STUDIES ON THE FUNCTION OF CELL SURFACE GLYCOPROTEINS

I. Use of Antisera to Surface Membranes in the Identification of Membrane Components Relevant to Cell-Substrate Adhesion

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ABSTRACT

An antiserum prepared against purified surface membranes of transformed BHK₂₁/C₁₃ cells (C₁₃/B₄) reversibly rounded and detached hamster cells from plastic tissue culture plates but did not affect cells of other species. Antiserum treatment did not alter the growth rate of C₁₃/B₄ or BHK₂₁/C₁₃ cells; however, NIL-8 cells exposed to the antiserum detached from the substrate and stopped growing, but remained viable for up to 72 h in the presence of the antiserum. Rounding and detachment were not inhibited by DNP or cycloheximide. Antiserum-detached cells did not reattach in the presence of these inhibitors. F(ab)' fragments also induced rounding, thus ruling out the involvement of complement and ligand-induced rearrangement of surface antigens in rounding and detachment.

Three different surface-reactive immunoglobulin preparations were used in indirect immunoprecipitation studies in an attempt to identify cell surface antigens involved in regulating adhesion and morphology. Antiserum against surface membranes (anti-M) and against material shed by the cells into serum-free medium (anti-SFM) caused rounding and detachment, but a third antiserum (anti-LIS) prepared against a partially purified glycoprotein did not. All three immunoglobulin preparations precipitated glycoproteins with an apparent mol wt of 120,000 daltons from a crude membrane preparation solubilized by Nonidet NP-40. The two immunoglobulin preparations that caused rounding precipitated an additional glycoprotein peak of 140,000 daltons. Extensive preabsorption of the extract with anti-LIS immunoglobulin enriched the anti-membrane and antiserum-free medium precipitates for the 140,000-dalton peak. Anti-M immunoglobulin eluted from intact cells and subsequently used to precipitate NP-40 solubilized membrane constituents also reacted with a group of glycoproteins of ~140,000 mol wt. Therefore, this group of glycoproteins was considered most likely to be the glycoproteins involved in substrate adhesion and maintenance of cellular morphology.
KEY WORDS antisurface membrane antibody · surface membrane · glycoproteins · adhesion · immunoprecipitation

Many changes occur in the structure of cell surface membranes as the result of malignant transformation (10, 17); however, it is not known how such alterations affect the biological behavior of the cell. One alteration that usually results from in vitro transformation is reduced adhesion to a substratum (7). Alterations in adhesive properties of cells are thought to be relevant to metastasis and perhaps to tumorigenesis, but the actual significance of reduced adhesion will probably be understood only after elucidation of the mechanism by which a normal cell adheres to a substratum.

The observation that a number of proteins, peripherally associated with the membrane, remain associated with the substratum after solubilization of the surface membrane with nonionic detergents or after complete removal of cells by chelating agents (3, 5, 19) has implicated these proteins in adhesion. The implicated proteins include the large external transformation-sensitive glycoprotein (LETS) described by several investigators (10), as well as cytoskeletal elements such as actin, myosin, and the monomeric subunit of 10-nm filaments (3, 5, 19). These studies suggest that control of adhesion must be a transmembrane process involving proteins on both sides of the lipid bilayer. However, none of the proteins mentioned above are considered to be integral components of the surface membrane. At some point in the adhesion process, an interaction must occur between these peripheral proteins and some component that is an integral part of the lipid bilayer.

Two recent reports have implicated integral surface proteins in cell-cell adhesion. Takeichi (22) has associated a trypsin-sensitive, iodinatable surface protein with a mol wt of ~150,000 daltons with the intercellular adhesion of V79 hamster cells. Thirty et al. (23), by using antisera prepared against neural retinal cells and against material shed from these cells into tissue culture medium, have identified a cellular protein with a subunit mol wt of ~140,000 as important in intercellular adhesion.

This paper reports on the ability of antisera against various surface components to reversibly disrupt substrate adhesion and describes the use of these antisera as probes to identify the surface molecules possibly involved in cellular adhesion.

MATERIALS AND METHODS

Cells and Cell Culture

Two fibroblastic cell lines of baby hamster kidney (BHK) origin were used for most of the experiments described below: a control cell line designated BHK/C13 and this same cell line transformed by the Bryan high-titer strain of Rous sarcoma virus, designated C57/Br. NIL-8 cells, also of hamster origin, and HT2 cells, an epithelial cell line derived from a human mammary carcinoma, were also used for some experiments. Cells were grown in Eagle's minimum essential medium supplemented with 5% fetal calf serum (Flow Laboratories, Inc., Rockville, Md.), 10% tryptose phosphate broth (Difco Laboratories, Detroit, Mich.), penicillin (50 U/ml), and streptomycin (100 µg/ml). Unless otherwise noted, cells were grown in Costar dishes containing six 9.5 cm² wells/dish (Costar, Data Packaging, Cambridge, Mass.).

Polycrylamide Gel Electrophoresis (PAGE)

Samples being analyzed by electrophoresis were run on a 1.5-mm-thick polycrylamide gradient slab gel, with a 10 cm 7-16% polycrylamide separating gel and a 2-cm stacking gel with a 3% polycrylamide concentration, with the buffer system described by Laemmli (13). Before they were applied to the gel, samples were boiled for 3 min in sample buffer. Gels were electrophoresed at 20 mA until the tracking dye reached the bottom of the gel (usually ~3 h). Samples run under nonreducing conditions were treated identically, except that they were dissolved in sample buffer without mercaptoethanol. The Laemmli system was also used for preparative gels; however, in this case 0.5 × 10-cm cylindrical gels were made with a straight 8.75% acrylamide concentration in the separating gel. Upon completion of a run, gels were fixed for 2 h in 10% acetic acid and 25% isopropanol, then stained overnight with 0.025% Coomassie blue in the same solution. After they were stained, gels were deolorized in a solution of 10% acetic acid and 5% methanol.

