Studies on Cell Adhesion and Recognition

II. The Kinetics of Cell Adhesion and Cell Spreading on Surfaces Coated with Carbohydrate-reactive Proteins (Glycosidases and Lectins) and Fibronectin

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ABSTRACT The kinetics of cell attachment and cell spreading on the coated surfaces of two classes of carbohydrate-reactive proteins, enzymes and lectins, have been compared with those on fibronectin-coated surfaces with the following results: (*a*) A remarkable similarity between the kinetics of cell attachment to fibronectin-coated and glycosidase-coated surfaces was found. In contrast, cell attachment kinetics induced by lectin- and galactose oxidase-coated surfaces, in general, were strikingly different from those on fibronectin and glycosidase surfaces. The distinction between fibronectin- or glycosidase- and lectin- or galactose oxidase (an enzyme with lectin-type characteristics)-coated surfaces was further supported by the finding that cytochalasin B and EDTA inhibited cell attachment to fibronectin- and glycosidase-coated surfaces but not lectin-coated surfaces. (*b*) Fibronectin, if labeled and added to a cell suspension, showed only low or negligible interaction with the cell surface. However, fibronectin absorbed on plastic surfaces showed a high cell-attaching activity. It is assumed that fibronectin coated on plastic surfaces may form polyvalent attachment sites in contrast to its lower valency in aqueous solution. (*c*) Various inhibitors of cell attachment to both fibronection-, galactose oxidase-, and lectin-coated surfaces were effective only during the first few minutes of the adhesion assay, after which time the attached cells became insensitive to the inhibitors. It is suggested that the initial specific recognition on either lectin-type or fibronectin-type surfaces is followed by an active cell-dependent attachment process. The primary role of the adhesion surface is to stimulate the cell-dependent attachment response. (*d*) Cells attached on tetravalent concanavalin A (Con A) spread very rapidly and quantitatively, whereas divalent succinyl Con A and monovalent Con A were effective stimulators of cell attachment but not cell spreading. Cross-linking of succinyl Con A restored the cell spreading activity. Tetravalent Con A surfaces specifically bind soluble glycoproteins, whereas succinyl Con A has a greatly reduced ability to bind the same glycoproteins. These results suggest that cross-linking of cell surface glycoproteins by the multivalent adhesive surface may trigger the cellular reaction leading to cell spreading.

The components and mechanisms involved in cell attachment on various adhesive surfaces have been studied extensively as a model of cell-cell and cell-substratum interactions in multicellular systems (see, for reviews, references 8, 9, 12, 14, 30, 34, and 46). In the preceding paper (40), two classes of carbohydrate-binding proteins, lectins and glycosidases, have been found to be active promoters for cell adhesion and spreading. Of various components at the cell surface that could promote cell attachment and spreading, fibronectin is the best characterized (21, 45, 46). This paper provides further information on

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the kinetics and the mechanisms involved in cell adhesion and spreading. The studies are focused on the following aspects: (*a*) The similarities and differences of cell attachment on fibronectin-, enzyme-, and lectin-coated surfaces were investigated. These studies include a comparative study of the kinetics of cell attachment, differential drug effects, and binding of soluble proteins to the cell surface. (*b*) Existence of two distinguishable phases during the process of cell adhesion, i.e., an initial reversible phase that is sensitive to various drug and haptens, and a later irreversible phase that is insensitive to drug and hapten inhibitors. (*c*) The influence of the valency of adhesion-promoting surfaces and of membrane receptors upon cell attachment and spreading.

Based on these studies, a possible mechanism involved in cell attachment, spreading, and their sequential events will be discussed. It is envisioned that an initial recognition reaction stimulates or triggers the subsequent processing of active cell attachment. A possible cross-linking of cell surface receptors by the adhesion surfaces could be an instrumental signal for cell spreading.

MATERIALS AND METHODS

Materials

Fibronectin was purified from hamster plasma and from conditioned medium of BALB/c 3T3 cells by gelatin affinity chromatography (10). The isolated proteins were electrophoretically homogeneous, as estimated on polyacrylamide gel electrophoresis in the presence of SDS (26). These two forms of fibronectin gave the same rate and extent of cell adhesion (at 10 μg/ml fibronectin) in trial experiments. Soybean agglutinin[1] (SBA) and peanut agglutinin (*Arachis hypogaea*) (PNA) were purified by published affinity-chromatography techniques (28, 29). Wheat-germ agglutinin (WGA) and succinyl concanavalin A (con A) (16) were from Vector Laboratories (Burlingame, Calif.). Monovalent Con A was a gift from Professor Toshiaki Osawa (Tokyo University, Tokyo, Japan). Monovalent Con A was prepared by photoaffinity labeling with azido-phenyl α-mannoside (3), followed by succinylation as previously described by Beppu et al. (4). β-Galactosidase was purified according to Li and Li (27) and was kindly donated by Dr. Michiko Fukuda. Trypsin (crystalline) was purchased from Worthington Biochemical Corp. (Freehold, N. J.). 2-Deoxy-2,3-dehydro-*N*-acetylneuraminic acid, described as a sialidase inhibitor by Meindl et al. (32), was kindly donated by Professor Roland Schauer (Christian Albrecht University, Kiel, Germany). Cytochalasin B and 1,3-diaminopropane were from Aldrich Chemical Company, Milwaukee, WI. Con A (Con A), soybean trypsin inhibitor, diamide (azodicarboxylic acid-*bis*-dimethylamide), phenylmethylsulfonyl fluoride (PMSF), dithiothreitol, glutathione (reduced form), *p*-chloromercuribenzene sulfonic acid, *N*-acetyl-D-galactosamine, 1-ethyl-3(3-dimethyl-amino-propyl)-carbodiimide HCl (EDC), NaN_3, *N*-ethyl maleimide (NEM), EDTA, and *Clostridium perfringens* sialidase (type IX, affinity purified) were obtained from Sigma Chemical Co. (St. Louis, Mo.) The sialidase was homogeneous on polyacrylamide gel electrophoresis with M_r 70,000. Galactose oxidase was obtained from Kabi (Stockholm, Sweden).

Cells and Cell Culture

BALB/c 3T3, hamster embryo fibroblasts (NIL cells) and baby hamster kidney (BHK) cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 5 or 10% fetal calf serum, 10^2 U of penicillin G/ml and 0.1 mg of streptomycin/ml in an atmosphere of 5% CO_2.

Adhesion Assays

The adhesion assays followed the same procedure as previously described (see preceding paper [40]). In assays for the study of the effect of sialic acid–containing components, Salt/Pi buffer was insufficient to buffer the adhesion medium, and therefore the cells were washed with a balanced salt solution buffered with 30 mM HEPES (modified from the Tris-citrate-buffered balanced salt solution of references 38 by replacing the dicarboxylic acids and Tris-citrate with 30 mM HEPES).

Cell Spreading

For studies on cell spreading, the adhesion assay was modified to permit photography of attached cells. Petri dishes (60 × 10 mm, no. 1007; Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) were incubated with 2 ml of protein solution (10 μg/ml unless otherwise specified) for 2 h at room temperature and then washed three times with Salt/Pi buffer. The plates were then incubated with bovine serum albumin (BSA)-Salt/Pi buffer for 1 h at room temperature. Cells were suspended by trypsinization as described under adhesion assay and then suspended in BSA-Salt/Pi buffer. 2×10^6 cells in 4 ml of BSA-Salt/Pi buffer were added to each plate, incubated at room temperature for 1 h, and then washed five times with Salt/Pi buffer before being photographed under a phase-contrast microscope at × 100 magnification.

Analytical Methods

Protein was determined by fluorescamine assay (5).

Labeling of Proteins

WGA was acetylated with [^{3}H]acetic anhydride (2.5 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), using the procedure of Miller and Great (33). The resulting labeled WGA (1×10^6 cpm/mg) was active and >85% of the radioactivity could be precipitated by reaction with hog blood group A+H substance, a receptor for WGA. On gel electrophoresis all the radioactivity comigrated with the WGA protein staining band.

