulin reacting with specific cell surface components. This is supported by the observation that although the cells bind equal quantities of anti-LIS and anti-M IgG uniformly over the entire cell surface, only binding of the anti-M IgG results in cell rounding and detachment (Figs. 9, 10, and 12). Thus, merely coating the cell with a layer of immunoglobulin is not sufficient to produce the changes reported here. This suggests that the anti-M preparation recognizes some surface constituents that are not recognized by anti-LIS and that binding of these surface molecules, presumably glycoproteins (32),
FIGURES 9-11 Unstained sections of cells treated with either immune or nonimmune serum followed by fixation and treatment with rabbit anti-goat IgG conjugated to peroxidase (see Materials and Methods).

FIGURE 9 Portion of a cell treated with anti-M serum for 3 h before processing. The cell is rounded and detached and has a highly convoluted surface. Dense reaction product is uniformly distributed over the surface of the cell. × 20,000. Bar, 1 μm.

FIGURE 10 Cell treated with anti-LIS serum for 3 h. Reaction product covers both the cell surface and the substrate. Cell remains adherent despite the presence of antiserum over its entire surface. × 15,000. Bar, 1 μm.

FIGURE 11 BHK cells treated with nonimmune goat serum for 3 h. There is very little reaction product present on cell surfaces or on the substrate. × 20,000. Bar, 1 μm.

...interferes with the maintenance of cell-substrate contacts.

The cytoskeletal changes noted when cells are treated with anti-M immunoglobulin resemble closely those found in vivo in BHK cells which have either just completed mitosis or have been plated onto a suitable substrate after trypsinization (11). Antiserum-treated cells clearly do not resemble cells treated with cytoskeletal disruptive agents such as colchicine or cytochalasin B. We have observed, as have others, that neither of these drugs causes cell detachment. In fact, colchicine promotes flattening of BHK cells (8) and completely depolymerizes microtubules. Anti-M...
The major similarity between antiserum-treated BHK cells (Wylie and Buck, unpublished observations) is that the antiserum does not affect the ability to form organized arrays (Fig. 8). The fact that BHK cells continue to divide in the presence of antiserum (32) is a further indication that the antiserum does not affect the integrity of microtubules themselves. The major difference between antiserum-treated and colchicine-treated BHKx/Ct cells is that both contain a perinuclear region packed with 10-nm filaments (Figs. 5 and 7; reference 8). In contrast to anti-M-treated cells, cytochalasin B-treated BHKx/Ct cells become partially rounded but remain attached to the substrate via long armlike processes which contain microfilament bundles (10). Other reagents, such as lectins, are known to bind to surface glycoproteins and trigger well-documented changes in the subsurface cytoskeleton of cells in suspension (2, 3, 30, 33). There has been one report that concanavalin A (Con A) will induce rounding of a mouse tumor cell line (6). However, Con A does not induce rounding of either normal (BHKx/Ct) or transformed (Ct/Br) BHK cells (Wylie and Buck, unpublished observations).

The mechanisms by which cells attach to and separate from a substrate in vivo (for instance, during the cell cycle) are clearly complex and not well understood. Several components have been shown to be relevant to adhesion, but their particular contributions to the process have not been identified clearly. Biochemical evidence for specific interactions between particular proteins on the external cell surface and components on the substrate (thought to be composed of both material shed from cells and adsorbed serum components) has been presented (14, 15). A critical role for cytoskeletal proteins, especially actin and associated microfilament proteins, in both the cell-substrate contact (14, 15) and spreading phases of adhesion has also been proposed (5, 11, 12, 14). Finally, ultrastructural and immunocytochemical data for the participation of microfilament-associated proteins in adhesion plaques (1, 5, 11, 26, 27) support the suggestion that cytoskeletal membrane interactions are fundamental to the maintenance of adhesive contacts between cell and substrate. In our experiments with anti-M serum, we have demonstrated that adhesion can be disrupted under conditions which permit continued viability and growth of BHK cells (32). The major drawback of our present antiserum is that it is very heterogeneous and may bind to some surface components which are not relevant to adhesion. However, this preparation has demonstrated the feasibility and potential of using an immunological approach for dissecting complex physiological processes such as adhesion, and rapid progress toward understanding adhesion at the molecular level should follow the development of antibodies specific for individual cell surface-associated proteins.

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