1 Abbreviations used in this paper: Con A, concanavalin A; DNP, 2,4 dinitrophenol; LETS, large external transformation-sensitive glycoprotein; PAGE, polycrylamide gel electrophoresis; PBS, phosphate-buffered saline, 0.15 M NaCl, 0.002 M KCl in 0.012 M phosphate buffer, pH 7.4; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline, 0.15 M NaCl in 0.01 M Tris HCl, pH 7.5.

2 The authors thank Dr. Richard Hynes for supplying us with these cells.

3 Obtained from the Mason Research Institute, Rockville, Md. Their designation: MCF-7.
Antisera Production

All antigens used to elicit antibody production were prepared from cultures of C13/B4 cells. For production of antimebrane antiserum, surface membranes from C13/B4 cells removed from the monolayer with ethylene diamine tetracetic acid (EDTA) (0.001 M in phosphate-buffered saline (PBS)) were prepared by the zinc ion method of Warren et al. (24). Goats were injected five times with the surface membrane preparation at monthly intervals and challenged 6 mo after the fifth injection. A total of 2 mg of membrane protein in complete Freund's adjuvant was distributed over four sites at each injection. Immune serum was collected 1 wk after the second and third injections and at eight 2-wk intervals after the final injection.

For preparation of an immunogen consisting of shed cellular material, C13/B4 cells were inoculated in glass roller bottles (640 cm²) at a density of 2 × 10⁶ cells/bottle. After incubation at 37°C for 24 h, the cells were radioactively labeled with 50 μCi I-1-¹⁴C-ribose (40-55 mCi/mmol) per bottle for 48 h. (All radioisotopes were purchased from New England Nuclear, Boston, Mass.) Then, the labeling medium was replaced with Eagle's minimum essential medium without fetal calf serum, trypose phosphate broth, or antibiotics (serum-free medium). After incubation for 4 h at 37°C, the initial sample of serum-free medium was removed from each bottle and 20 ml of fresh medium was added. This medium change was designed to minimize contamination of the serum-free medium with any adsorbed serum components that may have been shed from the cells into the medium during the first few hours of incubation. After cells had been incubated in serum-free medium for 24 h, the medium was removed, centrifuged at 50,000 g for 24 h. Digestion was stopped by raising the pH of the solution to 8.0 with solid Trizma base (Sigma Chemical Co., St. Louis, Mo.) equilibrated with 0.1 M Tris-HCl, pH 8.0, and deoxygenated under a stream of N₂ for 15 min. The solution was brought to 0.2 M with dithiothreitol and allowed to react for 30 min at room temperature under N₂. The solution was then brought to 0.3 M with iodoacetamide and allowed to react for 30 min at room temperature, during which time the pH was maintained between 7.8 and 8.4.

Preparation of F(ab')² Fragments

Purified immunoglobulin was digested by a modification of the method of Nisonoff et al. (18). 300 mg of immunoglobulin in 3 ml of PBS was dialyzed overnight at 4°C against 4 liters of 0.1 M acetate buffer, pH 4.5. After dialysis, 3 mg of pepsin (Sigma Chemical Co., St. Louis, Mo.) was added and the solution was incubated for 24 h at 37°C. Digestion was stopped by raising the pH of the solution to 8.0 with solid Trizma base (Sigma Chemical Co.). The digested antibody was then passed over a 2.5 × 150 cm AcA34 column (LKB Instruments, Inc., Rockville, Md.) equilibrated with 0.1 M Tris-HCl which contained 0.2 M NaCl and 0.002 M Na₂ EDTA, pH 7.7. 5-ml fractions were collected and the A₂₈₀ of each was measured. One major peak eluted in the inclusion volume of the column. When analyzed by SDS-PAGE, the peak consisted of a 100,000-dalton mol wt band under nonreducing conditions. Since these results would be expected if the material from this peak consisted of F(ab')² fragments, this peak was assumed to consist of these fragments and was subsequently used for reduction and alkylation.

100 mg of F(ab')² fragments was dialyzed overnight against 0.5 M Tris-HCl, pH 8.0, at 4°C, and deoxygenated under a stream of N₂ for 15 min. The solution was then brought to 0.2 M with dithiothreitol and allowed to react for 30 min at room temperature under N₂. The solution was then brought to 0.3 M with iodoacetamide and allowed to react for 30 min at room temperature, during which time the pH was maintained between 7.8 and 8.4.
with 2 N NaOH. This solution was passed over a G-100 column (2.5 x 100 cm) equilibrated with PBS containing 0.002 M NaCl. One peak was found in the inclusion volume of the column. That this material consisted entirely of F(ab) fragments is shown by the fact that, when analyzed by SDS-PAGE under nonreducing conditions, a single band of protein with a mol wt of 50,000 daltons was seen. The same material electrophoresed under reducing conditions produced a single band of protein with a mol wt of 25,000 daltons.

**Immunofluorescence**

Cells were grown on coverslips for 24 h and washed three times with PBS immediately before staining. The coverslips were incubated for 30 min at room temperature in undiluted anti-membrane serum, then washed three times with PBS. Rabbit anti-goat globulin labeled with fluorescein isothiocyanate (Grand Island Biological Co. [GIBCO], Grand Island, N. Y.) was added under the same conditions. The coverslips were then washed three times in PBS, mounted on microscope slides in a solution of 90% glycerol and 10% PBS, and examined with a Leitz fluorescence microscope (E. Leitz, Inc., Rockleigh, N. J.) which contained the appropriate filters for fluorescein fluorescence.

**Preparation of Radiolabeled Antigen for Immunoprecipitation**

BHK21/C13 and C13/B4 cells that had been grown in the presence of 50 μCi [1-14C]amino mixture and 25 μCi L-[1-3H]fucose (40-55 μCi/mmol) per 50 ml of tissue culture medium were removed from the monolayer with EDTA. They were then swollen in hypotonic saline for 10 min at 4°C and disrupted by homogenization with a GT 21 Laboratory Stirrer at a setting of 10 (Gerald Heller Co., Las Vegas, Nev.). The homogenate was centrifuged at 27,000 g for 30 min to pellet the membrane fragments. This crude membrane pellet was resuspended in 5 ml of 0.5% Nonidet P-40 (NP-40, Shell Chemical Co., Houston, Tex.) in 0.01 M Tris-HCl, pH 8.0, containing 0.002 M phenylmethylsulfonyl fluoride, and extracted for 2 h at 4°C. After centrifugation at 27,000 g for 30 min, the supernate was used as the source of solubilized membrane antigens for subsequent immunoprecipitation. NP-40 extraction as described resulted in the solubilization of 50% of the amino acid counts and 90% of the fucose counts from the crude membrane pellet of either cell line.