Fibronectin, *Clostridium perfringens* sialidase, Con A and succinyl Con A were iodinated, using the chloramine-T method (11). *Dolichos biflorus* lectin was labeled with [^{14}C]formaldehyde, using the reductive methylation procedure (42).

Cross-linking of Succinyl Con A

The carboxyl groups of succinyl Con A were reacted with free amines, using the basic procedures for protein carboxyl group modification described by Hoare and Koshland (18). Care was taken to avoid inactivation of the lectin as a result of modification of carboxyl groups required for carbohydrate binding as described by Hassing et al. (17).

Basically, the cross-linking of succinyl Con A proceeded as follows: Succinyl Con A (1 mg) was dissolved in 5 ml of 0.5 M NaCl containing 100 mM α-methyl-D-mannoside. EDC was dissolved in H_2O and added to the stirring solution to a final concentration of 25 mM. The pH of the solution was adjusted to 5 with 1 M HCl and a solution of 1,3-diaminopropane (2.0 M in H_2O, pH adjusted to 5 with HCl) was added to a final concentration of 10 mM. The pH of the stirring solution was maintained at 5 with additions of 1 N HCl until the solution became turbid (~20 min at room temperature), at which point, 2 ml of 1 M sodium acetate was added to stop the reaction. The cross-linked succinyl Con A solution was dialyzed against Salt/Pi buffer and centrifuged to remove small quantities of insoluble material before use. After cross-linking, the major proportion of the lectin possessed a relative subunit molecular weight of 50,000 as determined by polyacrylamide gel electrophoresis in the presence of SDS under reducing conditions (26), suggesting that dimerization of the succinyl Con A subunits of 26,000 mol wt (16) was the major cross-linking reaction. Succinylation of the cross-linked succinyl Con A and fibronectin followed the basic procedure described by Gunther et al. (16), and reduced the number of free amino groups by 57 and 63%, respectively, as determined by fluorescamine assay.

RESULTS

Comparison of Cell Attachment on Lectin-type and Fibronectin-type Adhesion Surfaces

KINETICS OF CELL ATTACHMENT: Kinetics of 3T3 cell attachment on fibronectin-coated surfaces and on Con A–coated surfaces are shown in Fig. 1*A*. All figures involving comparisons of cell attachment on different adhesion surfaces were obtained using the same cell preparation. This permits direct comparison of cell attachment results in any one figure. Fibronectin-mediated cell attachment clearly displays a sig-

[1] *Abbreviations used in this paper:* Con A, *Canavalia ensiformis* (jack bean) lectin; EDC, 1-ethyl-3(3-dimethyl-amino-propyl)-carbodiimide HCl; NEM, *N*-ethyl maleimide; Salt/Pi, 137 mM NaCl/2.7 mM KCl/0.7 mM $CaCl_2$/0.5 mM $MgCl_2$/8.1 mM Na_2HPO_4/1.5 mM KH_2PO_4; BSA-Salt/Pi, Salt/Pi containing 100 μg/ml of bovine serum albumin; PNA, *Arachis hypogaea* (peanut) lectin; SBA, *Glycine max* (soybean) lectin; WGA, *Triticum vulgaris* (wheat-germ) lectin.

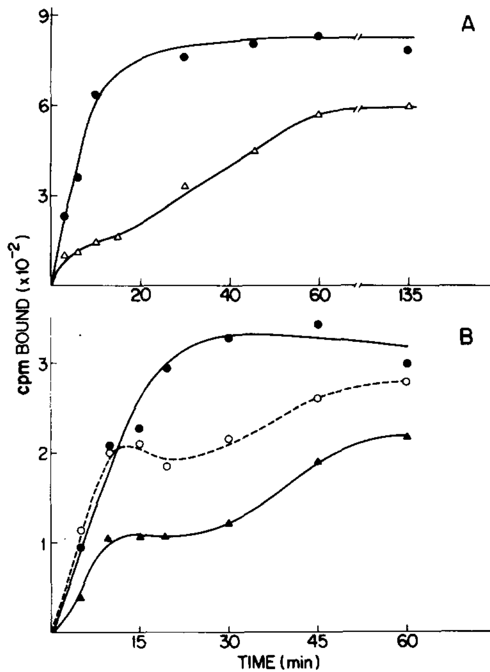

FIGURE 1 Time-course of 3T3 cell attachment at 25°C on fibronectin, Con A, monovalent Con A, and neuraminidase adhesion surfaces. (*A*) [^{3}H]thymidine-labeled cells from the same suspension were pipetted simultaneously on fibronectin (△) and Con A (●) surfaces, using a multiwell pipette (Titertek; Finnpipette, Helsinki, Finland). At the time intervals indicated, the adhesion medium was removed, and the nonattached cells were removed by washing the surfaces simultaneously (twice with 100 μl of phosphate-buffered saline [Salt/Pi]), using a multiwell pipette. The adhesion medium from each well was removed after the last time interval studied (135 min), and the radioactivities were solubilized for counting. Each measured radioactivity is an average of two determinations. (*B*) An analysis on Con A (●), monovalent Con A (○), and neuraminidase (▲) adhesion surfaces as described in *A* above.

moidal-type time-course. The initial rather rapid increase (in 3–5 min) of bound radioactivity was also observed on BSA-coated plates and therefore reflects unspecific or passive binding, which does not increase after the cells have settled upon the adhesion surface. Thereafter a lag period of up to ~15 min follows, during which there is only a slow increase in the number of attached cells. During the following 40–50 min, ~50% of the added cells were attached (estimated from the recovery of radioactivity), and after this rapid reaction the number of attached cells increased only slowly. Con A–mediated cell attachment showed strikingly different kinetics. In agreement with the microscope observation, a significant attachment was already observed in 5–10 min, with little increase after 15–20 min (Fig. 1*A*). Succinyl Con A (results not shown) and monovalent Con A (Fig. 1*B*) were also active in attaching cells, although the kinetic curves were different from that of either tetravalent Con A or fibronectin in that some increase in the number of attached cells occurred during the time period of 20–40 min. SBA showed the same kind of kinetics as Con A (Fig. 2*A*). Galactose oxidase, an enzyme capable of oxidizing but not hydrolyzing galactosyl and *N*-acetylgalctosaminyl residues has previously been shown to agglutinate sialidase-treated erythrocytes (19). As seen in Fig. 2*C*, cell attachment to galactose oxidase–coated surfaces exhibited kinetics very similar to tetravalent Con A or SBA surfaces. Galactose oxidase, therefore, has lectin-type binding qualities even for cells that have not been treated with sialidase. β-Galactosidase- (Fig. 2*B*) or sialidase- (Fig. 1*B*) mediated adhesion was fibronectin-like and exhibited an initial lag period and rather slow adhesion rate as compared with Con A–, SBA- or galactose oxidase–induced attachment. An increase of receptor sites containing terminal β-galactose residues on the cell surface by preincubating the cells with sialidase followed by washing did not remove the lag period, but considerably increased the rate and extent of active attachment on β-galactosidase-coated sur-

