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

DWANE E. WYLIE, CAROLINE H. DAMSKY, and CLAYTON A. BUCK

From the Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104. Dr. Wylie's present address is the Scripps Institute, La Jolla, California 92037.

ABSTRACT

An antiserum prepared against purified surface membranes of transformed BHK_{21}/C_{13} cells (C_{13}/B_4) 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 C_{13}/B_4 or BHK_{21}/C_{13} 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.

385

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)[1] 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. Thiéry 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_{21}/C_{13} and this same cell line transformed by the Bryan high-titer strain of Rous sarcoma virus, designated C_{13}/B_4. NIL-8 cells[2], also of hamster origin, and HT_3 cells[3], 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.).

Polyacrylamide Gel Electrophoresis (PAGE)

Samples being analyzed by electrophoresis were run on a 1.5-mm-thick polyacrylamide gradient slab gel, with a 10 cm 7–16% polyacrylamide separating gel and a 2-cm stacking gel with a 3% polyacrylamide 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 decolorized 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, polyacrylamide 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 C_{13}/B_4 cells. For production of antimembrane antiserum, surface membranes from C_{13}/B_4 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, C_{13}/B_4 cells were inoculated in glass roller bottles (640 cm^2) at a density of 2×10^7 cells/bottle. After incubation at 37°C for 24 h, the cells were radioactively labeled with 50 μCi L-[1-^{14}C]fucose (40–55 mCi/mol) 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, tryptose 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 60 min, dialyzed exhaustively against $0.1 \times$ Tris-buffered saline (TBS), then against water, and lyophilized. The sample was then dissolved in 0.05 M Tris-HCl buffer containing 1.4 grams $CsCl_2/cm^3$ and centrifuged for 24 h at 250,000 g. Fractions consisting of eight drops each were collected, and 10 μl of each fraction was used to measure radioactivity. Fractions corresponding to a density of 1.22–1.38 g/cm^3 were pooled, dialyzed against distilled water, and lyophilized. This sample was dissolved in TBS and used as the serum-free medium antigen. Goats were injected three times at 2-wk intervals in the manner described for the membrane antigen. The goats were given a final injection 9 mo later and bled 2 wk after this injection.

For preparation of an immunogen consisting of membrane material extractable with lithium diiodosalicylate (LIS; Eastman Organic Chemical Div., Eastman Kodak Co., Rochester, N. Y.), C_{13}/B_4 cells labeled with ^{14}C-glucosamine were extracted by the method of Marchesi and Andrews (15). The aqueous phase material was dialyzed, lyophilized, and examined by using sodium dodecyl sulfate (SDS)-PAGE. One of the gels was cut into 1-mm slices and counted for radioactivity. The radioactivity was found primarily in one peak with an apparent mol wt of 120,000 daltons. The region corresponding to this molecular weight region was cut from companion gels and homogenized in 2.5 ml of TBS and 2.5 ml of complete Freund's adjuvant and injected into goats. The goats were given four injections of LIS extract preparations at 2-wk intervals and bled 2 wk after the third and fourth injections. Preimmune serum was collected from all goats before injection.

Immunoglobulin Purification

Immunoglobulin was purified by a modification of the procedure of Kekwick (11) by precipitation with decreasing concentrations of sodium sulfate. 25% Na_2SO_4 was added by drops to whole serum at room temperature to a final concentration of 18%. After centrifugation at 12,000 g for 15 min at 25°C, the precipitate was resuspended in borate saline buffer (0.15 M NaCl, 0.16 M Na-borate, pH 8.0), and dialyzed overnight at 4°C against 4 liters of the same buffer. The same procedure was followed to a final Na_2SO_4 concentration of 14%. After centrifugation, the precipitate was redissolved in 0.0175 M phosphate buffer, pH 6.9, and dialyzed overnight at 4°C against 4 liters of this buffer. The preparation was centrifuged as described above to pellet the lipoproteins that precipitate during dialysis against the low ionic strength phosphate, then dialyzed against PBS and used as purified immunoglobulin.

Preparation of F(ab)' 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 Na_2 EDTA, pH 7.7. 5-ml fractions were collected and the A_{280} 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 N_2 for 15 min. The solution was brought to 0.2 M with dithiothreitol and allowed to react for 30 min at room temperature under N_2. 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 × 100 cm) equilibrated with PBS containing 0.002 M NaN_3. 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

BHK_{21}/C_{13} and C_{13}/B_4 cells that had been grown in the presence of 50 µCi L-³H(G)amino mixture and 25 µCi L-[1-¹⁴C]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 × 10⁸ 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 H_2O). 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 (IN-US 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

BHK_{21}/C_{13} 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, C_{13}/B_4 cells exhibited the characteristic epithelioid morphology of transformed fibroblasts (Fig. 1a). In Fig. 1b, C_{13}/B_4 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 antiserum 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 antiserum, 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 at as "reversal". Fig. 1d–f shows BHK_{21}/C_{13} treated in the same manner as described above for C_{13}/B_4 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, BHK_{21}/C_{13} cells were affected more rapidly than C_{13}/B_4 cells (Table I). 24 h after removal of the immunoglobulin, the majority of cells had reattached to the substratum, with 84% of the C_{13}/B_4 cells and 76% of the BHK_{21}/C_{13} 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 Antiserum