**Preparation of Bacterial Adsorbant for Immunoprecipitation**

Staphylococcus aureus Cowan I strain (Staph) was prepared for use as a bacterial adsorbant by the method of Kessler (12). Immediately before use, the bacteria were washed once with 0.5% NP-40 in NET buffer (0.15 M NaCl, 5 mM EDTA, 50 mM Tris, and 0.02% sodium azide, pH 7.4), once with NET buffer containing 0.05% NP-40, and once with PBS.

**Immunoprecipitation**

0.5 ml of a 5-ml NP-40 solubilized membrane extract from 5 x 10⁶ cells was allowed to react at 4°C overnight with 14 mg of preimmune immunoglobulin. The extract was mixed with a washed Staph pellet from 2 ml of a 10% bacterial suspension and allowed to stand at 4°C for 1 h. The mixture was then centrifuged at 1,200 g for 10 min. The Staph pellet was washed three times with cold PBS and eluted by boiling for 3 min in a 0.5% solution of SDS (wt/vol in H₂O). A 10-μl portion of the eluate was counted for radioactivity. The remainder was lyophilized and resuspended in 50 μl of Laemmli sample buffer for electrophoresis (13). The supernate from the preimmune adsorption was incubated with 10-15 mg of immunoglobulin at 4°C overnight. The immune complexes were then adsorbed to and eluted from Staph as described above, with 10 μl used to measure radioactivity.

After lyophilizing, the eluate was dissolved in Laemmli sample buffer and run on SDS-polyacrylamide slab gels. Each gel was sliced into 1-mm slices, which were dissolved by incubation overnight at 37°C in 3 ml of toluene containing 4% Concentrol (New England Nuclear), 4% Protosol (New England Nuclear), and 1% water. The gels were then counted in an Intertechnique SL 4000 liquid scintillation counter (INUS Service Corp., Fairfield, N. J. 07006).

**Protein Determination**

Protein was measured by the method of Lowry et al. (14) using bovine serum albumin fraction V (Sigma Chemical Co.) as the standard.

**Protease Assay**

The presence of proteases was assayed by using the general substrate Azocoll (Calbiochem, San Diego, Calif.) according to the manufacturers directions. Standards containing known amounts of trypsin (Worthington Biochemical Corp., Freehold, N. J., crystallized three times) were used to calibrate the reaction and establish lower limits of sensitivity.

**Absorption and Elution of Immunoglobulin from Cells**

BHK21/C13 cells in roller bottles were washed five times with PBS and fixed with 0.15% glutaraldehyde. After fixation, the monolayers were washed three times with PBS containing 0.15 M glycine and then three times with PBS. 20 mg of anti-M immunoglobulin in 5 ml of PBS was added to each of 10 roller bottles, and absorption was allowed to continue for 1 h at 37°C. Each roller bottle was washed five times with PBS to remove any residual immunoglobulin. The immunoglobulin absorbed to the monolayer was eluted in 5 ml of 3 M
sodium thiocyanate. The eluates from 10 bottles were pooled, dialyzed first against PBS, and then against water. The immunoglobulin was concentrated by lyophilization and dissolved in PBS.

RESULTS

Effect of Anti-Membrane (anti-M) Serum or Immunoglobulin on Cellular Adhesion and Morphology

Before the addition of heat-inactivated anti-M serum, C13/B4 cells exhibited the characteristic epithelioid morphology of transformed fibroblasts (Fig. 1a). In Fig. 1b, C13/B4 cells are shown after incubation for 2 h in medium containing a 1:10 dilution of anti-M serum. The cells have rounded and formed aggregates that are completely detached from the substratum. Incubation for an identical period of time in the same concentration of preimmune serum or in antisera prepared against whole tissue culture medium did not change the appearance of these cells from that of untreated cells (data not shown). Within 24 h after the removal of antisera, the majority of the cells reattached to the substratum and spread, leaving very few cells still in suspension (Fig. 1c). This phenomenon of immunoglobulin-detached cells reattaching and spreading onto the substratum when placed in antibody-free medium will subsequently be referred to as "reversal". Fig. 1d-f shows BHK21/C13 treated in the same manner as described above for C13/B4 cells. The antiserum exerted the same effect on this cell line as on the transformed cell line against which the antiserum was produced. Therefore, the anti-M serum is not directed against a transformation-specific surface membrane component.

The extent of the immunoglobulin-mediated detachment and reversal is quantitated in Table I, which shows the percent of cells detached at various times after addition of immunoglobulin. Although both cell lines were nearly completely detached by the immunoglobulin, BHK21/C13 cells were affected more rapidly than C13/B4 cells (Table I). 24 h after removal of the immunoglobulin, the majority of cells had reattached to the substratum, with 84% of the C13/B4 cells and 76% of the BHK21/C13 re-adhering. Both detached and adhering cells treated with anti-M immunoglobulin showed a viability of over 90% as measured by trypan blue exclusion.