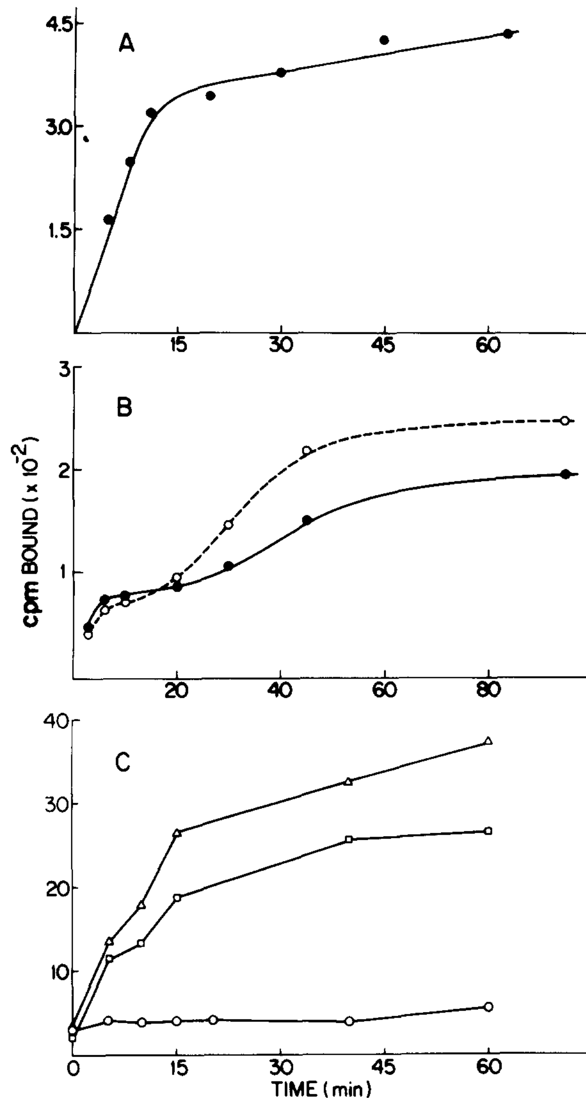

FIGURE 2 Time-course of 3T3 cell attachment at 25°C on SBA and β-galactosidase adhesion surfaces; effect of neuraminidase treatment of cells. (*A*) Cell adhesion experiments were performed as described in Fig. 1 on SBA (●) adhesion surface. (*B*) An analysis on β-galactosidase surface as described in Fig. 1. Control cells (●) and cells treated with neuraminidase (○) as follows: Neuraminidase treatment of the cells (1 × 10^6 cells) was carried out in Salt/Pi, pH 6.6 (1 part of phosphate-buffered saline, 1 part of 0.2 M phosphate), using 25 U of *Vibrio cholerae* neuraminidase (Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, Calif.) in 1 ml for 0.5 h at 37°C with shaking of the incubation mixture. Control cells were incubated in the same way without enzyme. The cells were washed three times, and analyzed for the attachment as described above. (*C*) Time-course of NIL cell attachment at 25°C on BSA, Con A, and galactose oxidase adhesion surfaces. Cell adhesion experiments were performed as described in Fig. 1 on BSA (○), Con A (△), and galactose oxidase (□) adhesion surfaces.

faces (Fig. 2 *B*). When sialidase-treated cells were analyzed for attachment ability on surfaces coated with *Clostridium perfringens* sialidase, a reduction of cell attachment to 46% of controls, as the mean of two different determinations, incubated in plain buffer was observed. No difference in cell attachment on plain plastic resulting from sialidase treatment of cells could be observed by microscopy or by counting of the radioactivity. It therefore appears that the adhesion-modulating molecules like Con A, SBA, and galactose oxidase give a steep hyperbolic kinetic curve, whereas the time-course of cell attachment on surfaces coated with fibronectin or glycosidases is less rapid, and shows a time lag. Cell attachment on divalent and monovalent Con A–coated surfaces proceeds with adhesion kinetics that have characteristics of both lectin- and fibronectin-surface kinetics. It is evident from the data given above that many different adhesion-mediating molecules may appear to be very similar when long-term (60-min) adhesion assays are used for comparisons. However, the same adhesion surfaces can be clearly differentiated when a kinetic analysis of cell attachment is performed.

EFFECT OF METABOLIC INHIBITION AND CHEMICAL MODIFICATION ON CELL ATTACHMENT: The distinction between lectin-type- and fibronectin-type-induced cell attachment was further emphasized by adhesion-inhibition studies using compounds that have previously been reported to inhibit cell adhesion (14, 22) or cell division (25). The reagents capable of inhibiting cell attachment to fibronectin-, enzyme-, and lectin-coated surfaces can be divided into two classes (see Table I): those factors that inhibit both lectin-type and fibronectin- or enzyme-type interactions (low temperature, diamide, and NEM) and those that efficiently inhibit only fibronectin-type adhesions (cytochalasin B, EDTA, sodium azide). Inhibition of oxidative energy production by sodium azide strongly inhibited cell attachment on fibronectin- and sialidase-coated surfaces (Table I). Although cell attachment on Con A surfaces was also reduced by sodium azide, the inhibition was clearly to a lesser degree than on either fibronectin or sialidase surfaces. Similarly, cytochalasin B and EDTA decreased cell attachment to fibronectin, sialidase, and β-galactosidase surfaces but not to lectin surfaces (Table I).

In contrast to the reagents described above, chemical modification of the cells with sulfhydryl-blocking agents such as NEM (Table I), iodoacetic acid, or *p*-chloromercuribenzene sulfonic acid greatly decreased cell attachment to all adhesion surfaces tested. Diamide, a nontoxic sulfhydryl oxidant (24) that inhibits mitosis and depletes cellular reduced glutathione levels (25), produced maximal inhibitory effects at concentrations of 100 μM on all surfaces tested. The inhibitory effect of diamide was totally neutralized by the addition of 5 mM glutathione to the adhesion medium, suggesting that the diamide inhibition was the result of an oxidation process. None of the inhibitors described had any effect if incubated with the adhesion surface and then washed off the plates before addition of the cells, suggesting that inhibition of cell attachment was the result of effects on the cells and not on the adhesion surface. In support of this idea, incubation of the cells with the sulfhydryl reagents (30 min at room temperature in 1 mM concentration), followed by washing, was as effective as addition of the reagents directly to the adhesion mixture in reducing cell attachment. Sulfhydryl-reducing agents (1–10 mM dithiothreitol or 5 mM reduced glutathione) had no effect on cell atachment (results not shown).

TABLE I

Inhibition of Cell Adhesion on Fibronectin, Sialidase (Clostridium perfringens), and Con A Adhesion Surfaces

Adhesion surface	Inhibitor*					
	NaN_3 (5 mM)	Cytochalasin B‡ (10 μM)	EDTA (10 mM)	Diamide (1 mM)	NEM (1 mM)	Cold (2°C)
Fibronectin	37	48	55	25	14	23
Sialidase	24	27	67	10	20	8
Con A	60	95	92	27	30	50

* The inhibitors were added directly to the adhesion reaction and were present throughout a 60-min incubation as described in detail in Materials and Methods, under Adhesion Assays. Values presented represent the percent (mean of two determinations) of cells bound in the presence of inhibitor relative to cells bound without inhibitor.

‡ Cells were incubated for 30 min with or without cytochalasin B before addition of the cells to the adhesion surface.

BINDING OF SOLUBLE FIBRONECTIN, ENZYMES, AND LECTINS TO CELLS: Incubation of cells (1.2×10^6) with [^{125}I] iodine-labeled fibronectin (21 μg/ml, 1 h, 37°C) or sialidase (10 μg/ml, 25°C, 0.5 h) in suspension, followed by washing and centrifugation, failed to result in any cell-bound radioactivity. To remove the possibility that the direct binding of soluble fibronectin or sialidase may not have been detected because of rapid dissociation of the bound material, we also performed competition studies. Addition of fibronectin or *Clostridium perfringens* sialidase to cells in suspension, followed by addition of the cells to fibronectin- and sialidase-coated surfaces, failed to inhibit the cell attachment assay by competition for or blocking of cell surface receptors. Because the adhesion assay is carried out at pH 7.4, well outside the pH optimum range for the hydrolytic activity of *Clostridium perfringens* sialidase (pH 4–6; see preceding paper [40] for detailed discussion of the pH dependence of enzyme activity), no appreciable loss of cell surface sialic acid can be expected as a result of hydrolysis during the adhesion assay. Neither soluble fibronectin nor sialidase exhibits any detectable cell surface affinity by these assays although both proteins effectively attach cells after immobilization on a plastic surface.

