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Adhesion of Cultured Fibroblasts to Insoluble Analogues of Cell-Surface Carbohydrates

(SV40-transformed 3T3 cells/sugar-derivatized Sephadex beads)

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ABSTRACT **Animal cells are coated with complex carbohydrates. Insoluble analogues of these substances were prepared by coupling monosaccharides to Sephadex beads (crosslinked dextran), and the interactions between these derivatives and three established cell lines were studied. A virally transformed fibroblast, simian virus 40-transformed 3T3 cells, adhered to the beads derivatized with D-galactose, but did not adhere to the corresponding beads derivatized with D-glucose or *N*-acetyl-D-glucosamine. Cells that adhered to the galactose-beads appeared to initiate a nucleation process in that they became more adhesive towards the cells in suspension, leading to the formation of large aggregates containing both cells and galactose-beads. The results suggest that specific carbohydrates are involved in the processes of cell recognition or cell adhesion, or both.**

The surfaces of eukaryotic cells are thought to play a dominant role in various complex physiological phenomena, including cell recognition and adhesion, contact inhibition of motility and of growth, morphogenesis, and metastasis. While the molecular mechanisms underlying these phenomena are unknown, it has been proposed that the surface glycocalyx, containing various complex carbohydrates, is involved in at least certain of these processes, such as cellular adhesion (1). This hypothesis was tested in the present studies by observation of the responses of cultured fibroblasts to synthetic, insoluble analogues of cell-surface carbohydrates. The analogues were synthesized by covalently linking monosaccharides to Sephadex beads. Specificity for the glycose moiety was exhibited by one of the cell lines, simian virus (SV) 40-transformed 3T3 cells, which formed large aggregates only with the beads derivatized with D-galactose. These results provide evidence for the role of carbohydrates in the processes of cell recognition or adhesion, or both.

METHODS

Ligands. Several procedures are available for linking monosaccharides or other ligands to insoluble matrices such as polyacrylamide, agarose, or crosslinked dextran (Sephadex) (2–4). Most of these procedures use a sequence of reactions to build an appropriate "spacer-arm" or linking group on the matrix. The desired ligands, such as glycosides, are coupled to the "spacer-arm" at the last step. We have found these methods to be unsatisfactory, primarily because the initial synthetic steps proceed in less than quantitative yield. The resulting matrices thus contain byproducts, such as ionic groups, that interact with or adsorb proteins nonspecifically. In the present studies, a molecule containing both the ligand and the "spacer-arm" is coupled to the activated polymer at the *last* step in the reaction sequence.* A similar approach has been used to couple *N*-acetylglucosamine (GlcNAc) to polysaccharides (5).

The ligands* were synthesized by methods reported elsewhere (ref. 6; manuscript in preparation). Each ligand was isolated in crystalline form, and purity was established by elemental analysis, optical rotation, nuclear magnetic resonance, thin-layer chromatography, and paper electrophoresis. Since hydrolases that cleave *O*-glycosides are present in serum and in cells, the ligands were synthesized as the thioglycosides (thioglycosides are not hydrolyzed by glycosidases). All of the ligands used in this study are described in Fig. 1.

Cyanogen Bromide Activation of Sephadex. Sephadex G-25 beads, superfine (10–40 μm in diameter, Pharmacia, Inc.), were permitted to swell in water for at least 6 hr. A 5-ml portion of the beads was suspended in a solution of 40 ml of water containing 1 g of cyanogen bromide (4). The suspension was stirred for 6 min at room temperature (23°), while the pH was maintained between 9.5 and 10.0 with 1 N NaOH. The mixture was then rapidly filtered through a coarse fritted-glass funnel, and the beads were washed at 4° with 1500 ml of 0.1 N sodium bicarbonate followed by 1500 ml of 0.1 M sodium borate buffer (pH 9.5). The filtration and washing procedures were effected in 6–8 min.