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 antiserum is caused by the recognition of species-specific surface antigens and not by the recogni-
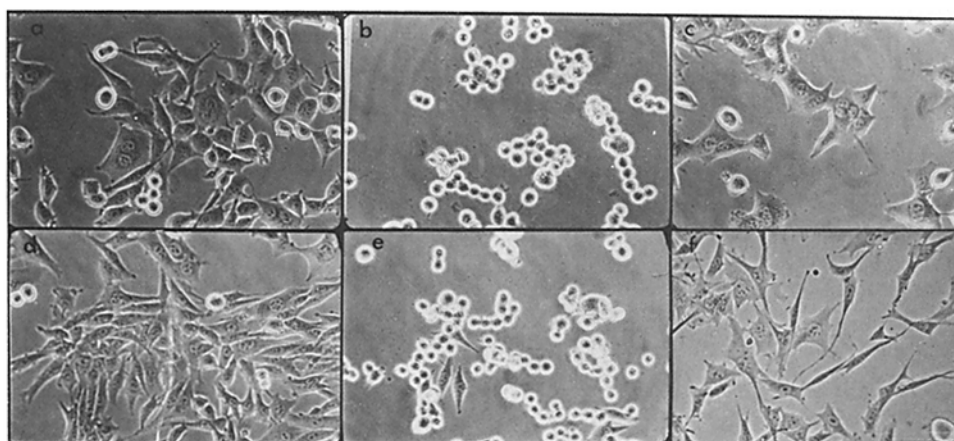
FIGURE 1   Effect of antiserum against C_{13}/B_4 plasma membranes on substratum adhesion and morphology of C_{13}/B_4 and BHK_{21}/C_{13} cells. Cells were treated with a 1:10 dilution of heat-inactivated antiserum as described in text. (a) Untreated C_{13}/B_4 cells; (b) C_{13}/B_4 cells 2 h after addition of antiserum; (c) C_{13}/B_4 cells 24 h after removal of antiserum; (d) untreated BHK_{21}/C_{13} cells; (e) BHK_{21}/C_{13} cells 2 h after addition of antiserum; (f) BHK_{21}/C_{13} cells 24 h after removal of antiserum. All figures × 100.

TABLE I

Quantitation of Cell Detachment by Anti-Membrane Serum

Cell line	Treatment	Detached cells		Attached cells	
		% of total	% viable	% of total	% viable
C13/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
BHK21/C13	Immune, 0 h	2	96	98	98
	Immune, 3 h	91	97	9	99
	Immune, 24 h	92	91	8	96
	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

$1-2 \times 10^6$ 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 C13/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 replated in a 25 cm² tissue culture flask. After 24

TABLE II

Species Specificity of Anti-Membrane Serum

Cell line	Species	Rounding	Loss of adhesion	Fluorescence
BHK21/C13	Hamster	+	+	+
C13/B4	Hamster	+	+	+
Primary embryo	Hamster	+	+	ND*
NIL-8	Hamster	+	+	+
Primary embryo	Chicken	-	-	-
Primary kidney	Mouse	-	-	-
3T3	Mouse	-	-	-
HT3	Human	-	-	ND

The antiserum against C13/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.

tion of anomalous antigens acquired as the result of long-term cultivation in tissue culture. In addition, detachment was shown not to be caused by recognition of an organ-specific antigen common to kidney cells, because there was no cross-reactivity with primary mouse kidney cells.

To determine whether the rounding and detachment of cells could be the result of antibody-induced release of soluble nonspecific factors such as proteases, human and hamster cells were co-cultivated in the presence and absence of anti-M serum. In Fig. 2a, islands of human cells are clearly distinguishable from the surrounding BHK21/C13 fibroblasts. Upon exposure to anti-M serum, the islands of human cells remained unaltered whereas the BHK21/C13 cells rounded and detached. The medium from cells which were rounded and detached with anti-M immunoglobulin was assayed for protease activity by using the general substrate Azocoll. No protease activity was detected under assay conditions in which as little as 0.02 μg/ml (0.005 U) of trypsin could be