In contrast, binding of soluble lectins to the cell surface is dependent on lectin concentration, inhibitable by specific hapten sugars, and saturable at high lectin concentrations (see preceding paper [40]). In addition, treatment of cells with monovalent Con A (50 μg/ml) before addition of the cells to Con A–coated plates effectively reduced cell adhesion to 42% of controls by competing for Con A receptors on the cell surface. The possibility that monovalent Con A inhibits cell attachment as a result of cell toxic effect is unlikely because preincubation of cells with monovalent Con A does not inhibit cell attachment to culture plates (39).

Sequence of Events in "Stimulation" of Cell Adhesion and Spreading

EFFECT OF DIAMIDE ON CELL-SUBSTRATE INTERACTIONS: As seen in Table I, preincubation of cells with diamide or addition of diamide directly to the adhesion medium at the beginning of the assay caused nearly complete inhibition of cell attachment to both lectin and fibronectin on coated surfaces. However, diamide treatment of cells did not inhibit the binding of soluble WGA to cells or WGA-induced cell agglutination (Fig. 3 *A*) under conditions that completely inhibit cell adhesion on WGA-coated surfaces (Fig. 3 *B*). Appar-

ently diamide does not greatly alter either the number or organization of cell surface receptors for WGA. In similar studies, diamide, at a concentration of 100–500 mM, inhibited 97% of NIL cell attachment to galactose oxidase–coated surfaces over BSA background. However, cell surface labeling of NIL cells, using the galactose oxidase–NaB[^{3}H]$_4$ method (13) in the presence or absence of 500 mM diamide, labeled cells to 5.4 × 10^3 cpm/μg of protein and 4.3 × 10^3 cpm/μg of protein, respectively. Also, there was no detectable difference in the cell surface labeling patterns of the cells as determined by polyacrylamide gel electrophoresis in the presence of SDS (26) (results not shown) and fluorography (6). These results indicate that diamide does not interfere with the interaction of galactose oxidase with the cell surface either quantitatively or qualitatively. In addition, the results from the studies with both WGA and galactose oxidase suggest that the interaction of these high-affinity attachment surfaces with their cell surfaces receptors is insufficient to maintain a stable cell attachment. Apparently some additonal cell-dependent stabilization is required for cell attachment and will be further discussed.

IRREVERSIBILITY OF CELL ADHESION AFTER THE INITIAL ATTACHMENT: The importance of the cell-dependent stabilization of cell attachment was confirmed by the following studies. Addition of diamide, after the initiation of an adhesion

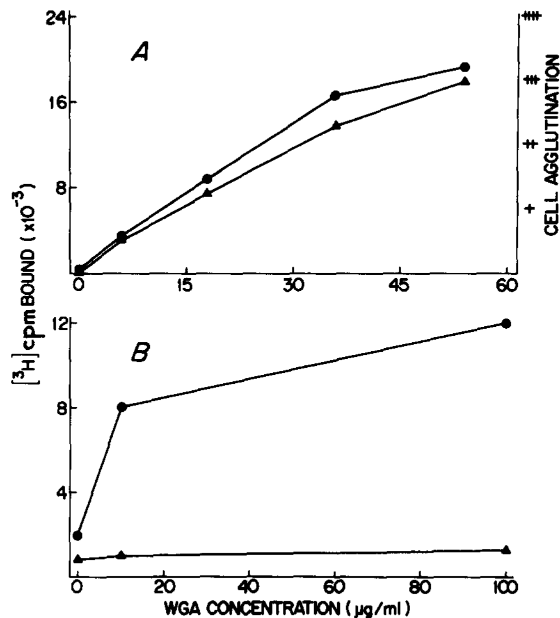

FIGURE 3 Comparison of the effects of diamide on (A) binding of soluble WGA to NIL cells and WGA-induced agglutination and (B) adhesion of NIL cells to WGA-coated plates. (A) Confluent cultures of NIL cells were suspended with trypsin and washed with soybean trypsin inhibitor as described in Materials and Methods, then suspended in Salt/Pi buffer containing BSA (100 μg/ml) at a final concentration of 5 × 10^6 cells/ml). Aliquots of cell suspension (0.5 ml or 2.5 × 10^6 cells/aliquot) were transferred into BSA-coated glass tubes, followed by either chitin oligosaccharide (a specific hapten inhibitor of WGA [10 mM final concentration]), diamide (500 μM final concentration), or nothing. [^{3}H]WGA (see Materials and Methods) and Salt/Pi buffer were added to a final volume of 1.0 ml and a concentration range of 0–60 μg WGA/ml. After a 30-min incubation at room temperature, with occasional mixing, the agglutination of the cells was recorded. Cell agglutination ranged from − (not detectable) to +++ (maximum). The cells were washed three times wiht Salt/Pi by centrifugation. The washed cell pellets were dissolved with 1% SDS in 0.5 M NaOH, transferred to liquid scintillation vials, and counted. WGA binding in the presence of chitin oligosaccharide never exceeded 3% of binding in the absence of inhibitor. ●, [^{3}H]WGA binding; ▲, [^{3}H]WGA binding in the presence of 500 μM diamide. (B) Hydrophobic plastic wells were coated with WGA at 0, 1, 10, and 100 μg/ml as described in Materials and Methods. The adhesion surfaces were then washed with Salt/Pi buffer and again incubated with Salt/Pi buffer containing BSA (100 μg/ml) for 60 min. [^{3}H]proline-labeled NIL cells were trypsinized and suspended in BSA-Salt/Pi as described in Materials and Methods for preparation of cells for adhesion assay. Labeled cells were incubated on the adhesion surface with and without 500 μM diamide for 60 min at room temperature. ●, Cell adhesion in the absence of diamide; ▲, cell adhesion in the presence of 500 μM diamide.

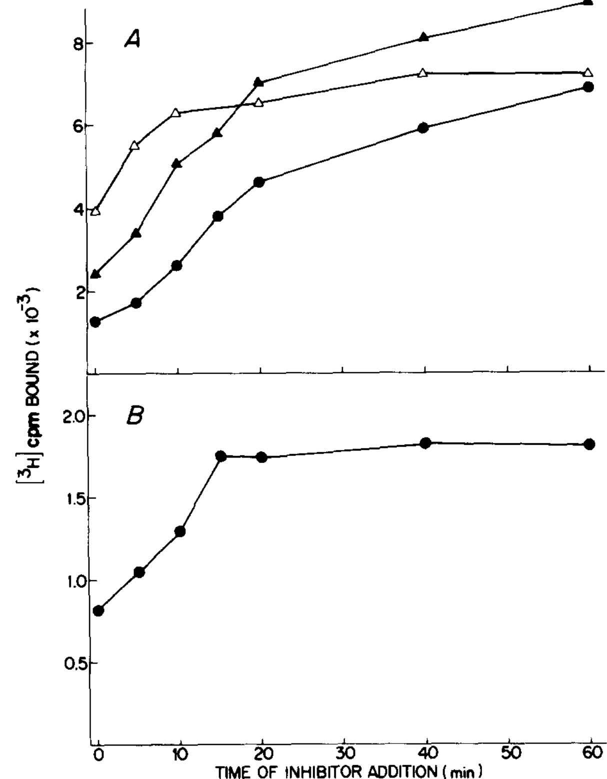

FIGURE 4 (A) Kinetics of diamide and N-acetylgalactosamine-induced inhibition of NIL cell attachment to fibronectin- and SBA-coated adhesion surfaces. Hydrophobic plastic wells were coated with either fibronectin (10 μg/ml) or SBA (10 μg/ml) as described in Materials and Methods. The adhesion surfaces then received equal numbers of [^{3}H]proline-labeled cells, prepared for cell adhesion experiments as described in Materials and Methods. At various time-points after the initial cell addition, an equal volume of 1 mM diamide in Salt/Pi buffer or 50 mM N-acetylgalactosamine in Salt/Pi was added to duplicate wells. The cells were incubated for 60 min at room temperature and adherent cells were counted for radioactivity. ○, Effect of diamide addition on cell adhesion to fibronectin surfaces; ▲, effect of diamide addition on cell adhesion to SBA surfaces; △, effect of N-acetylgalactosamine addition on cell adhesion to SBA surfaces. Each point represents the average of two separate determinations. (B) Kinetics of galactose-induced inhibition of NIL cell attachment to galactose oxidase–coated adhesion surfaces. In an experiment separate from that shown in A, plastic wells were coated with galactose oxidase (10 U/ml) as described in Materials and Methods and then incubated with [^{3}H]proline-labeled NIL cells. As described in A, 50 mM galactose in Salt/Pi was added at various times after the start of the incubation, and all wells were washed and counted for radioactivity after 60 min incubation at room temperature.