Coupling of Ligand to CNBr-activated Sephadex. CNBr-activated beads (5 ml) were added at 4° to 5 ml of 0.1 M sodium borate buffer (pH 9.5) containing 150 μmol of ligand. The suspension was gently shaken at 4° for 24 hr, and the beads were then filtered and washed over a 3-day period at room temperature with the following sequence of solutions: 0.1 M sodium borate buffer (pH 9.5), 3 liters; 0.1 M sodium bicarbonate, 6 liters; water, 500 ml; 0.1 M acetic acid, 6 liters; 1 M acetic acid, 1 liter; and 0.1 M acetic acid, 6 liters. The beads, designated as shown in Fig. 1, were stored in 0.1 M acetic acid at 4°.

Determination of Covalently Linked Ligand. Preliminary studies showed that the ligands could be removed from the beads with alkali (with negligible cleavage of the dextran),

Abbreviation: SV, simian virus; for other abbreviations see the legend to Fig. 1.

* In this paper, the compounds containing *both* the ligands and the "spacer-arms" are designated as *ligands*.

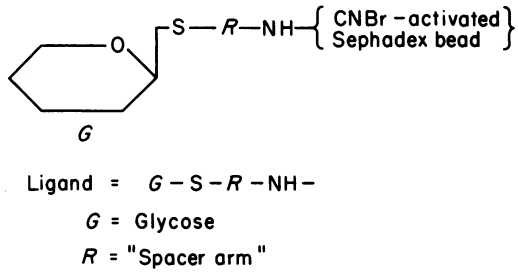

FIG. 1. General structure of derivatized Sephadex beads. The abbreviations and nomenclature used are the following (all sugars are D-glycopyranosides): *Gal-beads*, *G* = galactose, *R* = $(CH_2)_6$; *Glc-beads*, *G* = glucose, *R* = $(CH_2)_6$; *GlcNAc-beads*, *G* = *N*-acetylglucosamine, *R* = $(CH_2)_6$; *Gal-Phe-beads*, *G* = galactose, R = —⬡—NHCO—$(CH_2)_5$. *CNBr-beads*, which were used as controls, are Sephadex beads activated with CNBr and treated under the same conditions as those used in the coupling reactions but without ligands. As an additional control, *AmHex-beads* were used. These are CNBr-activated beads treated with 6-aminohexanol, giving beads linked to the —$(CH_2)_6$— "spacer-arm", i.e., HO—$(CH_2)_6$—NH-bead.

and kinetic experiments established the optimal time and temperature for complete hydrolysis. Packed derivatized beads (1.0 ml) were suspended in water to a final volume of 2.0 ml, and the suspension was gently shaken at 37° with 2.0 ml of 2 N NaOH. Aliquots (0.7 ml) were withdrawn at various intervals over a 24-hr period and added to 1 ml of 1 N acetic acid. The beads were removed by centrifugation, and the ligand content of the supernatant fluid was determined. For Gal-beads and Glc-beads (Fig. 1), the quantity of ligand was determined by a modified phenol–sulfuric acid method (7). For GlcNAc–beads, the GlcNAc was cleaved from the ligand with mercuric acetate (M. Krantz and Y. C. Lee, unpublished experiments) and the hexosamine content was determined by the Morgan–Elson method (8). In all cases, the corresponding ligand was used to obtain standard curves. Alkaline hydrolysis of the Gal– and Glc–beads appeared to be complete in 6–8 hr. The GlcNAc-ligand cleavage and analyses were kindly performed by Dr. Mark Krantz. Most of the experiments were performed with Gal– and Glc–beads containing 27 μmol of ligand per ml of packed beads or GlcNAc–beads containing 13–16 μmol of ligand per ml of packed beads. In the studies with the SV40/3T3 cells reported below, essentially the same results were obtained with five separate preparations of Gal–beads and three separate preparations of Glc–beads, each containing from 12 to 27 μmol of ligand per ml of packed beads. 6-Aminohexanol was also coupled to the beads under conditions identical with those used for synthesis of the glycoside beads. In every case, the beads were washed with the appropriate medium just before use.