Species Specificity of Antisera

The effect of antiserum used at these concentrations appears to be restricted to cells of hamster origin (Table II). In fact, immunofluorescence revealed no binding of the anti-M serum to the surface of cells from other species. The antiserum caused the same morphological change in primary hamster cells as in established hamster cell lines. This suggests that the effect induced by the antisera is caused by the recognition of species-specific surface antigens and not by the recogni-

**Figure 1** Effect of antiserum against C13/B4 plasma membranes on substratum adhesion and morphology of C13/B4 and BHK21/C13 cells. Cells were treated with a 1:10 dilution of heat-inactivated antisera as described in text. (a) Untreated C13/B4 cells; (b) C13/B4 cells 2 h after addition of antisera; (c) C13/B4 cells 24 h after removal of antisera; (d) untreated BHK21/C13 cells; (e) BHK21/C13 cells 2 h after addition of antisera; (f) BHK21/C13 cells 24 h after removal of antisera. All figures x 100.
### Table I

**Quantitation of Cell Detachment by Anti-Membrane Serum**

| Cell line | Treatment | % of Detached cells | % of Attached cells |
|-----------|-----------|---------------------|---------------------|
|           |           | % via- total ble    | % via- total ble    |
| C12/B4    | Immune, 0 h | 1 99 99 98          |                     |
|           | Immune 3 h  | 28 99 72 98         |                     |
|           | Immune, 24 h | 90 96 10 99    |                     |
|           | Immune, reversed for 24 h | 16 94 84 98 |                     |
| Preimmune, 0 h | 2 98 98 98 |                     |                     |
| Preimmune, 3 h  | 2 99 98 98 |                     |                     |
| Preimmune, 24 h | 20 90 80 98 |                     |                     |
| Preimmune, reversed for 24 h | 9 96 91 99 |                     |                     |
| Immune, 0 h  | 2 96 98 98 |                     |                     |
| Immune, 3 h  | 91 97 9 99 |                     |                     |
| Immune, 24 h | 24 91 76 99 |                     |                     |
| Immune, reversed for 24 h | 24 91 76 99 |                     |                     |
| Preimmune, 0 h | 1 95 99 98 |                     |                     |
| Preimmune, 3 h  | 2 94 98 98 |                     |                     |
| Preimmune, 24 h | 5 96 95 99 |                     |                     |
| Preimmune, reversed for 24 h | 11 87 89 99 |                     |                     |

1–2 × 10^6 cells in 25-cm² Falcon tissue culture flasks were incubated at 37°C with either 10 mg/ml of preimmune or immune globulin from antiserum against C12/B4 surface membranes. To determine the rate of immunoglobulin-induced detachment of cells from the flask, medium and detached cells were removed from the monolayer at the times stated above. Any cells still attached to the flask were removed by trypsinization. Cells removed by immunoglobulin detachment and trypsinization were separately suspended in PBS containing 0.05% trypan blue and counted with a hemocytometer. Total cells equals the sum of immunoglobulin detached and trypsinized cells.

Re-adhesion of immunoglobulin-detached cells was monitored after exposure of cells to anti-M immunoglobulin for 24 h. The detached cells were removed and pelleted by centrifugation. The pelleted cells were resuspended in tissue culture medium without immunoglobulin and replate in a 25 cm² tissue culture flask. After 24 h the number of cells remaining detached and the number that settled and attached to the flask were determined as described above.

### Table II

**Species Specificity of Anti-Membrane Serum**

| Cell line | Species | Rounding | Loss of adhesion | Fluorescence |
|-----------|---------|----------|-----------------|-------------|
| BHK21/C12 | Hamster | +        | +               | +           |
| C12/B4    | Hamster | +        | +               | +           |
| Primary   | Hamster | +        | +               | ND*         |
| NIL-8     | Hamster | +        | +               | +           |
| Primary   | Chicken | –        | –               | –           |
| kidney    |         |          |                 |             |
| Primary   | Mouse   | –        | –               | –           |
| 3T3       | Mouse   | –        | –               | –           |
| HT3       | Human   | –        | –               | ND          |

The antisera against C12/B4 surface membranes was tested for reactivity with cell lines of other species. Immunofluorescence was performed as described in Materials and Methods. Cells were grown for 24 h on coverslips and washed with PBS immediately before staining. For detection of morphological alterations, cells were grown in Costar dishes in the presence of a 1:5 dilution of either preimmune or immune serum and examined at 2 and 24 h. Cells were evaluated as positive for the presence or negative for the absence of each parameter.

* ND, not determined.
Effect of anti-membrane serum on co-cultured hamster and human cells. BHK21/C13 cells and HT-3 cells, which are epithelial cells derived from a human mammary tumor, were cultured together in the same well of a Costar dish. A 1:10 dilution of heat-inactivated anti-membrane serum was added, and the cells were observed for rounding and detachment after 24 h. (A) Cells before addition of antiserum; (B) cells after 24 h in the presence of antiserum. Both figures × 100.

detected. 10 times this amount of trypsin will not induce rounding or detachment of the BHK21/C13, C13/B4, or the human HT3 cells shown in Fig. 2.

Effect of Immunoglobulin on Cell Growth

The growth rate of cells cultured in the presence of anti-M immunoglobulin was determined for both C13/B4 and BHK21/C13 cells (Fig. 3a and b). Each cell line grew at approximately the same rate and to the same saturation density as cells growing in the presence of preimmune globulin, indicating that these cells were able to grow in the absence of a substrate even though neither cell line had been adapted for growth in suspension.

To determine whether other cell lines detached by the immunoglobulin concomitantly acquired the ability to grow in suspension, the growth of NIL-8 hamster fibroblasts was measured in the presence of anti-M immunoglobulin (Fig. 3c). In the first 24 h, the anti-M-treated cells increased in number at approximately the same rate as cells exposed to the same quantity of preimmune globulin. Subsequently, the total number of treated cells remained constant for 72 h, whereas the control cells continued to grow. When the immunoglobulin was removed, cells resumed growth at a rate similar to that of the control cells. In contrast to BHK cells, the NIL cells, although viable, were unable to grow in suspension for an extended period of time in the presence of anti-M immunoglobulin. Thus, release from substrate-dependent growth by anti-M immunoglobulin is clearly not a general phenomenon.

Effect of Metabolic Inhibitors on Immunoglobulin-Mediated Cell Detachment and Reversal

To determine whether cellular metabolism was required for the detachment of cells by immunoglobulin, detachment was carried out in the presence of various metabolic inhibitors (Fig. 4). Neither cycloheximide nor 2,4 dinitrophenol (DNP) inhibited detachment of cells by the immunoglobulin (Fig. 4d and g, respectively). However, when the same inhibitors were added at the time of reversal, cells that had been previously detached by immunoglobulin would not reattach to the substratum (Fig. 4e and h). Cells growing in the presence of cycloheximide (Fig. 4f) or DNP and preimmune globulin (Fig. 4i) for 24 h resemble control cells (Fig. 4c). Over 90% of the cells in cycloheximide and 75% of the cells in DNP were still viable after 24 h. It seems that an energy source and protein synthesis were not needed for
antibody-induced rounding and substrate detachment, but were required for reattachment.