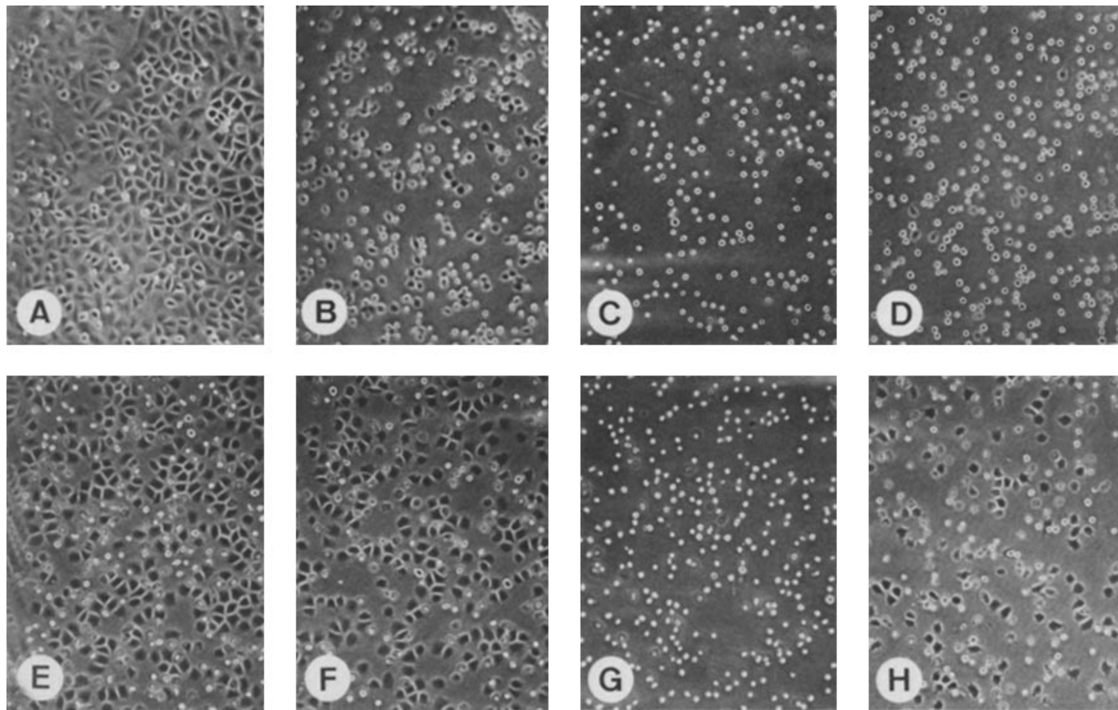

FIGURE 5 NIL cell spreading on fibronectin, neuraminidase, Con A, PNA, and commercial cell culture adhesive surfaces. Confluent cultures of NIL cells were washed with Salt/Pi buffer and then incubated with Salt/Pi buffer or buffer containing sialidase (*Vibrio cholerae*, 10 U/ml, pH 7.0) for 40 min at room temperature. The plates of cells were then extensively washed with Salt/Pi buffer and then suspended by trypsinization as usual. The two cell suspensions were adjusted to the same cell concentration and applied to the adhesion surfaces. After a 60-min incubation period, the nonadherent cells were washed off and the adherent cells photographed through a phase-contrast microscope, × 100 magnification. The protein-coated adhesion surfaces were prepared as described in Materials and Methods under Cell Spreading. (*A*) NIL cells on fibronectin surface. (*B*) NIL cells on sialidase (*Clostridium perfringens*) surface. (*C*) NIL cells on plastic tissue-culture plate (Falcon Labware). (*D*) Sialidase-treated NIL cells on plastic tissue-culture plates. (*E*) NIL cells on Con A surface. (*F*) Sialidase-treated NIL cells on Con A surface. (*G*) NIL cells on PNA surface. (*H*) Sialidase-treated NIL cells on PNA surface.

assay to fibronection- or SBA-coated plates, was progressively less effective in inhibiting cell attachment (Fig. 4*A*). In similar experiments, added GalNAc, a specific inhibitor of SBA-induced cell attachment (Fig. 4*A*), and added galactose, a specific inhibitor of cell attachment induced by galactose oxidase (Fig. 4*B*), also lost their ability to inhibit cell attachment if not present from the very beginning of the attachment assay. The adhesion reactions rapidly became insensitive to these inhibitors after active contact formation with the affinity surface, suggesting that some secondary attachment process, possibly cell spreading or secretion of substrate attachment material (8) follows the initial stimulation by the adhesion surface. Lectin-type and fibronectin-type proteins in cell attachment may act primarily as stimulators of a cell-dependent attachment response and not as the primary adhesion force.

Possible Mechanism for Stimulation of Cell Spreading

During our investigation of the capabilities of various lectins in modulating cell attachment to coated surfaces, we observed that lectins with poor or moderate cell-attaching potential, such as PNA or *Lotus tetragonolobus*[2] lectin, were also inadequate in inducing cell spreading. Cells attached to Con A–, SBA-, or fibronectin-coated surfaces spread during a 60-min adhesion experiment (Fig. 5), whereas PNA required 4 or 5 h of incubation for cells to spread. Cells attached to sialidase-coated surfaces spread quite readily in 60 min but not as well as on fibronectin surfaces (Fig. 5). In contrast, the low number of cells that bound to BSA-coated surfaces did not spread, even after addition of 10% fetal calf serum.

Enzymatic digestion of NIL cells with sialidase exposes at least two cell surface glycoprotein receptors for PNA termed GP37 and GP100.[2] The use of sialidase-treated cells in a cell adhesion assay increased the number of cells bound to a PNA-coated surface to 140% of control PNA surfaces receiving cells that were not treated with sialidase. In addition, many of the attached cells began to spread during a 60-min incubation period (Fig. 5). Sialidase treatment did not alter cell spreading on either plastic cell culture plates or Con A coated plates. Therefore, the ability of an adhesion surface to induce cell attachment and spreading requires one or both of the following conditions present on the cell surface: (*a*) a minimal number or density of cell surface receptors, and/or (*b*) a specific receptor capable of inducing cell spreading, such as GP37 or GP100.

The valency or density of binding molecules on the adhesion surface may also affect cell spreading. To test this possibility, native (tetravalent) Con A, succinyl (divalent) Con A, and monovalent Con A were coated on plates and incubated with cells. In the range of 1–50 μg of lectin/ml used to coat the plates, Con A, succinyl Con A, and monovalent Con A all induced cell attachment. Succinyl Con A and monovalent Con A were clearly less effective than Con A in inducing stable cell attachments even at high lectin concentrations (50 μg/ml) (Fig.

[2] Carter, W. G., and S. Hakomori. Unpublished results.