h the number of cells remaining detached and the number that settled and attached to the flask were determined as described above.
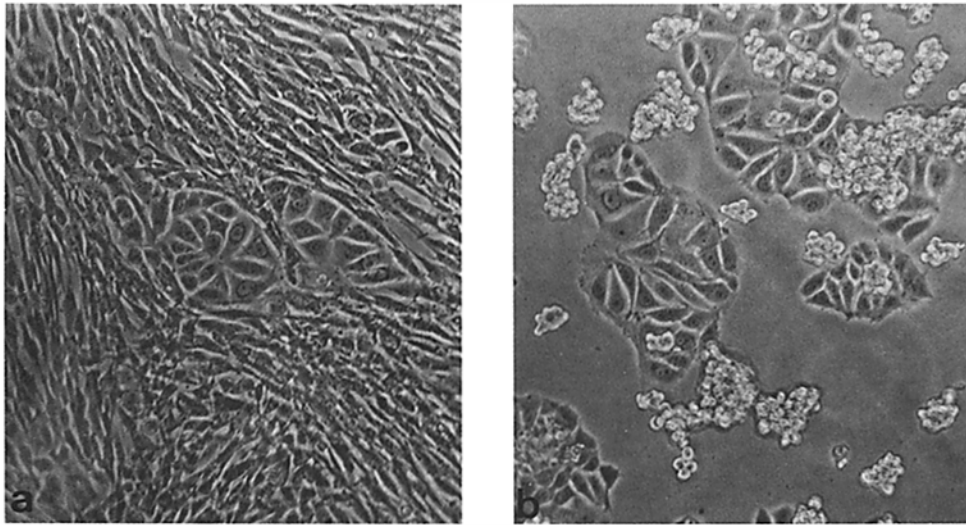
FIGURE 2   Effect of anti-membrane serum on co-cultured hamster and human cells. BHK_{21}/C_{13} cells and HT-3 cells, which are epithelial cells derived from a human mammary tumor, were cultured together in the same well of a Costar dish. A 1:10 dilution of heat-inactivated anti-membrane serum was added, 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 BHK_{21}/C_{13}, C_{13}/B_4, 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 C_{13}/B_4 and BHK_{21}/C_{13} cells (Fig. 3*a* 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. 3*c*). In the first 24 h, the anti-M-treated cells increased in number at about one-half the rate of 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. 4*d* 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. 4*e* and *h*). Cells growing in the presence of cycloheximide (Fig. 4*f*) or DNP and preimmune globulin (Fig. 4*i*) for 24 h resemble control cells (Fig. 4*c*). 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
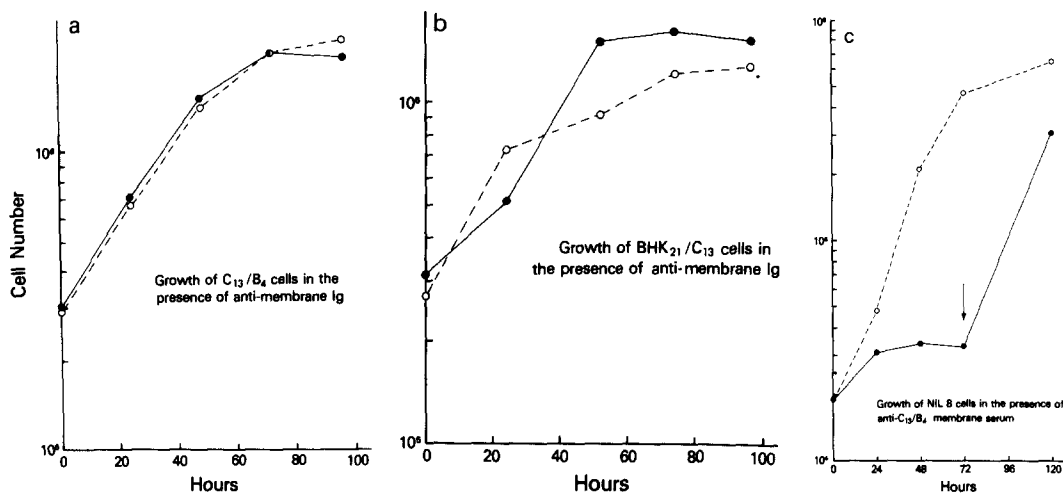
FIGURE 3   Effect of anti-membrane immunoglobulin on cell growth. Cells were inoculated into the wells of Costar dishes at a density of 1×10^5 (C_{13}/B_4 and BHK_{21}/C_{13} cells) or 1×10^4 (NIL-8 cells), and the dishes were incubated at 37°C overnight. Immune or preimmune globulin was added to each well at a concentration of 10 mg/ml. At designated intervals the medium was removed from each well and saved to retain any cells detached by the antibody. Adherent cells were removed from the monolayer with 0.25% trypsin and added to the corresponding medium sample. After centrifugation at 1,000 g, cells were resuspended in PBS containing 0.05% trypan blue and counted with a hemacytometer. Each point is the average of three individual cell counts. (a) C_{13}/B_4 cells; (b) BHK_{21}/C_{13} cells; (c) NIL-8 cells. O- - -O, Cells in the presence of preimmune immunoglobulin; ●—●, cells in the presence of anti-membrane immunoglobulin.