Maintenance of Cells. Balb/c 3T3 cells (highly contact-inhibited, clone 3, designated 3T3) and SV40-transformed Balb/c 3T3 cells (9) (clone 22, designated SV40/3T3) were obtained from Dr. Stephen Roth. BHK-21 cells (10) were obtained from Dr. Ian MacPherson. Except as specified below, cell lines were grown in BHK-21 medium supplemented with 10% calf serum (both obtained from Gibco, Inc.; the serum was heat-inactivated at 57° for 30 min before use), 50 units/ml of penicillin, 50 μg/ml of streptomycin sulfate, and 1% Gibco 100× nonessential amino acids (Medium A). Cells were grown at 37° in Falcon tissue culture plastic petri plates in a water-saturated atmosphere of 5% CO_2–95% air and passed just before confluence. For passage, cell layers were washed twice with Ca^{++}- and Mg^{++}-free phosphate-buffered salts solution (PBS, Gibco, Inc.), treated for 3 min at 37° with 1 ml of 0.25% Gibco trypsin solution, and diluted with 9 ml of fresh growth medium; single-cell suspensions were obtained by repeated pipetting with a 10-ml pipette. Aliquots (1.0 ml) were then transferred to 177-cm^2 Falcon plates to permit growth. Unless indicated otherwise, suspensions of single cells used for adhesion experiments were obtained by trypsin dissociation of cell layers, that had just reached confluence.

Adhesion of Cells to Beads. Suspensions of single cells were obtained as described above, harvested by centrifugation, and resuspended in BHK-21 medium buffered with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; sigma) (pH 7.3) without serum or sodium bicarbonate (Medium B). Hanks' balanced salt solution buffered with 10 mM HEPES (pH 7.3), or BHK-21 medium (with or without nonessential amino acids) buffered with bicarbonate (pH 7.3) gave similar results. Cell suspensions were counted and examined in a hemocytometer and preparations were used only when the population consisted primarily of single cells (>95%). The results described below were obtained with more than 25 different preparations of SV40/3T3 cells.

In addition, cell suspensions obtained from cells at different stages of confluency were studied. Aliquots of SV40/3T3 cells were plated on Falcon tissue-culture dishes and permitted to grow for 7 days. Confluency was attained at about 5 days, after which the layers became superconfluent. Single-cell suspensions prepared each day were tested with the beads by the standard procedure. Each of the suspensions gave similar results, indicating that aggregate formation was independent of the stage of confluency of the cells in culture.

Adhesion experiments were conducted in 60-mm Falcon tissue-culture dishes coated with 2% agarose, to which neither the cells nor the beads adhered. Mixtures of cells (100,000) and beads (50,000) in final volumes of 3 ml of Medium B were added to the dishes and incubated at 37°. To obtain a uniform suspension, the mixtures were gently shaken before incubation. Adhesion was observed over a 2-hr period. The beads settled rapidly to the agarose, while the cells settled more slowly. Aggregates containing cells and beads (Figs. 2 and 3) were readily distinguished from mixtures of beads, single cells, and small-cell aggregates by gentle shaking of the plate on the microscope stage. This process resulted in resuspension of most of the single cells and small aggregates, leaving the cell-free beads and the cell–bead aggregates on the agarose.

It should be noted that during the course of these experiments, the supernatant fluids above the derivatized beads contained single cells and small aggregates. Formation of large aggregates (containing only cells) was prevented by use of low concentrations of single cells in the suspension, about 33,000 cells per ml, which minimizes cell–cell adhesion.

RESULTS

Adhesion of cells to derivatized Sephadex beads

After incubation of SV40/3T3 fibroblasts with Gal–beads for 30 min at 37°, large aggregates were formed containing

beads and cells. No such aggregates were observed in the presence of Sephadex itself, CNBr-beads, AmHex-beads (CNBr-activated beads treated with 6-aminohexanol), Glc-beads, or GlcNAc-beads, although a few single cells occasionally attached to some of these beads (Fig. 2). The formation of large aggregates between SV40/3T3 cells and Gal-beads over a 1-hr period is illustrated in Fig. 3. Within about 5 min, single cells begin to attach to the beads (*A*). As the incubation proceeds, more cells attach to the beads (*B*), and the process appears to become autocatalytic, in that the cells that are bound to the beads seem to become more "sticky," facilitating the binding of new cells to their surfaces (*C*), until very large aggregates are observed (*D*). Such aggregates were not observed with any of the other derivatized or control beads even after several hours of incubation, suggesting that the autocatalytic or nucleation process is initiated by the Gal-beads. Cells also attached to Gal-Phe-beads, but the aggregates were fewer and smaller in size, possibly because of steric interference with the benzene ring in the "spacer-arm." As indicated above, the formation of cell-bead aggregates was independent of the stage of confluency of the cells.