6); however, all three lectin surfaces were more effective than fibronectin (fibronectin control not shown). A probable explanation for the decreased number of attached cells was observed. Cells attached to tetravalent Con A at a concentration range of 1–50 μg of lectin/ml were all well spread after a 60-min incubation period (Fig. 7). In contrast, cells attached to either monovalent or divalent Con A at the same lectin concentration range did not spread nearly as much in a 60-min incubation period (Fig. 7) and required 2–3 h of incubation before spreading started. The poorly spread cells detached much more readily than well-spread cells, thus accounting for the decreased ability of succinyl and monovalent Con A to attach cells stably. In further studies, succinyl Con A was cross-linked, using 1,3-diaminopropane and water-soluble carbodiimide (see Materials and Methods for details). The resulting cross-linked polyvalent succinyl Con A, both attached and spread cells, as did tetravalent Con A (Fig. 7). Further modification of the cross-linked polyvalent succinyl Con A with succinic anhydride did not reduce the cell-spreading capabilities (results not shown), suggesting that the increased negative charge of succinyl Con A was not the cause of the decreased cell spreading. Similarly, succinylation of fibronectin did not inhibit its ability to cause cell spreading. Previous studies (16) have indicated that tetravalent and succinyl Con A have similar affinities for low molecular weight haptens. However, we observed that succinyl Con A coated on plastic had a reduced ability to bind soluble, high molecular weight glycoprotein receptors, as compared with tetravalent Con A (Table II). Cross-linking of the succinyl Con A recovered most of the ability to bind glycoproteins. The failure of succinyl Con A to bind glycoproteins was not attributable to reduced lectin quantities on the plastic surface, because similar quantities of tetravalent and divalent Con A adsorb to the plastic surface (Table II). Similarly, the succinyl Con A was active, as indicated by its ability to attach cells. In addition, cross-linking of succinyl Con A restored its ability to stimulate cell spreading and to bind glycoproteins. Thus, a minimal number of cell surface receptors, as well as a minlaeqaffinity of those receptors for the adhesion surface, is required for effective cell spreading on lectin-coated surfaces. It is suggested that cross-linking of the surface receptors may be involved in initiating cell spreading.

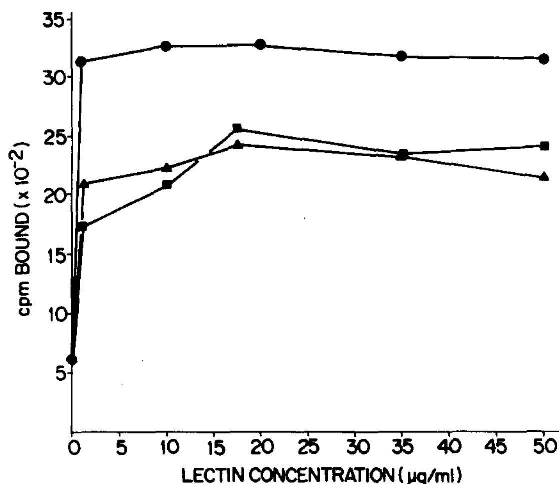

FIGURE 6 Attachment of NIL cells to adhesive surfaces prepared from different concentrations of Con A, succinyl Con A, and monovalent Con A. Solutions of Con A, succinyl Con A, and monovalent Con A were prepared at 1, 10, 20, 35, and 50 μg protein/ml Salt/Pi and adsorbed on microtiter wells at the concentrations indicated in 50 μl/well for 3 h at room temperature. The wells were washed three times with Salt/Pi; then 75 μl of BSA-Salt/Pi was added, and incubation continued for an additional 60 min. NIL cells were metabolically labeled with [^{3}H]proline, suspended by trypsinization as described in Materials and Methods, and then added to the lectin-coated wells. The cells were incubated 60 min at room temperature and washed; the adherent cells were then counted for radioactivity. Each point represents duplicate determinations. ●, Con A; ■, succinyl Con A; ▲, monovalent Con A. The experiment was repeated three times with similar results.

The ability of an adhesion surface to induce cell spreading is a function of the binding specificity of the adhesion protein. In Fig. 7 *G* and *H* we see that sialic acid, which is a poor inhibitor of cell adhesion induced by *Clostridium perfringens* sialidase (35, 40), is also a poor inhibitor of cell spreading. In contrast, however, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid, a potent sialidase inhibitor (32), is a good inhibitor of cell spreading.

DISCUSSION

To gain insight into the mechanism of fibronectin-mediated adhesion and spreading, we compared the adhesion-promoting activities of several lectins that possess cell surface affinity and might therefore promote cell attachment. We also observed that glycosidases, when adsorbed on hydrophobic surfaces, are highly effective in promoting cell adhesion (see the preceding paper [40]). Therefore, it appeared that proteins having specific affinity for various cell surface receptors can effectively mediate cell adhesion when adsorbed as a polyvalent monolayer on a hydrophobic surface.

Comparison of Cell attachment on Lectin-type and Fibronectin-type Adhesion Surfaces

We have made a comparison of cell attachment to fibronectin-, glycosidase-, lectin-, and galactose oxidase-coated surfaces, with the following conclusions:

(*a*) A kinetic study of cell attachment to fibronectin- and glycosidase-coated surfaces detected an initial lag period (up to ~15 min) and thereafter a rather rapid rise in the number of attached cells for ~60 min at 25°C. Therefore, early phases in cell attachment to fibronectin and glycosidases involve and depend on relatively slow, active cell events. In contrast, kinetic analysis of lectin- and galactose oxidase-induced cell attachment did not detect an initial lag period, and cell attachment was essentially complete in 30 min. The lectin-induced cell attachment required less time and therefore fewer active cell events during the initial stages of attachment than were observed for fibronectin- and glycosidase-coated surfaces.

(*b*) Drugs, metabolic inhibitors, and chemical modifying reagents were far more effective in inhibiting cell attachment when applied to the cells rather than to the adhesion surfaces. Factors that were tested and found to inhibit cell attachment, by their effects on the cells, can be classified into two categories: those that inhibit cell attachment to both fibronectin and glycosidases as well as lectins (NEM, reduced temperature, diamide) and those that efficiently inhibit only fibronectin- and glycosidase-modulated attachment (sodium azide, EDTA, cytochalasin B). Sulfhydryl-blocking agents like NEM have previously been reported by Grinnell (14) to be potent inhibitors of cell attachment to serum-coated surfaces. In our study, diamide, a mild sulfhydryl-oxidizing agent that has previously been reported to inhibit or dissociate microtubule assemblies