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 immuno-

globulin 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
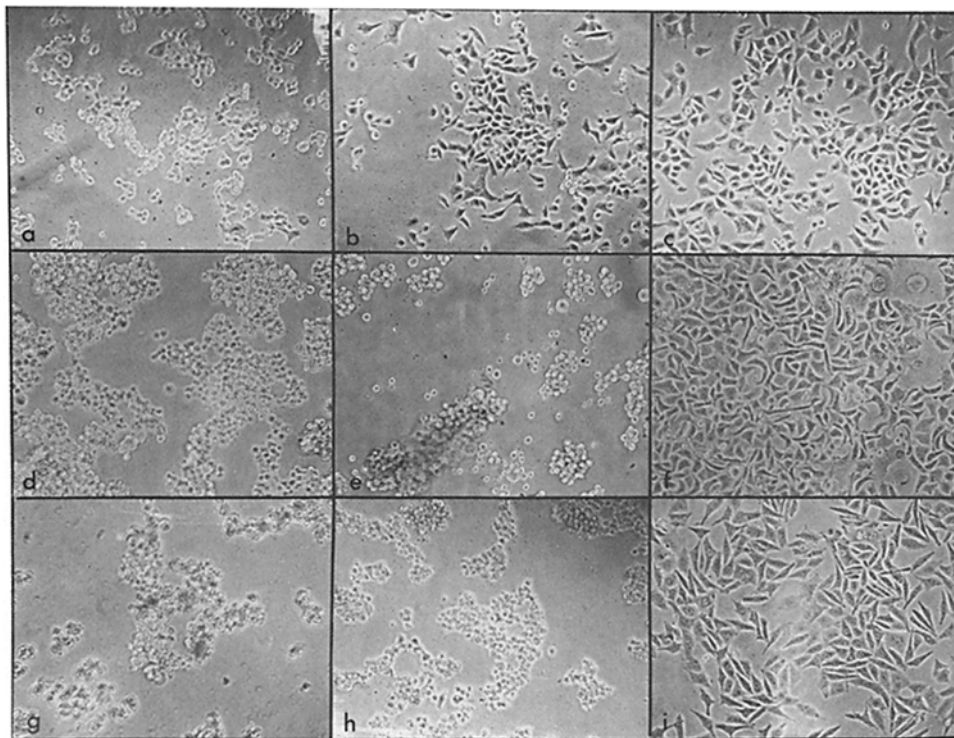
FIGURE 4   Effect of cycloheximide and DNP on rounding and reversal. C_{13}/B_4 cells were inoculated into Costar dishes at a concentration of 5×10^5 cells/well and incubated overnight at 37°C. Medium containing inhibitors was added 2 h before immunoglobulin or 2 h before reversal. Purified immunoglobulin was used at a concentration of 10 mg/ml. Cycloheximide and DNP were used at concentrations of 10 µg/ml and 1 mM, respectively. (a) Control C_{13}/B_4 cells 24 h after addition of immunoglobulins; (b) control cells reversed for 24 h; (c) control cells cultured without immunoglobulins or inhibitors for 24 h; (d) cells after 24 h in immunoglobulins plus cycloheximide; (e) cells detached with immunoglobulins in the absence of inhibitor, then reversed for 24 h in cycloheximide; (f) untreated cells grown in cycloheximide for 24 h; (g) cells after 24 h in immunoglobulin plus DNP; (h) cells detached with immunoglobulin without inhibitor then reversed for 24 h in DNP; (i) untreated cells grown in DNP for 24 h. All figures × 100.

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
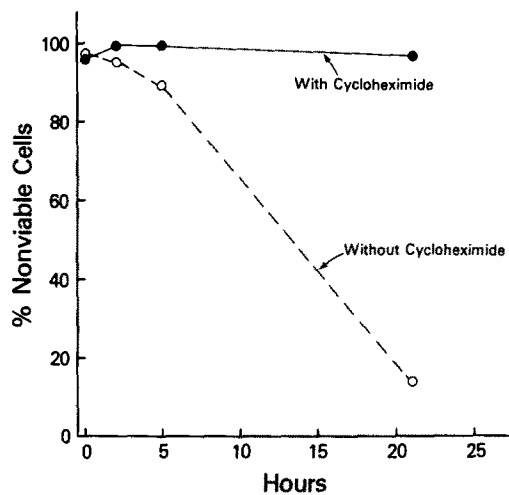
FIGURE 5 Loss of cell-bound immunoglobulin during reversal in the presence of cycloheximide. C_{18}/B_4 cells growing in Costar dishes were detached by treatment with anti-M serum for 3 h. 2 h before reversal, 10 µg/ml of cycloheximide was added to each well. At designated intervals the number of cells with immunoglobulin still attached to the cell surface was determined by complement-mediated cytotoxicity performed as follows. The medium was removed and saved. Adherent cells were detached with 0.001 M EDTA in Ca^{++} and Mg^{++}-free PBS. After centrifugation at 1,000 g cells were incubated in a 1:80 dilution of guinea pig complement for 30 min at 37°C. Cells were again centrifuged, resuspended in 0.05% trypan blue in PBS, and counted with a hemacytometer. ●—●, Cells in the presence of cycloheximide; O- - -O, cells in the absence of cycloheximide.

