STUDIES ON CELL ADHESION

III. Adhesion of Baby Hamster Kidney Cells

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ABSTRACT

Normal and transformed baby hamster kidney (BHK) cells attach to Falcon polystyrene with the same first order rate constant. The longer the cells are attached to the bottles, the more difficult they are to remove. Sulfhydryl (—SH) binding reagents inhibit both the attachment of BHK cells and the increase in adhesive strength of attached cells. Attached BHK cells bind fewer molecules of [1-^{14}C]*N*-ethylamleimide (an —SH binding reagent) than do suspended cells. Incubation of cells with high concentrations of trypsin results in a reversible loss of cell adhesiveness. The recovery of adhesiveness of trypsin-treated cells is inhibited by cycloheximide.

INTRODUCTION

Cell adhesions are involved in cell aggregation (1), cell mobility (2), and intercellular communication (3). It is also believed that intercellular adhesiveness is stronger in normal than in malignant cells (for a review see reference 4) and that malignant cells may be less sensitive to contact inhibition than are normal cells (5) because of differences in cellular adhesiveness (6).

The adhesion of suspension-grown hepatoma cells to a substratum is inhibited by sulfhydryl (-SH) binding reagents (7, 8). In this report, we show that adhesion of baby hamster kidney (BKH) cells is also sensitive to —SH binding reagents and that attached cells have fewer exposed —SH groups than do suspended cells.

Experiments are reported in which the detachment of adhered cells is studied. These studies indicate that adhesive bond formation is a continuing process and that the strength of the attachment (the number of bonds formed) increases with time. This latter process is also inhibited by —SH binding reagents.

Trypsin treatment of cells results in the loss of adhesion. Recovery of adhesion by trypsin-treated cells is a time-dependent process which can be inhibited by cycloheximide.

MATERIALS

BHK cells which were suspension culture-adapted (BHK-21-13s) were the gift of Dr. Adrian Chappel, and polyoma-transformed baby hamster kidney cells (BHK-py) were the gift of Dr. Walter Eckhart. Eagle's minimal essential medium (MEM) ("spinner modified"), MEM amino acids, MEM vitamins, fetal calf serum, and bovine serum were obtained from Grand Island Biological Co., Grand Island, N.Y. Human serum albumin (25%, salt poor) was obtained from Armour Pharmaceutical Co., Chicago, Ill. HEPES buffer was purchased from California Biochemical Co., La Jolla, Calif. [2-^{14}C]leucine, [5-T] uridine, and [1-^{14}C]*N*-ethylmaleimide (NEM) were purchased from Schwarz Bio Research Inc., Orangeburg, N. Y. Trypsin (type XI), cycloheximide, and chloramphenicol were obtained from Sigma Chemical Co., St. Louis, Mo. Actinomycin D was the gift of Dr. Jon Nishimura.

METHODS

BHK cells were grown in suspension culture in Wistar medium which is Eagle's MEM ("spinner modified") with double the concentration of amino acids (except 1 × glutamine) and vitamins and supplemented with 0.1 mg/liter ferric nitrate, 2.0 g/l glucose, 10% tryptose phosphate broth, and 10% fetal calf serum.