To determine whether cycloheximide and DNP blocked reattachment by inhibiting the release of surface-bound immunoglobulin, the percent of cells with bound antibody was determined by complement-mediated cytotoxicity. At designated intervals after removal of the immunoglobulin, cells were placed in medium containing guinea pig complement. After incubation for 30 min in the presence of complement, the number of viable cells was determined by trypan blue staining. As shown in Fig. 5, most of the cells reversed in the absence of cycloheximide lost the antibody from their surface. This release was slow for the first 5 h, then more rapid between 5 and 21 h. At the end of this time only 16% of the cells still contained enough antibody to be lysed by the addition of complement. In contrast, >90% of the cells in cycloheximide still had complement-fixing antibody bound to their surface after 21 h.

Cell Detachment with F(ab)’ Fragments

To determine whether the rounding and substrate detachment induced by anti-M immunoglobulin requires rearrangement of surface molecules and is therefore dependent on the presence of a divalent reagent, cells were exposed to monovalent F(ab)’ fragments prepared from anti-M serum. As seen in Fig. 6, these fragments cause cells to round in a reversible manner. Therefore, it seems likely that the critical event initiating cell rounding is the binding of antibody to the cell surface, and not any subsequent arrangement caused by cross-linking of antigens by a divalent antibody.

Indirect Immunoprecipitation of Membrane Antigens

Indirect immunoprecipitation using the immunoglobulin fraction from three separate antisera (Table III) was performed in an attempt to identify the membrane components with which our immunoglobulins were reacting and which therefore might be involved in regulating cellular adhesion and morphology. The reaction of all three immunoglobulin preparations with the cell surface was detected by complement-mediated cytotoxicity. However, as shown in Table III, anti-LIS
immunoglobulin did not induce rounding. It was reasoned that any antigens precipitated by the anti-LIS serum should not be directly involved in the process of cell adhesion, but common antigens precipitated by the anti-M and antiserum-free medium (anti-SFM) immunoglobulins and distinct from the LIS antigens might play a role, either directly or indirectly, in this process. The antigens used in these experiments were prepared by solubilizing crude membrane fractions from either cell line with the nonionic detergent NP-40. The radioactive gel profiles for the membrane material solubilized by detergent extraction of cells grown in the presence of \(^{3}H\)-amino acids and \(^{14}C\)-l-fucose are shown in Fig. 7a for BHK\(_{21}/C_{13}\) cells. Most of the fucose-labeled material migrated in several broad bands in the 50,000- to 150,000-dalton mol wt regions. There was some amino acid radioactivity in these regions, but the majority of this label was found at 75,000 daltons and below.

The antigens precipitated by all three immunoglobulin preparations were found to be enriched in glycoproteins of a more restricted molecular weight range than noted in the whole NP-40 extract. The anti-LIS immunoglobulin precipitated a more nearly homogeneous group of glycoproteins (Fig. 7b) than did the anti-SFM or anti-M immunoglobulins (Fig. 7c and d). The major group of glycoproteins precipitated by the anti-M and anti-SFM immunoglobulins contain, in addition to the 120,000-dalton glycoproteins, a group with an apparent mol wt of 140,000 daltons. All
im munoglobulin preparations precipitated to some extent a glycoprotein or group of glycoproteins of ~50,000 daltons.

It appears that all three immunoglobulin preparations preferentially precipitated glycoproteins rather than nonglycosylated proteins. To quantify the enrichment of glycoproteins in the immune precipitates, the ratio of fucose to amino acid radioactivity was calculated for the whole membrane extract and compared to the ratios of immunoprecipitates from each of the three immune globulin preparations (Table IV). In both cell lines, the precipitates from the specific immunoglobulin showed a higher $^{14}$C/$^3$H ratio than did that of the whole extract.

Because anti-LIS immunoglobulin had no morphological effect on cells (see Table III), attempts were made to remove the glycoproteins precipitated by the anti-LIS globulin from NP-40 extracts by exhaustively preprecipitating the extracts with anti-LIS immunoglobulin and Staph A. After preprecipitation with anti-LIS immunoglobulin, the extracts were precipitated with either anti-M or anti-SFM immunoglobulin. The precipitates were then run on SDS-polyacrylamide gels. The results of such an experiment using NP-40 extracts from BHK$_{21}$/C$_{11}$ cells are shown in Fig. 8. A comparison of Fig. 8a with Fig. 8d and c shows that preabsorption with anti-LIS immunoglobulin greatly reduced the heterogeneity of the material precipitated by either anti-M or anti-SFM immunoglobulin. In both cases, the 120,000-dalton peak was significantly reduced.

To further document that the 120,000-mol wt group of glycoproteins precipitated by the anti-LIS immunoglobulin were distinct from the 140,000-mol wt glycoproteins remaining in the NP-40 extracts after anti-LIS preadsorption, the following double-label experiments was performed. BHK$_{21}$/C$_{11}$ cells were labeled with either $^3$H- or $^{14}$C-L-fucose. Crude membrane fractions were extracted with NP-40 and subjected to immunoprecipitation. The $^{14}$C-labeled glycoproteins that were immunoprecipitated with anti-M immunoglobulin were mixed with $^3$H-labeled glycoproteins immunoprecipitated with anti-LIS immunoglobulin. The mixture was subjected to SDS-PAGE. The results are shown in Fig. 9. The major peak of glycoproteins precipitated by the anti-LIS immunoglobulin is distinct from the 140,000-mol wt group of proteins and comigrates with the leading edge of the 120,000-mol wt group of glycoproteins precipitated also by the anti-M immunoglobulin.