immunoglobulin 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 quantitate 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 $^{14}C/^3H$ 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_{13} 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_{13} cells were labeled with either ^3H- 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 ^3H-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_{13} 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_{13} cells labeled with ^3H-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
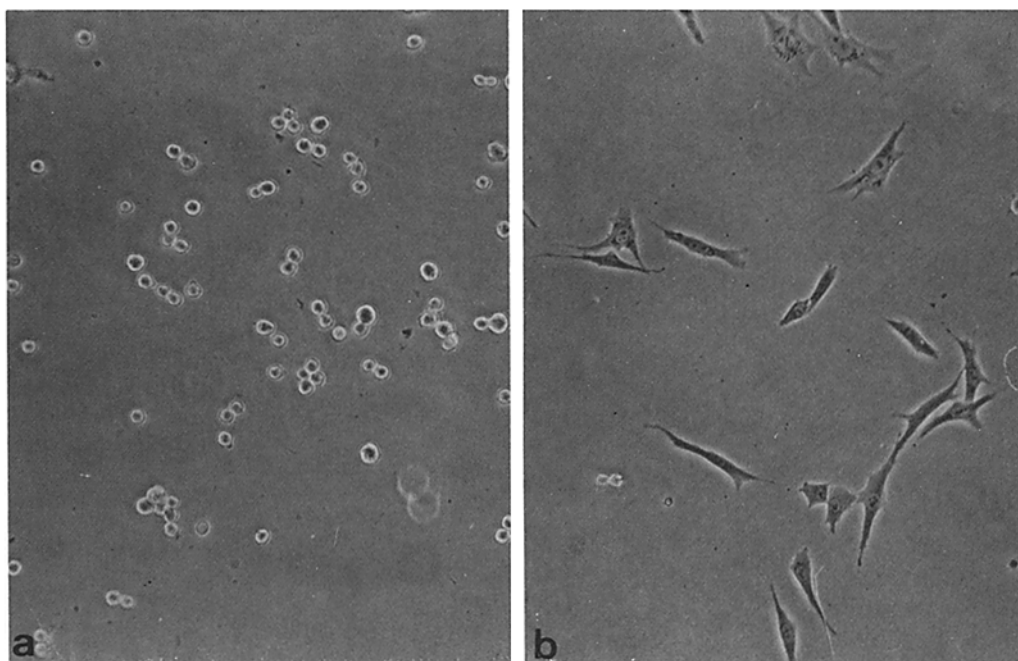
FIGURE 6 Effect of anti-membrane F(ab)' fragments on cell morphology and adhesion. C_{13}/B_4 cells growing in Costar dishes were treated with F(ab)' fragments at a concentration of 5 mg/ml of culture medium. (A) Cells after 2 h in the presence of F(ab)' fragments; (B) cells previously detached with F(ab)' fragments after 24 h reversal. Both figures × 100.

TABLE III

Characteristics of Antisera Used in Immunoprecipitation

Antiserum	Antigen	Rounding	Complement-mediated cyto-toxicity	Fluorescence
Anti-LIS	Lithium diiodosalicylate extracts of C_{13}/B_4 cells	−	+	+
Antiserum-free medium	C_{13}/B_4 cellular components shed into serum-free medium	+	+	+
Anti-membrane	C_{13}/B_4 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
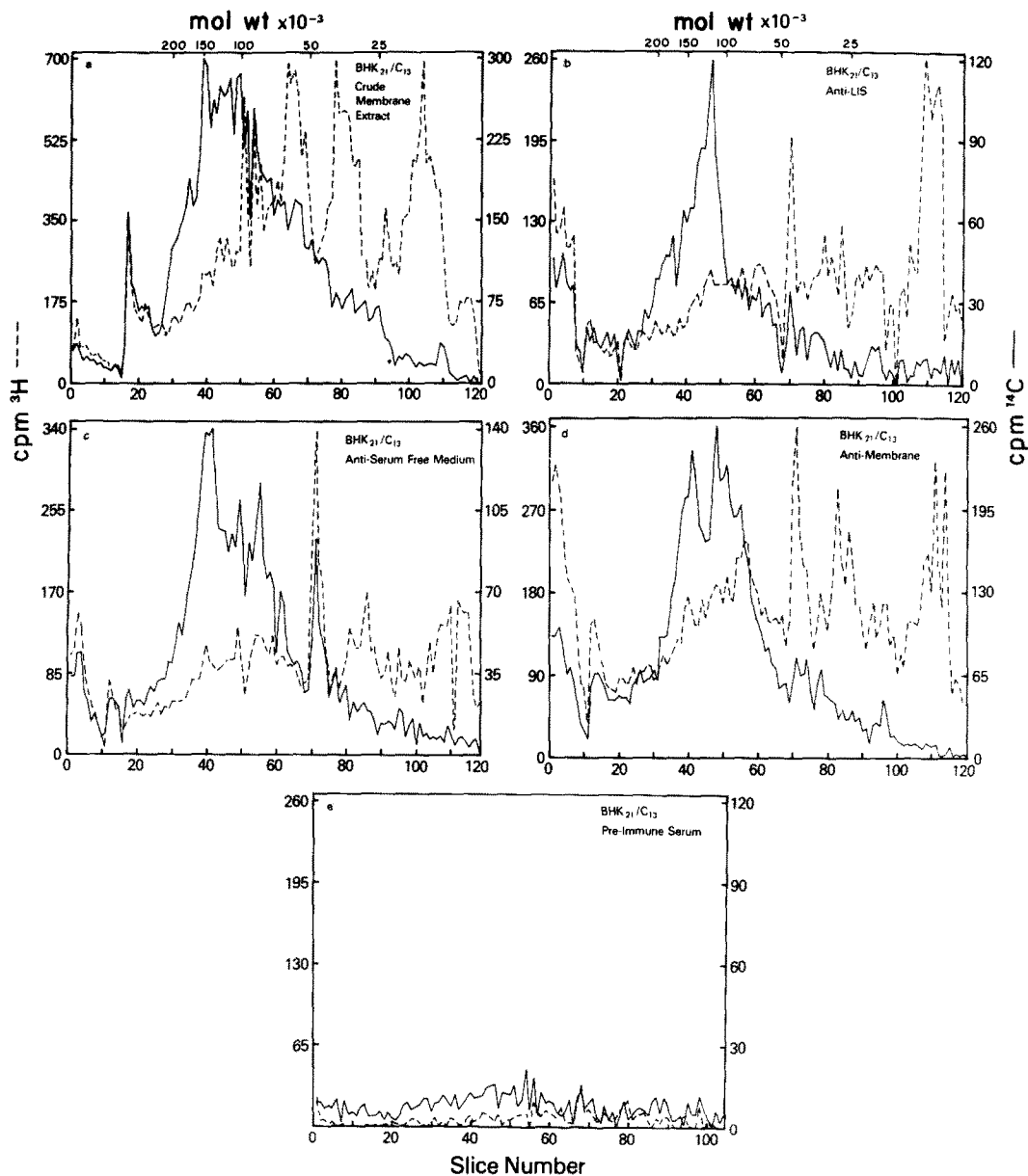
WYLIE, DAMSKY, AND BUCK *Surface Membrane Glycoproteins and Adhesion* **395**