The adhesion of cells was routinely measured in Falcon 3012 polystyrene culture bottles (Falcon Plastics, Division of B-D Laboratories, Inc., Los Angeles, Calif.) (30 ml capacity, 25 cm^2 surface area). Suspension culture cells in the logarithmic growth phase were collected by centrifugation at 1,000 *g* for 5 min, resuspended in 5.0 ml of adhesion medium (Earle's balanced salt solution, minus calcium and bicarbonate, containing 20 mM HEPES (pH 7.5) and 5.0% bovine serum), and placed in the Falcon bottles. The bottles were placed in a 30°C incubator for the time periods designated in the experiments. At the end of the incubations, the nonadherent cells were resuspended by swirling the medium in the bottles, and this suspension was carefully removed with a pipette. 5.0 ml of detachment medium (adhesion medium minus bovine serum minus $MgCl_2$, containing 0.5 mM EDTA and 0.5% human serum albumin) were added to the attached cells, which remained in the bottles. Attached cells became detached from the surfaces of the culture bottles after incubation in detachment medium for 10 min at 37°C. The recovered attached and nonattached cells were each collected by centrifugation at 1,000 *g* for 5 min and each resuspended in 1.0 ml of adhesion medium. The turbidity of the suspensions was measured at 650 nm in a Gilford Model 2000 spectrophotometer, and cell concentrations were calculated from a previously determined relationship between cell number and absorbancy (7, 9). Recovery of cells at the end of the incubations varied as described in the text.

RESULTS

Rate of Attachment of BHK Cells

The data in Fig. 1 show the time course of attachment of BHK-21-13s and BHK-py to Falcon polystyrene culture bottles. When the data in Fig. 1 were replotted as log of percent nonadherent cells vs. time, straight lines were obtained. The first order rate constant (slope) for adhesion of BHK-py cells was 0.014/min and of BHK-21-13s cells was 0.015/min. Thus, although we observed an initial burst of attachment of BHK-py cells, the first order rate constants for adhesion of both cell types are essentially the same. Attached cells were observed to be rounded in phase-contrast microscopy even after 1 h of attachment; flattening of cells was observed only after the cells had been attached for several hours.

Table I shows the percent of adhesion and the recoveries of cells incubated under various conditions. Tryptose phosphate had no effect on cell

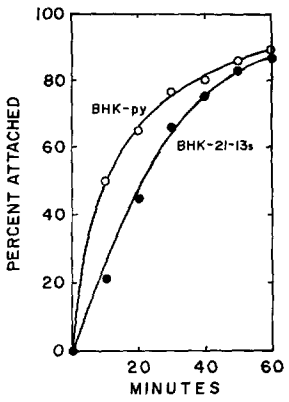

FIGURE 1 Time dependence of adhesion of BHK-21-13s and BHK-py. The incubations contained 10^6 cells and were carried out for the time periods indicated. Other details are described under Methods.

TABLE I

*Adhesion of BHK Cells to Falcon Polystyrene**

Exp	Incubation medium	Serum	% adhesion	% recovery‡
1	Adhesion medium	+	64	90
	" "	−	52	56
2	BHK growth medium (− tryptose phosphate)	+	68	96
	" " "	−	64	43
3	Adhesion medium (+ 10% tryptose phosphate)	+	67	87
	" "	−	47	67
4	BHK growth medium	+	66	94
	" " "	−	60	60

* The incubations contained 10^6 cells and were carried out for 30 min. Adhesion medium and BHK growth medium are the media described under Methods, minus serum. The final serum concentration was 5% where added. Other details are described under Methods.

‡ $\frac{\text{Total cells recovered at end of experiment}}{\text{Starting no. of cells}} \times 100.$

adhesion in either adhesion medium or growth medium. Adhesion in the presence of serum was slightly increased. Recovery of cells at the end of the experiments was usually about 90%; in the absence of serum, recovery was only 50%. In every case, recovered cells were examined microscopically and found to be morphologically intact and able to exclude trypan blue.

Detachment of BHK Cells

BHK-py and BHK-21-13s cells were attached to Falcon polystyrene bottles for 10 min, after which the attached cells were subjected to shaking on a New Brunswick G-2 shaker at 100 rpm for 1 min. This treatment did not cause attached cells to detach, which indicates the firmness of attachment. When the cells were subjected to a shaker speed of 450 rpm, attached cells were sheared off the surface of the bottles. The data in Table II show the percentage of attached cells that were detached by 450 rpm treatment as a function of the time that they were attached before treatment. At longer times of attachment, the percent detachment of both BHK-21-13s and BHK-py cells decreased, indicating that the cells became more firmly attached.