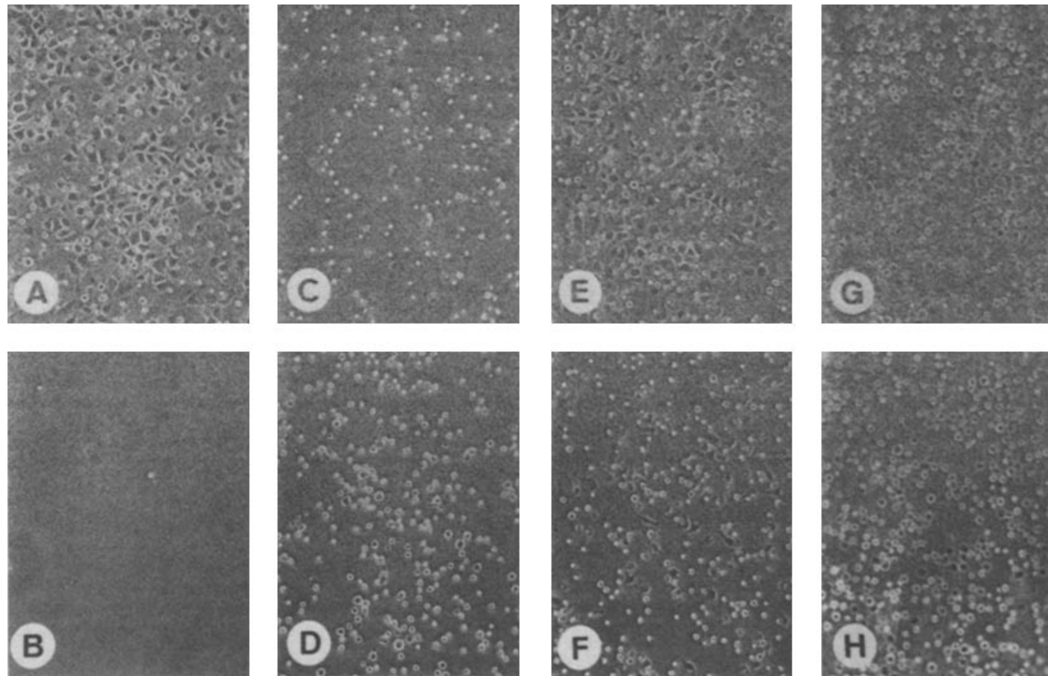

FIGURE 7 The spreading of BHK cells on monovalent, divalent, and tetravalent Con A, cross-linked succinyl Con A, sialidase, and BSA adhesion surfaces. The adhesion surfaces were prepared as usual, using protein solutions at 10 μg/ml unless otherwise indicated. Confluent cultures of BHK cells were suspended as usual by trypsinization and applied to the adhesion surfaces, incubated for 60 min at room temperature, rinsed with Salt/Pi buffer, and photographed through a phase-contrast microscope, × 100 magnification. To facilitate the comparison of the effects of *N*-acetylneuraminic acid and 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid on spreading, the wells were photographed without washing off the nonadherent cells (in the case of the deoxyneuraminic acid, >50% of the cells do not attach). (*A*) Native Con A, tetravalent. (*B*) BSA. (*C*) Monovalent Con A. (*D*) Succinyl Con A, divalent. (*E*) Cross-linked succinyl Con A, polyvalent. (*F*) Succinyl Con A, divalent (50 μg/ml used for coating). (*G*) *Clostridium perfringens* sialidase in the presence of 10 mM *N*-acetylneuraminic acid. (*H*) *Clostridium perfringens* sialidase in the presence of 1 mM 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid.

TABLE II

Binding of Glycoproteins by Con A and Con A Derivatives Adsorbed on Plastic Surfaces *

Protein Surface	α-Methylmannoside (50 mM) −	α-Methylmannoside (50 mM) +	Glycoprotein specifically bound	Lectin adsorbed‡	Cell attachment§	Cell spreading§
	cpm bound/plate		*ng/plate*	*μg/plate*		
Con A	5,555	822	315	1.1	+++	+++
Succinyl Con A	700	585	8	0.9	++	−
Cross-linked succinyl Con A¶	2,840	638	148	ND	++	++
Cross-linked succinyl Con A (resuccinylated)¶	2,100	660	96	ND	++	++
BSA	307	302	0	ND	−	−

* Con A and Con A derivatives were adsorbed on petri dishes (3.5 × 15 mm, #1008, Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) in 1 ml Salt/Pi buffer at a concentration of 10 μg protein/ml for 3 h at room temperature, washed with Salt/Pi and incubated with BSA-Salt/Pi for 60 min. [^{14}C]*Dolichos biflorus* lectin (150,000 cpm/10 μg protein), a high-mannose-type glycoprotein that has previously been shown to be specifically bound by Con A (7), was dissolved in BSA-Salt/Pi or BSA-Salt/Pi containing 50 mM α-methylmannoside at a final concentration of 20 μg/ml. 500 μl of the diluted glycoprotein solution was added to each plate and incubated for 30 min on a gyrating mixer. Each plate was washed three times with Salt/Pi, and the adherent proteins were dissolved in 500 μl of 1% SDS in 0.5 M NaOH and counted. *ND*, no determination.

‡ Determined by quantitation of [^{125}I]Con A (1.23 × 10^6 cpm/50 μg protein) and [^{125}I]succinyl Con A (1.01 × 10^6 cpm/50 μg protein) adsorbed to petri dishes as described above.

§ Qualitative presentation of adhesion and spreading results obtained in Fig. 6 and 7, for comparison.

¶ See Materials and Methods for preparation.

(37), was a potent inhibitor of cell attachment on all adhesion surfaces tested. The inhibitory effects induced by sulfhydryl-blocking and -oxidizing agents were not observed for sulfhydryl-reducing agents. Dithiothreitol (1–10 mM) and glutathione (5 mM) had no inhibitory effect on fibronectin- or lectin-induced cell attachment. This observation is surprising in light of previous reports that dithiothreitol is capable of causing release of fibronectin from the cell surface (1).

(*c*) Soluble sialidase and fibronectin both exhibited low or negligible affinity for the cell surface, either in direct-binding studies or adhesion-competition studies. In contrast, soluble lectins exhibit both high affinity and specificity for carbohydrate structures on the cell surface as detected by direct-binding studies and adhesion-competition studies. Despite these differences, lectins, glycosidases, and fibronectin, when coated on hydrophobic surfaces, can all induce cell attachment and spreading. Lectins can be clearly distinguished from glycosidases and fibronectin in terms of their ability to induce an

increased rate of cell attachment and decreased sensitivity to various inhibitors. It has been suggested that in adhesive reactions, contact formation from cell to cell and cell to substratum is modulated by both cell surface and cytoskeletal components (8). Grinnell (14) has shown that the requirement for divalent cations and the effect of cytochalasin B in serum-mediated cell attachment can be overcome by centrifugation of cells upon the adhesion surface, suggesting that cytochalasin B and the lack of divalent cations impair contact formation from the cell to the substratum. From these considerations, fibronectin- and glycosidase-mediated attachment is strongly dependent on cellular functions involved in the attachment process, whereas lectin-mediated reactions are less dependent on cellular activity, at least during initial stages of attachment. The simplest explanation for the differences between lectin- and fibronectin/glycosidase-type cell adhesions is based on the apparent lower affinity of fibronectin and glycosidase for the cell surface. Therefore, multiple low-affinity contacts functioning in a cooperative manner between the cell and the adhesion surface are needed before the cells can attach. In contrast, lectins, having a high cell affinity, are less dependent on active contract formations that require energy-dependent cell activities and therefore exhibit no lag period in cell attachment. The kinetic, inhibition, and direct-binding studies discussed above are all in agreement with this interpretation.

It has been suggested that fibronectin undergoes a conformational change upon adsorption on hydrophobic surface, and may even have a high cell affinity (8, 14) after adsorption. However, from the results discussed above, the fibronectin surface may be a low-affinity surface comparable to glycosidase surface and quite different from lectin-type high-affinity protein layers on plastic. Also, a high cell-attaching activity without high cell affinity in solution is not a specific property of fibronectin alone, because sialidase behaves in the same way. Therefore, it is not necessary to assume a conformational change upon adsorption on a hydrophobic surface, because the polyvalency of fibronectin and sialidase surfaces would seem sufficient for high cell-attaching activity. Recent studies by Grinnell (15) also support the idea that valency is a key requirement in fibronectin binding to cells. Adhesion on fibronectin and glycosidase surfaces is strongly dependent on cell activities, as discussed above. However, this does not mean that fibronectin and glycosidases would not be suitable candidates as adhesion molecules in vivo. On the contrary, the apparent low affinity of fibronectin and glycosidases for the cell surface, with the resulting requirement for multivalency and cell-dependent attachment activities, may have a closer resemblance to the type of adhesion described for mammalian cells in vivo (34, 44) than the high-affinity, lectin-mediated reactions studied.

Reversibility of Cell Attachment on Lectin-type and Fibronectin-type Adhesion Surfaces

The effective inhibition of cell attachment on various adhesion surfaces by various drugs and haptens was rapidly lost unless the inhibitors were present from the beginning of the attachment assay. In contrast to the differences observed in the kinetics of cell attachment on lectin and fibronectin surfaces, the kinetics of inhibition of cell attachment were remarkably similar on both lectin-type and fibronectin-type adhesion surfaces. These results suggest that cell attachment on both types of adhesion surfaces may stimulate a secondary cell-dependent attachment response that progresses with similar kinetics on both types of surfaces. The biochemical mechanism of this cell-dependent attachment response is not defined at this time. However, cell spreading with the formation of filopodia and excretion of substratum adhesion material (8, 9) are processes that are probably either continuous with or subsequent to cell-dependent attachment. At the present time we cannot say that either process is directly responsible for the cell-dependent attachment.