FIGURE 7   Immunoprecipitation with surface-reactive immunoglobulins of NP-40 solubilized crude membrane preparation from BHK$_{21}$/C$_{13}$ 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

Enrichment of Glycoproteins in
Immunoprecipitations

BHK$_{21}$/C$_{13}$	Total ^{3}H cpm	Total ^{14}C cpm	^{14}C/^{3}H	Enrichment in glycoproteins
NP-40 extract	16,301	12,995	0.8	—
Anti-LIS	459	1,278	2.8	3.5 ×
Anti-SFM	550	2,274	4.1	5.1 ×
Anti-M	1,176	2,858	2.4	3.0 ×
C$_{13}$/B$_4$				
NP-40 extract	37,424	15,724	0.4	—
Anti-LIS	685	1,836	2.6	6.5 ×
Anti-SFM	841	2,553	3.0	7.6 ×
Anti-M	3,205	4,779	1.5	3.7 ×

The enrichment of glycoproteins over proteins in the immunoprecipitates from each antiserum was determined by using NP-40 extracts labeled with ^{3}H-amino acids and ^{14}C-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 ^{14}C/^{3}H ratio for each antiserum by the ratio for the whole extract.

suggests that this group of glycoproteins may be involved in regulating cellular adhesion. A summary of the immunoprecipitation data in light of the morphological effect of the antisera on cell shape is presented in Table V.

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 C$_{13}$/B$_4$ 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 C$_{13}$/B$_4$ cells or mate-

rial 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 BHK$_{21}$/C$_{13}$ or C$_{13}$/B$_4$ 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
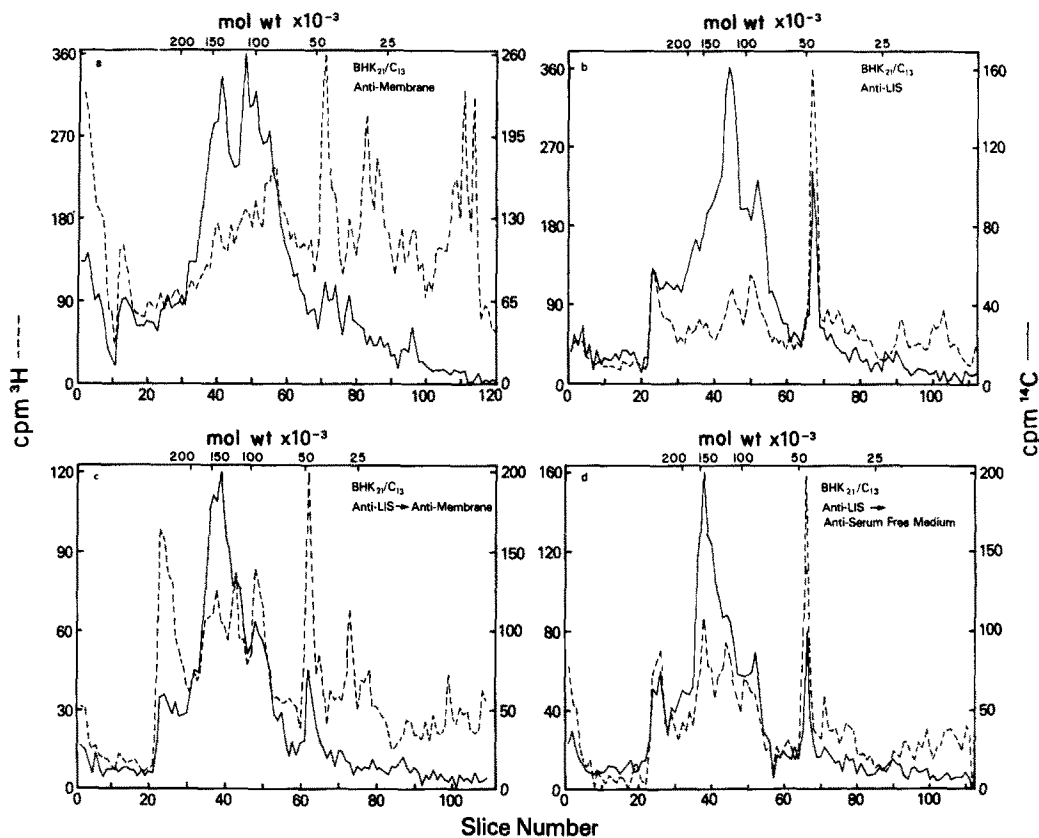
FIGURE 8 Immunoprecipitation of BHK$_{21}$/C$_{13}$ membrane extract with anti-SFM or anti-M after preabsorption with anti-LIS. An NP-40 extract from cells grown in ^{3}H-amino acids and ^{14}C-fucose was prepared as in Fig. 7. The extract was preabsorbed once with preimmune globulin and S. aureus, followed by three absorptions with anti-LIS immunoglobulin. Absorption with S. aureus followed each anti-LIS preabsorption. The supernate from these absorptions was then precipitated with either anti-SFM or anti-M. The immunoprecipitates were electrophoresed on SDS-polyacrylamide gels as in Fig. 7. (A) Material precipitated with anti-M immunoglobulin; (B) material precipitated with anti-LIS; (C) material from preabsorbed extract precipitated with anti-M immunoglobulin; (D) material from preabsorbed extract precipitated with anti-SFM. Molecular weight positions determined as stated in Fig. 7. - - -, ^{3}H-amino acids, —, ^{14}C-fucose.