The process was temperature dependent, proceeding more rapidly at 37° than at room temperature, with no binding or aggregation being observed at 4°. However, incubations maintained at 4° for 1 hr formed aggregates at the normally observed rate when warmed to and maintained at 37°.

The Gal-beads gave different results with the three cell lines tested. 3T3 cells gave similar results to those obtained with the SV40/3T3 cells, except that the 3T3 cell-bead aggregates were smaller in size and fewer in number. By contrast, three separate preparations of BHK-21 cells failed to form aggregates with the Gal-beads, although occasional single cells attached to the Gal- and control beads.

Adhesion of SV40/3T3 cells to collagen

The formation of aggregates between SV40/3T3 cells and Gal-beads may mimic normal intercellular adhesion or may reflect some other phenomenon, such as binding of cells to collagen. Adhesion of blood platelets to collagen is inhibited by chlorpromazine (11). We found that SV40/3T3 cells also adhered to collagen and the process is inhibited by chlorpromazine. However, the adhesion of these cells to Gal-beads was not chlorpromazine-sensitive, suggesting that formation of cell-bead aggregates is not comparable with cell-collagen binding.

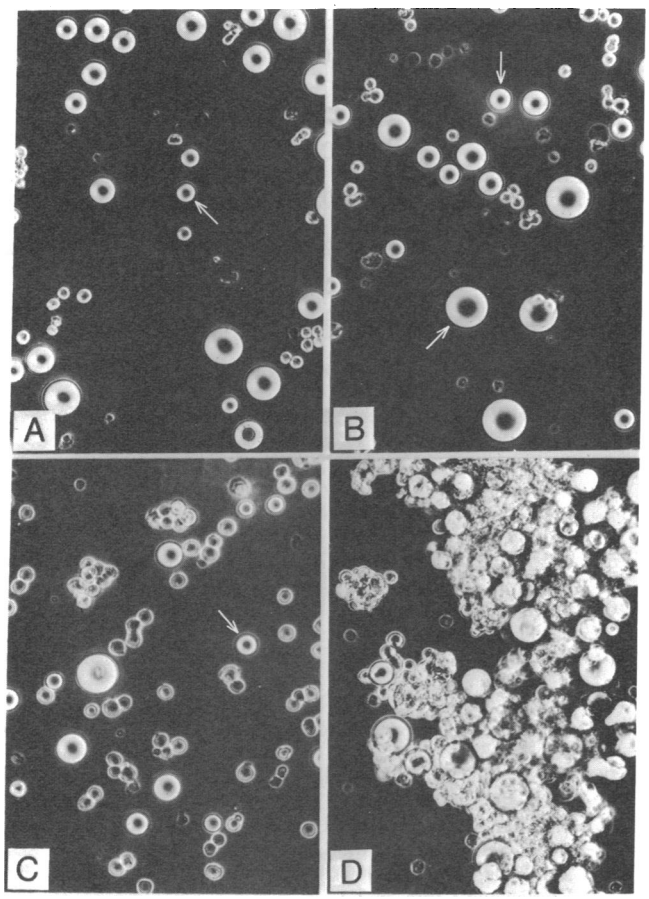

FIG. 2. Adhesion of SV40/3T3 cells to derivatized Sephadex beads. Cells were grown in Medium A. Suspensions of single cells were prepared from confluent monolayers (see *Methods*). Cells and beads were incubated at 37° for 30 min in Medium B, and examined by phase contrast microscopy (magnification, ×49), with the focus set slightly above the agarose gel. The diameter of the beads varied from 10 to 40 μm. *A*, CNBr-beads; *B*, Glc-beads; *C*, GlcNAc-beads; *D*, Gal-beads. Representative beads are indicated by the *arrows*.