The Adhesion Surfaces as a Stimulator of a Cell-dependent Attachment

Diamide, which was found to inhibit cell attachment on all adhesion surfaces tested, failed to inhibit binding of soluble WGA to the cell surface or to inhibit cell agglutination induced by WGA. In addition, diamide failed to quantitatively or qualitatively alter the interactions of galactose oxidase with the cell surface as detected by agglutination of cells or oxidation of cell surface receptors. The studies suggest that even high-affinity adhesion surfaces such as WGA and galactose oxidase are incapable of attaching cells stably without the aid of a cell-mediated attachment process, which can be inhibited by diamide. We further suggest that the primary role of the adhesion surface is to stimulate a cell-dependent attachment response. This stimulation phenomenon may partially explain the effectiveness of multivalent enzyme surface, because the attachment would occur even if the cell surface receptors were slowly hydrolyzed or altered by the adhesion surface itself. For example, as previously published (19), the agglutination of sialidase-treated erythrocytes by soluble galactose oxidase is a transient process. The cells rapidly agglutinate after addition of the galactose oxidase; however, the aggregates begin to dissociate after a few minutes, presumably as the membrane-associated substrate is converted to the oxidized form. In sharp contrast, cell attachment to galactose oxidase–coated surfaces does not reverse itself even hours after the cells have attached, indicating the initiation of cell-dependent attachment response.

Possible Mechanisms for Stimulation of Cell Attachment and Spreading

The progression from an initial, reversible adhesion phase to a second, more stable attachment was accompanied by cell spreading. We have concluded that the initial attachment of cells to a particular attachment surface precedes cell spreading. For example, PNA surfaces were found to stimulate only a moderate cell attachment reaction with no cell spreading. However, after sialidase treatment of the cells, the ability of cells both to attach and spread on PNA-coated surfaces increased dramatically. Therefore, either a minimal number or density of cell surface receptors or a specific cell surface receptor is required for cell spreading to occur on PNA-coated surfaces.

The quantity of cells that attaches to lectins, enzymes, and fibronectin is dependent on the density of protein molecules immobilized on the adhesion surface (20, 40, 43). However, in the concentration range of 1–50 μg of Con A/ml used to coat plates, all attached cells spread very rapidly. So the density of Con A molecules on the adhesion surface does not greatly affect cell spreading once cell attachment has occurred. However, the valency of the immobilized lectins greatly affects the cell spreading but not cell attachment, as evidenced by the fact that tetravalent Con A or cross-linked, divalent Con A, but not divalent or monovalent Con A, were effective in inducing cell

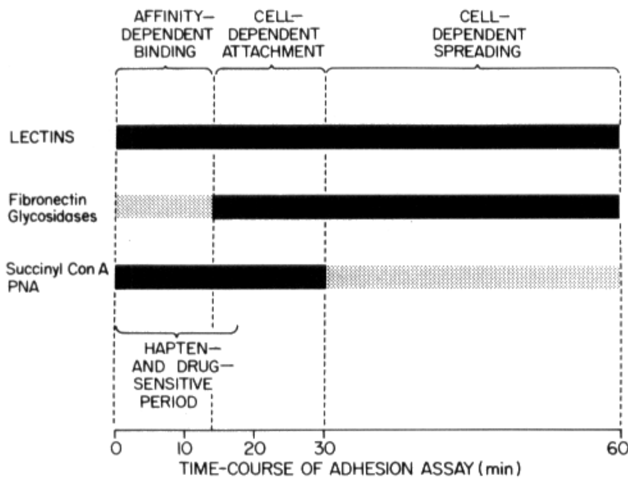

FIGURE 8 Schematic presentation of the sequence of events involved in cell attachment and spreading on lectin-type and fibronectin-type adhesion surfaces. Black bars represent periods of major cell attachment or spreading on various adhesion surfaces. Dotted bars represent periods of minor or barely detectable activity. Cell attachment on most high-affinity, lectin-type adhesion surfaces is rapid. Specific inhibition of the cell attachment, using hapten sugars, is effective only during the initial attachment period, suggesting that it is "affinity-dependent binding." Cell attachment on fibronectin-type surfaces proceeds with an initial lag period, followed by cell attachment that can be inhibited by drugs affecting cellular function (like diamide). Cell attachment on lectin-type and fibronectin-type surfaces becomes insensitive to drug inhibition at the same time, suggesting that "cell-dependent attachment" is stimulated by both surfaces at similar rates. At some time-point after stimulation of cell-dependent attachment, drugs are no longer effective inhibitors of cell attachment. It is not possible to say, at this time, whether the initial drug-sensitive period is attributable to a single cellular function occurring at the end of the drug-sensitive period or whether the sensitive cellular functions are functioning throughout the drug-sensitive period. Cell spreading is apparent on most lectin-type and fibronectin-type surfaces after cell-dependent attachment. However, some lectins with reduced numbers of cell surface receptors (PNA) and/or reduced binding valency (succinyl Con A) stimulate cell-dependent attachment but not "cell-dependent spreading."

spreading. Because succinylation of Con A does not reduce the ability of the lectin to interact with its cell surface receptors (16) or to attach cells, or greatly reduce the association constants for low molecular weight haptens (3, 4, 16), the decreased spreading we observed may result from the decreased valency of the adhesive protein. Saturating the adhesion surfaces with either divalent or monovalent Con A only partially restored the cell spreading induced by even low quantities of tetravalent or cross-linked succinyl Con A. Studies on the ability of the lectin surfaces to bind soluble glycoproteins suggest that decreasing the lectin valency reduces the glycoprotein binding ability of the lectin. These combined results suggest that reduction in lectin valency causes a decrease in the number of stable interactions with membrane glycoproteins, with a resulting decrease in cell spreading but no major decrease in cell attachment. Thus, decreased cell spreading may result from the (*a*) decreased numbers of cross-linked lectin receptors on the membrane or (*b*) from a total decrease in the number of receptors interacting with the lectin-coated surface. Apparently this decrease is not adequate to alter drastically the cell-dependent attachment, but is sufficient to inhibit cell spreading.

Our studies using tetravalent, divalent, monovalent, and cross-linked succinyl Con A suggest that cross-linking of cell surface lectin receptors accelerates the rate of spreading. Cell attachment without cross-linking, as carried out by monovalent and divalent Con A and PNA, is insufficient to accelerate cell spreading. A number of reports have proposed that cross-linking of cell surface receptors is a signal for a transmembrane attachment to the cytoskeleton (2, 23, 31, 36, 41). The coupling of the cytoskeleton to the membrane may facilitate the cellular recognition observed in cell attachment and spreading.

In summary (Fig. 8), we have concluded that surfaces coated with high-affinity, lectin-type proteins can induce a more rapid initial rate of cell attachment than low-affinity, fibronectin-type adhesion surfaces. However, both adhesion surfaces stimulate the cells to form their own cell-dependent attachments with the substratum, and these attachments are independent of the stimulating adhesion surface. The differences in the initial rate of cell attachment to the lectin-type and fibronectin-type adhesion surfaces most likely are attributable to the differences in affinity the two surfaces possess for the cell membrane. The stimulation of cells to form their own attachment processes may precede or simply be the beginning of cell spreading. At the present time, much more work is required to delineate further the cell-dependent attachment and spreading response. However, it is reasonable to assume that the cell attachments induced by PNA or succinyl Con A are adequate to induce the cell-dependent attachment responses (because both are inhibited by diamide) but inadequate to stimulate the cell-spreading response. Thus, the cell-dependent attachment mechanism is independent from and precedes cell spreading.

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