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$_{21}$/C$_{13}$ and C$_{13}$/B$_4$ 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 C$_{13}$/B$_4$ 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$_{21}$/C$_{13}$ cells adhere to the substratum more tightly and clone much less efficiently in soft agar than do the transformed C$_{13}$/B$_4$ 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
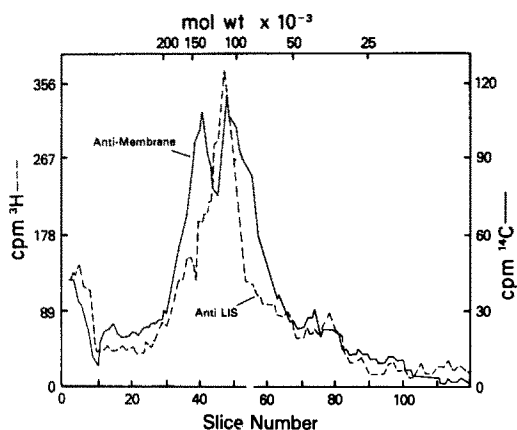
FIGURE 9 Co-electrophoresis of glycoproteins bound to anti-M and anti-LIS immunoglobulins. Cells were grown in the presence of ^{3}H- or ^{14}C-L-fucose. NP-40 extracts were prepared from membrane fractions as described in Fig. 7. After absorption with preimmune immunoglobulins and Staph A, 10 mg of anti-M immunoglobulin was added to the ^{14}C-L-fucose-labeled extract and 10 mg of anti-LIS immunoglobulin was added to the ^{3}H-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. – – –, ^{3}H-L-fucose, —, ^{14}C-L-fucose.

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
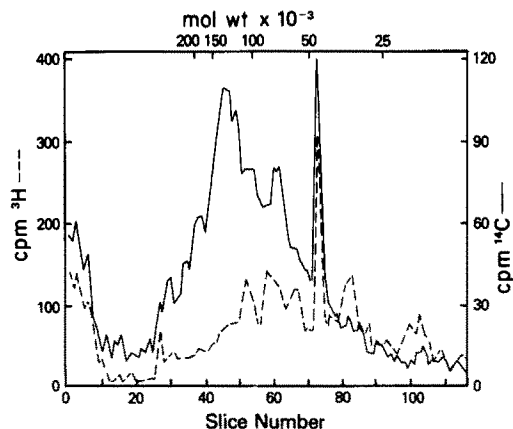

FIGURE 10 Immunoprecipitation using anti-M Immunoglobulin eluted from intact cells. Anti-M immunoglobulin was absorbed to 2×10^8 BHK$_{21}$/C$_{13}$ cells which had been fixed in 0.15% glutaraldehyde. The cells were washed and the absorbed immunoglobulin was eluted with 3 M sodium thiocyanate. An NP-40 extract of a membrane preparation of BHK$_{21}$/C$_{13}$ cells labeled with ^{3}H-amino acids and ^{14}C-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. – – –, ^{3}H-amino acids. —, ^{14}C-L-fucose.

TABLE V

Comparison of Effects of Antisera on Cellular Morphology with the Antigens Found in Immunoprecipitates

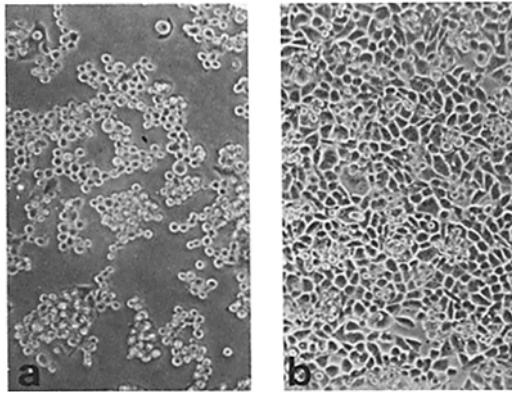| Antiserum	Round-ing	Fluores-cence	Complement-mediated cy-totoxicity	Mol wt major glycoproteins on SDS-PAGE × 10^{-3}		
				50	120	140
Pre-immune	–	–	–	+	–	–
Anti-LIS	–	+	+	+	+	–
Anti-SFM	+	+	+	+	+	+
Anti-M	+	+	+	+	+	+

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 C_{13}/B_4 crude membrane preparations were added to C_{13}/B_4 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 C_{13}/B_4 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 immunoglobulin 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 BHK_{21}/C_{13} 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, antisera 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 because it is missing the 210,000-dalton iodinatable protein that is present in BHK_{12}/C_{13} cells (data not shown). Fourth, the antigen 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 BHK_{21}/C_{13} or C_{13}/B_4 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.[4] 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).