During the course of measuring adhesion, some suspended cells are becoming initially attached simultaneously as attached cells become more firmly attached. To show the increase in attachment strength apart from the problem of the newly attached cells, an experiment was performed in which cells were allowed to attach for 30 min (first incubation) and then loosely or nonattached cells were removed. Duplicate flasks of these attached cells either were subjected to treatment at various shaker speeds on the shaker or were allowed to incubate for an additional 30 min (second incubation). After the second incubation, these cells were also tested for detachment. The data in Table III show that fewer cells detached after the second incubation than after the first incubation, at all the shaker speeds tested. These results show that the firmness of attachment increased during the second incubation period. Similar results were seen for both BHK-21-13s and BHK-py.

TABLE II

*Detachment of BHK Cells**

Time of attachment	% detachment	
	BHK-21-13s	BHK-py
min		
10	52	41
20	34	34
40	20	16
70	13	14
100	10	12

* 10^6 cells were attached for the time periods indicated as described under Methods; after the incubations the nonattached cells were detached and to the attached cells were added 5 ml fresh medium. These cells were then subjected to shaking at 450 rpm for 1 min on the New Brunswick G-2 shaker, and the percentage of attached cells released by this treatment was determined.

TABLE III

*Detachment of BHK Cells at Various Times and Shaker Speeds**

Cell type	Shaker speed	% of cells detached‡	
		After 1st incubation	After 2nd incubation
	rpm		
BHK-21-13s	0	None (61)§	None (93)
	150	8	6
	250	26	6
	350	44	25
	450	57	27
BHK-py	0	None (41)	None (80)
	150	5	1
	250	22	4
	350	46	10
	450	71	14

* The incubations contained 10^6 cells. The first incubation was 30 min for BHK-21-13s and 20 min for BHK-py. After the first incubation nonattached cells were removed and 5 ml of fresh adhesion medium were added to the attached cells. The attached cells were then either subjected to shaking on the New Brunswick G-2 shaker for 1 min at room temperature or incubated for an additional 30 min at 30°C. The attached cells after the second incubation were then subjected to shaking as above. Other details are described under Methods.

‡ Percent of cells detached is the percent of attached cells freed from the surface during the shaking process, an average of duplicate determinations.

§ The number in parentheses is the percent of cells in the bottles attached at the end of incubations before further treatment.

Inhibition of Adhesiveness by —SH Binding Reagents

Several different —SH binding reagents inhibit the adhesiveness of rat hepatoma (HTC) cells (7, 8). The data in Table IV show that such reagents also inhibited the attachment of BHK-21-13s cells, in both the presence and absence of serum. —SH reagents did not cause attached cells to detach, which is a result similar to that observed previously with HTC cells.

Treatment of attached BHK-21-13s cells with NEM inhibited the strengthening of the attachment that occurs with time. Cells were allowed to attach for 15 min (first incubation), after which loosely and nonattached cells were decanted and the remaining cells were subjected to NEM or other treatments. These cells were then tested for detachability by shaking or they were incubated for an additional 30 min (second incubation) and then tested for detachability. The results are shown in Table V and indicate that after NEM treatment the percent of cells detached by shaking did not decrease during the second incubation. In fact, NEM-treated cells detached more easily than untreated cells.

HTC cells attached to a substratum are less sensitive to inhibition by —SH binding reagents than cells in suspension treated with these reagents (10). In these experiments cells were attached, treated with an —SH reagent, detached by EDTA treatment, and tested for reattachment. We have performed a similar experiment with BHK-21-13s cells, using radiolabeled NEM. Cells, either in suspension or attached to Falcon bottles, were treated with [1-^{14}C]NEM. The reactions were stopped by addition of an excess amount of dithiothreitol (DTT) which reacts with unused NEM but does not affect the NEM that is bound to the cells. The cells were then recovered (the attached cells were detached by EDTA treatment) and washed and determinations were made of the ^{14}C-incorporation that had occurred into the cell membranes and of the adhesiveness of the cells. In parallel experiments using nonradioactive NEM, we determined the ability of the cells to take up [2-^{14}C]leucine and [5-T]uridine after NEM treatment. A representative experiment is shown in Table VI. Attachment of the cells before NEM treatment resulted in decreased labeling of the cells by NEM and decreased inhibition of cell adhesiveness and uridine uptake; however, leucine