Although surface membranes were used as immunogens in preparing the anti-M immunoglobulin, such membrane preparations always have portions of the underlying cytoplasmic material stuck to their cytoplasmic surface (24). The question then arises as to whether the glycoproteins present in the immunoprecipitations are representative of the cell surface. This question was addressed by first adsorbing anti-M immunoglobulin to the surface of fixed, intact BHK$_{21}$/C$_{11}$ cells. The adsorbed immunoglobulin, which should now be specific for molecules found on the cell surface, were eluted and reacted with an NP-40 extract of membranes from BHK$_{21}$/C$_{11}$ cells labeled with $^3$H-amino acids and $^{14}$C-fucose. The immune complexes were absorbed with Staph A, eluted, and electrophoresed on SDS-polyacrylamide gels. The results are shown in Fig. 10. The immunoprecipitate contains primarily glycosylated proteins. It is
Effect of anti-membrane F(ab′) fragments on cell morphology and adhesion. C13/B4 cells growing in Costar dishes were treated with F(ab′) fragments at a concentration of 5 mg/ml of culture medium. (A) Cells after 2 h in the presence of F(ab′) fragments; (B) cells previously detached with F(ab′) fragments after 24 h reversal. Both figures x 100.

**TABLE III**

**Characteristics of Antisera Used in Immunoprecipitation**

| Antiserum               | Antigen                                      | Rounding | Complement-mediated cytotoxicity | Fluorescence |
|-------------------------|----------------------------------------------|----------|----------------------------------|--------------|
| Anti-LIS                | Lithium diiodosalicylate extracts of C13/B4 cells | -        | +                                | +            |
| Antiserum-free medium   | C13/B4 cellular components shed into serum-free medium | +        | +                                | +            |
| Anti-membrane           | C13/B4 surface membranes prepared by zinc-ion method of Warren and Glick (21) | +        | +                                | +            |
| Anti-tissue culture medium | Tissue culture medium containing 10% TBS and 7.5% fetal bovine serum | -        | -                                | -            |

Interaction of the antisera with the cell surface was tested by three different methods. Rounding was detected by observation of cells that had grown in Costar dishes in the presence of a 1:5 dilution of heat-inactivated antiserum for 3 h. For determination of complement-mediated cytotoxicity, cells grown in Costar dishes in the presence of a 1:5 dilution of antiserum were observed after 2 h, and antisera were evaluated as positive if cells were lysed and negative if cells remained intact. Immunofluorescence was performed as described in Materials and Methods. Cells were evaluated as positive for the presence or negative for the absence of fluorescence on the cell surface.

Enriched in a group of glycoproteins of between 130,000 and 150,000 mol wt, suggesting that the glycoproteins which react primarily with anti-M and anti-SFM immunoglobulin (Fig. 8c and d) are found on the cell surface. This is further corroborated by the fact that both anti-M and anti-SFM react with a group of iodinatable proteins or glycoproteins on the cell surface which migrate in the 130,000- to 150,000-mol wt range on SDS-polyacrylamide gels (Damsky and Buck, unpublished observations).

These data show that the materials precipitated by anti-M and anti-SFM immunoglobulin are enriched in glycoproteins of ~140,000 daltons which...
FIGURE 7 Immunoprecipitation with surface-reactive immunoglobulins of NP-40 solubilized crude membrane preparation from BHK21/C13 cells. Cells grown in \(^{3}H\)-amino acids and \(^{14}C\)-fucose were harvested and homogenized to prepare crude membrane fraction as described in Materials and Methods. Membranes were extracted with 0.5% NP-40 in Tris-HCl pH 8.0 for 2 h at 4°C, and the solubilized fraction was used as the antigen in immunoprecipitation. The extract was preabsorbed once with preimmune globulin and *S. aureus* before being precipitated with immunoglobulin. Immunoprecipitates were electrophoresed on 7-16% polyacrylamide gradient gels under reducing conditions in the presence of SDS. Gels were sliced and counted for radioactivity. (A) Whole antigen extract; (B) membrane material precipitated with anti-LIS; (C) membrane material precipitated with anti-SFM immunoglobulin; (D) membrane material precipitated with anti-M immunoglobulin; (E) nonspecific material from immunoprecipitation with preimmune globulin. Molecular weight positions were extrapolated from curves constructed on the basis of the migration rates of standard proteins run in adjacent wells on the same slab gel. ---, \(^{3}H\)-amino acids, ---, \(^{14}C\)-fucose.
TABLE IV

|                 | BHK₂₁/C₁₅ | NP-40 extract | Anti-LIS | Anti-SFM | Anti-M |
|----------------|------------|---------------|----------|---------|--------|
| Total H cpm    | 16,301     | 459           | 550      | 1,176   | 37,424 |
| Total ¹³C cpm  | 12,995     | 1,278         | 2,274    | 2,858   | 15,724 |
| Enrichment in glycoproteins | 0.8 | 2.8 | 4.1 | 2.4 | 0.4 |

The enrichment of glycoproteins over proteins in the immunoprecipitates from each antiserum was determined by using NP-40 extracts labeled with ³H-aminos acids and ¹³C-fucose. The total specific radioactivity precipitated by an antiserum was calculated by subtracting the amount of an isotope found in the preimmune precipitate from that found in the immune precipitate. The glycoprotein enrichment was determined by dividing the ¹³C/H ratio for each antiserum by the ratio for the whole extract.

The enrichment of glycoproteins in immunoprecipitations

The possibility still exists that the NP-40 extraction procedure used to prepare antigens for the above immunoprecipitation experiments failed to extract the components relevant to the control of cellular adhesion. To eliminate this possibility and to strengthen our assertion that glycoproteins are responsible for the phenomena observed, two antisera were prepared; one against the material extracted by NP-40 treatment and the other against the residue insoluble in NP-40. The effect of these antisera on the morphology and adhesion of intact C₁₀/B₄ cells is shown in Fig. 11. Rounding and detachment occurred only in the presence of the antiserum directed against the NP-40 soluble material (Fig. 11a) even though both antisera were capable of reacting with the cell surface as demonstrated by complement-mediated lysis at equivalent dilutions.

DISCUSSION

The results presented here demonstrate that immunoglobulins from antisera prepared against either surface membranes of C₁₀/B₄ cells or material shed by these cells into tissue culture medium are capable of affecting markedly both the morphological and adhesive properties of hamster cells. The immunoglobulins react only with hamster cells and not with other rodent cells such as mouse 3T3 fibroblasts or mouse kidney cells. The immunoglobulins are not specific for transformed cells because they react with nontumorigenic hamster fibroblasts such as NIL-8 and primary hamster embryo cells, as well as with transformed cells. The effect of the immunoglobulins was completely reversible: cells that rounded and detached from the substratum quantitatively re-adhered to the substratum upon removal of the immunoglobulin.