---

[4] Buck, Knudsen, Rao, and Damsky; manuscript in preparation.

REFERENCES

1. ABERCROMBIE, M., J. HEAYSMAN, and S. PEGRUM. 1971. The locomotion of fibroblasts in culture. IV. Electron microscopy of the leading lamella. Exp. Cell Res. 67:359-367.
2. ALI, I. U., V. MAUTNER, R. LANZA, and R. O. HYNES. 1977. Restoration of normal morphology, adhesion and cytoskeleton in transformed cells by addition of a transformation-sensitive surface protein. Cell. 11:115-126.
3. BROWN, S., W. LEVINSON, and J. A. SPUDICH. 1976. Cytoskeletal elements of chick embryo fibroblasts revealed by detergent extraction. J. Supramol. Struct. 5:119-130.
4. CHOWDHURY, T. K. 1974. Effects of Concanavalin A on cellular dynamics and membrane transport. Adv. Exp. Med. Biol. 55:187-205.
5. CULP, L. A. 1976. Electrophoretic analysis of substrate-attached proteins from normal and virus-transformed cells. Biochemistry. 15:4094-4104.
6. FARB, R. M., R. DYKES, and G. S. LAZARUS. 1978. Anti-epidermal-cell-surface pemphigus antibody detaches viable epidermal cells from culture plates by activation of proteases. Proc. Natl. Acad. Sci. U. S. A. 75:459-463.
7. GAIL, M. H., and C. W. BOONE. 1972. Cell-substrate adhesivity. Exp. Cell Res. 70:33-40.
8. GRAHAM, J. M., R. O. HYNES, E. A. DAVIDSON, and D. F. BRINTONG. 1975. The location of proteins labeled by the ¹²⁵I lactoperoxidase system in the NIL-8 hamster fibroblast. Cell. 4:353-365.
9. GRINNELL, F., M. MILAM, and P. A. SRERE. 1973. Studies on cell adhesion. III. Adhesion of baby hamster kidney cells. J. Cell Biol. 56:659-665.
10. HYNES, R. O. 1976. Cell surface proteins and malignant transformation. Biochim. Biophys. Acta. 458:73-96.
11. KEKWICK, R. A. 1940. The serum proteins of multiple myelomatosis. Biochem. J. 34:1248-1259.
12. KESSLER, S. 1976. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: Parameters of the interaction of antibody antigen complexes with protein A. J. Immunol. 115:1617-1624.
13. LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (Lond.). 227:680-685.
14. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265-275.
15. MARCHESI, V. T., and E. P. ANDREWS. 1971. Glycoproteins: Isolation from red cell membranes with lithium diiodosalicylate. Science (Wash. D. C.). 174:1247-1248.
16. NATH, K., and P. A. SRERE. 1977. Effects of temperature, metabolic, and cytoskeletal inhibitors on the rate of BHK cell adhesion to polystyrene. J. Cell. Physiol. 92:33-42.
17. NICOLSON, G. L. 1976. Trans-membrane control of the receptors of normal and tumor cells. Biochim. Biophys. Acta. 458:1-72.
18. NISONOFF, A., F. C. WISSLER, L. N. LIPMAN, and D. L. WOERNLEY. 1960. Separation of univalent fragments from the bivalent rabbit antibody molecule by reduction of disulfide bonds. Arch. Biochem. Biophys. 89:230-244.
19. OSBORN, M., and K. WEBER. 1977. The detergent-resistant cytoskeleton of tissue culture cells includes the nucleus and the microfilament bundles. Exp. Cell Res. 106:339-349.
20. RIEBER, M. and E. ROMANO. 1976. Transformation dependent modifications in released and cell-bound proteins detected by antisera to shed antigens. Cancer Res. 36:3568-3573.
21. SHEARER, W. T., G. W. PHILPOTT, and C. W. PARKER. 1975. Humoral immunostimulation. Cell. Immunol. 17:447-462.

22. Takeichi, M. 1977. Functional correlation between cell adhesive properties and some cell surface proteins. *J. Cell Biol.* **75**:464–474.

23. Thiery, J. P., R. Brackenbury, U. Rutishauser, and G. Edelman. 1977. Adhesion among neural cells of the chick embryo. II. Purification and characterization of a cell adhesion molecule from neural retina. *J. Biol. Chem.* **252**:6841–6845.

24. Warren, L., M. C. Glick, and M. K. Nass. 1966. Membranes of animal cells. I. Methods of isolation of the surface membrane. *J. Cell. Physiol.* **68**:269–287.

25. William, A. F., G. Galfre, and C. Milstein. 1977. Analysis of cell surfaces by xenogeneic myeloma-hybrid antibodies: differentiation antigens of rat lymphocytes. *Cell.* **12**:663–673.