TABLE IV

*Inhibition of BHK-21-13s Cell Adhesion by —SH Binding Reagents**

Inhibitor	Concentration	% inhibition	
		+ serum	− serum
	mM		
NEM	0.1	>90	88
$NaAsO_2$	1.0	52	40
$HgCl_2$	0.1	>90	>90
p-Mercuribenzoate	1.0	>90	>90

* The incubations contained 10^6 cells and were carried out for 30 min. Inhibitors were added at the concentrations indicated. Other details are described under Methods.

TABLE V

Effect of NEM and DTT on Detachment of BHK-21-13s Cells

Exp	Addition	% of cells detached	
		After 1st incubation (15 min)	After 2nd incubation (30 min)
1	None	39	26
	NEM	55	71
	NEM/DTT	52	64
	DTT	33	23
2	None	56	23
	NEM/DTT	68	73
	DTT	50	26

The incubations contained 10^6 BHK-21-13s cells, in 5 ml adhesion medium. At the end of the first 30°C incubation (15 min) the nonattached cells were removed and 5 ml fresh medium were added to the attached cells. These cells were treated at room temperature with NEM (final concentration 0.1 mM) for 5 min and then DTT (final concentration 1.0 mM) for 1 min as indicated. The medium in all the bottles was then replaced with fresh adhesion medium. Attached cells were then either subjected to 1 min shaking at 400 rpm on the New Brunswick G-2 shaker or incubated an additional 30 min at 30°C. After the second incubation the cells were also subjected to shaking. In both cases the cells shaken loose were collected and the cells that remained attached were detached as described under Methods and collected. The percent of attached cells detached by the shaker was determined. Other details are described under Methods.

TABLE VI

*Binding of [^{14}C]NEM by Attached and Suspended BHK-21-13s**

Cell treatment	Adhesiveness (% attached)	Exposed —SH (10^8 NEM/cell)	Uptake (cpm/10_6 cells) L-Leucine	Uridine
Attached				
Control	83	—	15,300	69,700
DTT	77	—	17,200	66,600
NEM/DTT	57	3.9	4,000	65,400
Suspended				
Control	74	—	17,300	59,400
DTT	72	—	15,500	57,300
NEM/DTT	33	7.4	5,700	45,200

* For each experiment 25×10^6 BHK-21-13s were incubated in 25 ml adhesion medium in 75 cm^2 falcon bottles for 60 min at 37°C. At the end of the incubations, nonattached cells were decanted. For experiments designated "attached," the bottles with cells attached were then treated as described below. For experiments designated "suspended" the detached cells were detached as described under Methods, resuspended in fresh media, and then treated as described below.

5 ml of adhesion salts (adhesion medium minus serum) were added to the attached cells and the suspended cells were also placed in 5 ml of adhesion salts. The cells were then treated with NEM (final concentration 0.1 mM) for 1 min at 30°C. The reactions were stopped by the addition of DTT (final concentration 1.0 mM). Controls were treated with 1.0 mM DTT alone or with nothing. In experiments with radiolabeled NEM, we used [1-^{14}C]NEM (10.2 Ci/M).

After the incubations the attached cells were detached as described under Methods, and all cells were washed and suspended in 2.0 ml of fresh adhesion salts. Portions of the cells were used to determine the following:

(*a*) *Cell number* by turbidity and direct counts with a hemocytometer.