The immunoglobulin-induced rounding reported here is distinct in several respects from the concanavalin A (Con A)-induced rounding of cells reported by Chowdhury (4). Con A induced rounding of tumor cells, but not of normal fibroblasts; Con A induced rounding, but not the detachment of cells; Con A-induced rounding was maximal after removal of the lectin; and Con A treatment markedly reduced the growth rate of the rounded tumor cells. Like the immunoglobulin-induced rounding and detachment reported here, the effects of Con A on tumor cells was reversible. However, Con A will not induce rounding or detachment of either the BHK₂₁/C₁₅ or C₁₀/B₄ cells used in these experiments (Buck and Wylie, unpublished observations).

It is unlikely that the effect of these immunoglobulin preparations on cells is caused by the presence of protease in the goat antiserum or immunoglobulin preparations, or by the release of a protease by the cells in response to the binding of immunoglobulin. The finding that the immunoglobulins will induce morphological alterations only of hamster cells argues against the presence of a protease in the immunoglobulin preparation itself, because no cell-specific protease has yet been described. The fact that the changes occur in the presence of such protease inhibitors as TPCK, PMSF, and soybean trypsin inhibitor (Buck and Knudson, unpublished results) further suggests there is no protease in the immunoglobulin and also serves to distinguish the loss of adhesion reported here from that seen with pemphigus antiserum in which the loss of adhesion is probably protease mediated (6). In addition, the effect is not seen with cells exposed to preimmune globulins from the same goat used to prepare the specific antiserum or from goats inoculated with other immunoglobulins. Finally, the Azocoll assay
revealed no protease activity in the tissue culture medium from cells undergoing rounding, and non-hamster cells co-cultivated with hamster cells in the presence of anti-M globulins are not induced to round. These observations indicate that no protease-like activity is released by the hamster cells as a result of immunoglobulin binding.

Both BHK/Cl3 and C13/B4 cells continued to replicate in the presence of anti-M immunoglobulin (Fig. 2). Because neither cell line is normally capable of growing in suspension culture, it appears that the immunoglobulin released these cells from substrate-dependent growth. It is not surprising that C13/B4 cells were able to continue growing after detachment from the substrate by the immunoglobulins, because they normally adhere poorly and are capable of growth in soft agar. On the other hand, BHK/C13 cells adhere to the substratum more tightly and clone much less efficiently in soft agar than do the transformed C13/B4 cells. Other cells that rounded and detached in the presence of anti-M immunoglobulins were tested for the ability to grow in suspension. NIL-8 hamster fibroblasts failed to grow in suspension (Fig. 3c) as did primary hamster embryo cells, although both cell types could be reversibly detached by the anti-M immunoglobulins and remained viable in the presence of immunoglobulins for 72 h. The
ability to grow independent of a substratum is not a property that is acquired by all cell lines as a direct result of immunoglobulin-mediated detachment.

To define more precisely the requirements for rounding and reversal, experiments with inhibitors and immunoglobulin fragments were undertaken. Neither DNP nor cycloheximide inhibited rounding and detachment, which indicates that these phenomena do not require protein synthesis or the continuous generation of ATP. Reversal, on the other hand, will not take place in the presence of either inhibitor. This finding contrasts with reports that DNP and cycloheximide at the concentrations used here have only a limited effect on cellular attachment (9, 16). Failure of our cells to undergo morphological reversal and attachment in the presence of cycloheximide is probably caused by the inability of the cell to turn over its surface membrane and thus release bound immunoglobulin (Fig. 5) rather than to a direct effect of the inhibitor on cellular adhesion.

Treatment of cells with monovalent immunoglobulin fragments produced reversible rounding and detachment (Fig. 6). This indicates that the phenomena reported here do not require the crosslinking of surface receptors that is necessary for capping in lymphocytes. These experiments also eliminate the possibility that the alterations require the interaction of complement or the binding of the FC portion of the antibody molecule to the cell surface. This is important because the effect on cell growth of antisera against L cells (21) and the effect on morphology of antisera

![Figure 9](image1.png) Co-electrophoresis of glycoproteins bound to anti-M and anti-LIS immunoglobulins. Cells were grown in the presence of ³H- or ¹⁴C-L-fucose. NP-40 extracts were prepared from membrane fractions as described in Fig. 7. After absorption with preimmune immunoglobulins and Staph A, 10 mg of anti-M immunoglobulin was added to the ¹⁴C-L-fucose-labeled extract and 10 mg of anti-LIS immunoglobulin was added to the ³H-L-fucose-labeled extract. The immune complexes were absorbed to Staph A, eluted, and pooled. The pooled eluates were subjected to SDS-polyacrylamide gel electrophoresis on 7-16% polyacrylamide gel gradients as stated in Fig. 7. The gel was sliced and counted for radioactivity. Mol wt positions were estimated as stated in Fig. 7. --, ³H-L-fucose, --, ¹⁴C-L-fucose.

![Figure 10](image2.png) Immunoprecipitation using anti-M Immunoglobulin eluted from intact cells. Anti-M immunoglobulin was absorbed to 2 × 10⁶ BHK₂₁/C₁₀ cells which had been fixed in 0.15% glutaraldehyde. The cells were washed and the absorbed immunoglobulin was eluted with 3 M sodium thiocyanate. An NP-40 extract of a membrane preparation of BHK₂₁/C₁₀ cells labeled with ³H-amino acids and ¹⁴C-L-fucose was prepared as described in Fig. 7. After preabsorption of the extract with preimmune immunoglobulin and Staph A, the eluted immunoglobulin was added to the NP-40 extract. Immune complexes were absorbed to Staph A, eluted, and electrophoresed on SDS-polyacrylamide gels as described in Fig. 7. --, ³H-amino acids, --, ¹⁴C-L-fucose.