(*b*) *Cell adhesion* in which 10^6 cells were suspended in fresh adhesion medium and 30 min adhesion was measured as described under Methods (duplicates).

(*c*) *[1-^{14}C]NEM bound per cell* in which 10^7 cells were subjected to freeze thawing twice followed by 10 strokes with a Tenbroeck homogenizer (Kontes Glass Co., Vineland, N.J.). The cells were then centrifuged at 3,500 *g* and the pellets were washed twice and treated with 5% TCA. The precipitates were filtered on 5 μm Millipore filters, and radioactivity was determined by scintillation spectrophotometry.

(*d*) *Uptake* in which 10^5 cells were suspended in adhesion salts containing 2% fetal calf serum and 1:10,000 MEM amino acids. 0.5 μCi of [2-^{14}C]L-leucine (57.6 Ci/M) or 5 μCi of [5-T]uridine (5 Ci/M) were added as indicated. The reactions were incubated for 30 min at 37°C and stopped by chilling the reaction mixtures. The cells were centrifuged at 1,000 *g*, washed twice and filtered on 5 μm Millipore filters, and radioactivity was determined with a scintillation spectrophotometer.

uptake was inhibited to the same extent whether cells were suspended or attached during treatment.

When the [1-^{14}C]NEM-labeled cell membrane fractions were subjected to chromatography by sodium dodecyl sulfate-gel electrophoresis, no differences were observed in the labeling pattern of the membrane proteins whether or not the cells were suspended or attached during NEM treatment.

Inhibition of Cell Adhesiveness by Trypsin Treatment

In order to subculture BHK cells, stationary stock cultures of BHK-21-13s and BHK-py are routinely removed from flasks by treatment with 0.025% (0.25 mg/ml) trypsin (Grand Island Biological Co.). Cells detached in this way and washed once are subsequently as adherent as suspension-grown cells. Trypsin has been previously shown to reversibly inhibit intercellular aggregation (11), and this reagent is commonly known to detach attached cells.

The data in Table VII show the effect of increasing concentrations of crystalline trypsin on the adhesiveness of BHK-21-13s cells. At concentrations of 0.5% and higher, there was a decrease in the percent of cells that became attached. Since cells treated with 0.4% (4 mg/ml) trypsin under these conditions appeared to be intact, their ability to regain adhesiveness was tested. The data in Fig. 2 show that trypsin-treated cells regained adhesiveness in several hours when incubated in fresh growth medium. The regain of adhesiveness was inhibited by 10^{-4} M cycloheximide. During this time period cycloheximide had no effect on untreated cells. In other experiments it was found that 10^{-5} M cycloheximide also inhibited the recovery of adhesiveness but that 2.5 μg/ml of chloramphenicol had no effect. Actinomycin D at 1.0 μg/ml partially inhibited the recovery of adhesion.

TABLE VII

*Effect of Trypsin Treatment of BHK Cells on Their Adhesion**

Trypsin concentration	Adhesiveness (% of cells attached)
mg/ml	
0	68
0.25	63
0.5	46
1.0	35
2.0	27
4.0	19

* The incubations contained 5×10^6 cells suspended in 5 ml of adhesion salts containing 1% serum. Trypsin (8,000 U/mg) was added at the concentrations indicated. The incubations were carried out for 5 min at 30°C after which the cells were centrifuged at 1,000 *g* and washed with adhesion medium. Portions of cells were suspended in 5.0 ml adhesion medium and 30 min adhesion was determined as described under Methods.

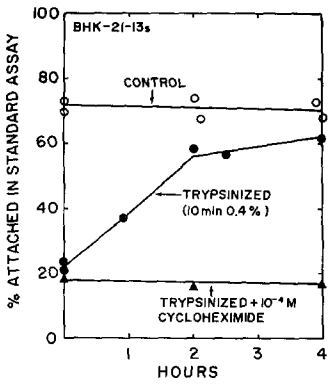

FIGURE 2 Trypsin treatment of BHK cells. BHK-21-13s cells were suspended in adhesion salts containing 1% fetal calf serum at a concentration of 5×10^6 cells/ml. For trypsinization experiments, trypsin (8,000 U/mg) was added to the cells at a final concentration of 4 mg/ml and incubated 10 min at 30°C. Trypsinized cells were centrifuged at 1,000 × *g*, washed in BHK growth medium, and suspended in fresh BHK growth medium at a final concentration of 10^6 cells/ml. Cycloheximide was added as indicated. The incubations were placed at 37°C and, at the times indicated, 1 ml samples were tested with 4.0 ml adhesion medium for 30 min adhesiveness as described under Methods.

DISCUSSION

The results reported in this paper can be discussed in terms of the following model. Adhesive sites are uniformly distributed on the cell's surface. When a cell settles and contacts the substratum, initially only a few adhesive sites interact with the substratum surface. With time, more adhesive sites approach and interact with the surface, and the cells become more firmly bound. The sites contain or are protein components which must be dynamically maintained.

The first order kinetics observed during attachment indicate that the ability of each cell to attach is the same as every other cell, and that the probability of a cell attaching does not change during the course of the experiment. This is consistent with the proposal of uniformly distributed adhesive sites on the cells and suggests that the sites are identical on all cells. Our data do not provide any insights concerning the arrangement of sites on the cell surface (random or in localized areas) or the mobility of the sites.

There was increased resistance of the cells to detachment as attachment time was increased, indicating that cells were becoming more firmly attached. Since —SH binding reagents inhibited both cell attachment and strengthening of cell attachment (without causing cell detachment), it seems likely that these two processes are the same. Thus, we have suggested that cell strengthening results from the continuous interaction of more adhesive sites with the substratum.

The effects of —SH binding reagents on cellular adhesion appear to be quite specific, suggesting that either the maintenance of adhesive sites or formation of the adhesive bond between cell and substratum requires an —SH group. The specificity of inhibition is indicated by the ability of different classes of —SH binding reagents (mercurials, alkylating agents, and arsenicals) to inhibit adhesion (see also reference 7); the ability of —SH binding reagents to inhibit adhesion to various substrata varying in chemical composition (8); the inaccessibility of the critical —SH groups once the adhesive bond is formed, since NEM titrates fewer —SH groups on the cell membrane of attached cells and neither this nor other —SH binding reagents cause attached cells to detach; and the differences in the NEM-inhibition of subsequent adhesiveness but not leucine-uptake ability of attached vs. suspended cells. Thus it seems unlikely that the effects of the —SH binding reagents is a result of a change at some membrane site other than the adhesive site.

The involvement of a critical —SH group in

adhesion suggests the involvement of a protein component in adhesion. The loss of adhesiveness by trypsin treatment and its recovery, which is cycloheximide inhibited, lends further support to the presence of surface adhesive sites that either are or contain proteins. The fact that isolated membranes do not have adhesive properties indicates that intact cell metabolism is necessary to maintain the sites.

This model is an elaboration of the one presented by Taylor (12) in which the cells progressively flatten onto a substratum. It is consistent with the theoretical argument by Pethica that a few cell microvilli initially make contact with the substratum followed by increasing contact by other regions of the cell surface with the substratum surface (13).

It is currently felt by some investigators that cell surface glycoproteins and mucopolysaccharides are involved in adhesion (14), and these have been implicated in intercellular aggregation (15, 16). Our data do not allow us to speculate on the chemical nature of the adhesive bond. Using the system of trypsin-treated cell recovery of adhesiveness, we hope to make some progress on this aspect of the problem.

F. Grinnell is a National Institutes of Health Postdoctoral Trainee (Training Grant T01 AM 05567) and a Fellow of the Texas Cancer Society. This research was supported by the United States Public Health Services.

Received for publication 18 July 1972, and in revised form 19 October 1972.

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