**Table V**

| Antiserum | Rounding | Fluorescence | Complement-median cytotoxicity | Mol wt major glycoproteins on SDS-PAGE x 10⁻³ |
|-----------|----------|-------------|-------------------------------|---------------------------------------------|
| Pre-immune | -        | -           | -                            | -                                           |
| Anti-LIS  | -        | +           | +                            | +                                           |
| Anti-SFM  | -        | +           | +                            | +                                           |
| Anti-M    | +        | -           | +                            | +                                           |

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FIGURE 11 Effect of antisera against NP-40 crude membrane fractions on cellular adhesion and morphology. 1:50 dilution of antisera directed against NP-40 soluble and insoluble fractions from C12/B4 crude membrane preparations were added to C12/B4 cells in culture. (A) Cells after 4 h in heat-inactivated antiserum against NP-40 soluble membrane fraction; (B) cells after 4 h in heat-inactivated antiserum against NP-40 insoluble membrane fractions. All figures × 100.

against material shed from rat kidney cells transformed by Rous sarcoma virus has been shown to depend upon or be augmented by the presence of complement (20).

The antisera used in these studies were produced against relatively crude antigens containing a mixture of proteins and glycoproteins, any of which, singly or in combination, might be responsible for mediating the reported effects. Hence, it was important to attempt to characterize the antigens with which the immunoglobulins were reacting. To do this, crude membrane fractions prepared from cells grown in the presence of radioactive fucose and amino acids were solubilized with NP-40. This procedure solubilized 90% of the fucose label and 50% of the amino acid label found in the membrane preparations. The antigens responsible for mediating the described effects could not have been in the detergent-insoluble residue because antiserum to this material did not induce cell rounding and detachment (Fig. 11). In addition, NP-40 extracts from C12/B4 cells will block anti-M or anti-SFM-induced rounding (Knudson and Buck, unpublished observations). Therefore the NP-40 soluble material was used in immunoprecipitation experiments involving all three immunoglobulins preparations, two of which induce rounding and detachment, and one which binds to the cells but elicits no change in morphology or adhesion. PAGE of immune precipitates revealed that glycoproteins of ~50,000, 120,000, and 140,000 daltons were the predominant groups of molecules precipitated by the immunoglobulins (Fig. 7). Because all of the immunoglobulin preparations reacted with the glycoproteins migrating in the 50,000-dalton region of the gel, this group was not considered to be directly involved in substrate adhesion. Pre-absorption of NP-40 extracts with anti-LIS immunoglobulins before immunoprecipitation with anti-M or anti-SFM immunoglobulins showed that the shoulder of 120,000-dalton glycoproteins was greatly decreased in these precipitates (Fig. 8). By a process of elimination, the 140,000-dalton group glycoproteins appear to contain the components most likely involved in the control of cellular adhesion and cytoskeletal organization. That such a group of glycoproteins is, in fact, on the cell surface was demonstrated by immunoprecipitation experiments in which glycoproteins were reacted with anti-M immunoglobulin which had been absorbed and subsequently eluted from the surface of intact cells. Among the glycoproteins precipitated by this anti-M immunoglobulin was a group migrating on SDS-polyacrylamide gels at ~140,000 daltons (Fig. 10). Immunoprecipitation of NP-40 extracts of membranes from BHK21/C13 cells iodinated by the lactoperoxidase method also revealed iodinatable surface proteins or glycoproteins migrating in this same 140,000-mol wt region on gels (Damsky and Buck, unpublished observations). Thus, the group of 140,000-mol wt glycoproteins reacting with anti-M and anti-SFM immunoglobulins appear to be represented on the cell surface.

Nonglycosylated proteins in the NP-40 extract are considered less likely to be involved in the adhesion and morphological alterations reported here for the following reasons. First, our immunoprecipitates were enriched three- to sevenfold in glycosylated proteins. Second, no particular groups of nonglycosylated proteins were reproducibly found in our immunoprecipitates. Third, immune complexes formed with either anti-M or anti-SFM immunoglobulin after careful, exhaustive preadsorption of NP-40 extracts with preimmune immunoglobulin and/or anti-LIS immunoglobulin (Fig. 8b and d) contained no radioactive amino acids except those that could be accounted for as glycoproteins.

Other proteins that have been implicated in the adhesion process include LETS molecules deposited by cells onto the substratum (3, 5, 19). There are several lines of evidence that suggest that
LETS does not play any part in cell rounding caused by our anti-M serum. First, no specific 210,000-dalton material was found in the immunoprecipitates. Second, antiserum directed against LETS show a high degree of interspecies cross-reactivity (12), whereas the anti-M serum was species specific. Third, the transformed cell line used as a source of antigen has little or no LETS noprecipitates. The sera used to prepare the anti-SFM was centrifuged on CsCl density gradients, which cause LETS to pellet with glycosaminoglycans (8). In addition, Ali et al. (2) found that antiserum prepared against purified hamster-derived LETS had no effect on cell morphology. Finally, antiserum against LETS will not induce detachment and rounding of the BHK21/C13 or C13/Ba cells used here (Buck and Avdalovic, unpublished observations).

The data presented here do not rule out the possibility that integral cell surface glycoproteins other than the ones present in our immunoprecipitates may be involved in regulating adhesion and morphology, or that one of the minor components present in the immunoprecipitates may be the actual surface molecules involved in adhesion. However, recent experiments have shown that the material in NP-40 extracts capable of blocking immunoglobulin-induced rounding and detachment is a 140,000-mol wt glycoprotein which is identical on PAGE with the major 140,000-mol wt glycoprotein present in anti-M immunoprecipitates. It is also not evident from these experiments whether the anti-M and anti-SFM immunoglobulins exert their effects on adhesion by interacting directly with molecules in the adhesion plaques involved in anchoring the cell to the substratum (1), or indirectly by perturbing the membrane structure outside these specific adhesion sites. Finally, because the antisera used here are not monospecific, it is difficult to know whether the phenomena of rounding and detachment are caused by the interaction of immunoglobulins with the same or different surface glycoproteins. These determinations will require the development of highly specific reagents such as monoclonal antibodies which will allow the localization and perturbation of individual cell surface molecules (25).

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