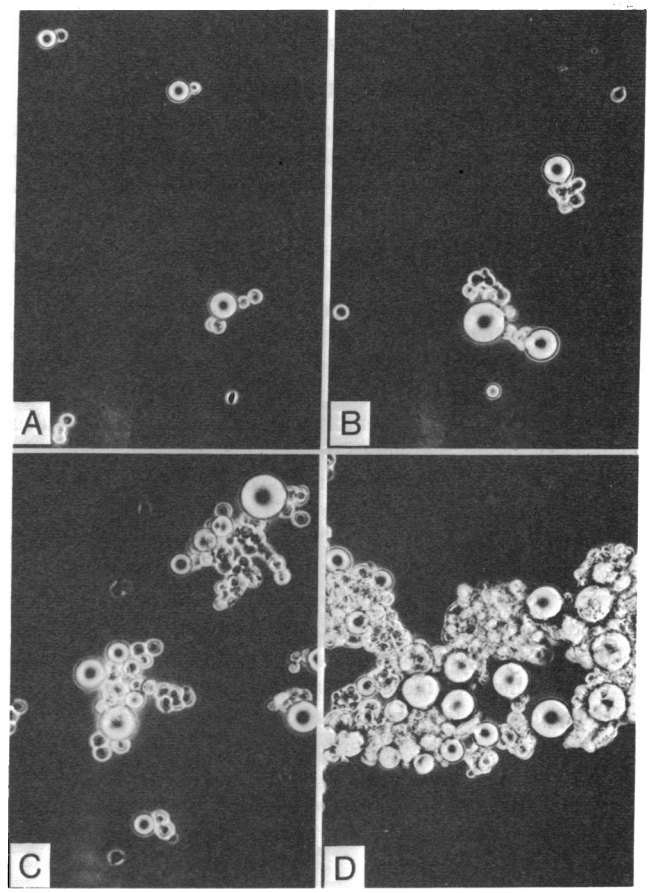

FIG. 3. Effect of time of incubation of SV40/3T3 cells with Gal-beads. The Gal-beads were incubated with SV40/3T3 cells at 37° under the standard conditions described in the *text*. The surfaces of agarose-coated Falcon plates were carefully examined by microscopy (×49 magnification), and the micrographs are representative of the course of aggregate formation. *A*, 5 min; *B*, 15 min; *C*, 30 min; *D*, 60 min.

Potential role of serum proteins

An alternative explanation for cell–bead adhesion is that galactose-binding proteins in the serum in the growth medium (such as antibodies directed against β-galactosyl groups) coat the cells, and that these interact with the galactosyl groups on the beads. The following series of experiments indicate that this is unlikely. (*a*) Attempts to prevent the interaction of the beads with cells, by "saturating" the Gal–beads with the presumed binding proteins (in complete growth medium), gave normal aggregate formation. (*b*) SV40/3T3 cells were grown for 24 generations (eight passages, 3 days each) in immunoglobulin-free serum (IPT fetal-calf serum, Gibco, Inc.) in place of calf serum, and gave the same results as did cells grown under the usual conditions. (*c*) While antibodies generally react almost as well at 4° as at 37° (12), the cells did not adhere to the Gal–beads at 4° (see above). (*d*) The Gal–beads did not adsorb more protein from the growth medium than did any of the nonreactive beads. (*e*) Direct attempts to detect Gal- or Glc- or GlcNAc-binding proteins in the growth medium gave negative results. In these experiments, the amino groups of the ligands (Fig. 1) were acetylated with [^{3}H]- and [^{14}C]acetic anhydride. The labeled ligands were then mixed with the growth medium and subjected to gel filtration chromatography; the protein fraction was unlabeled. The hypothetical serum proteins would also be expected to induce aggregate formation in the absence as well as the presence of beads, but this was not observed.

DISCUSSION

While the molecular mechanisms responsible for cell recognition and adhesion are not known, recent work suggests that carbohydrates are involved in these processes. Initial studies implicated cell-surface carbohydrates in the intercellular adhesion of mouse teratoma cells (13), a finding that led to the suggestion that intercellular adhesion may occur by the binding of surface complex carbohydrates to each other by hydrogen bonds, or by complex formation between the carbohydrates and the respective cell-surface glycosyltransferases (1). Evidence was then offered for the presence of cell-surface glycosyltransferases on embryonic cells (14), tissue culture cells (15), blood platelets (16, 17), and intestinal cells (18). Galactosyl residues may be involved in the adhesion of embryonic neural retina cells to each other (19), and of blood platelets to collagen (16, 17). A definitive series of experiments have shown that liver cells can recognize galactosyl end groups in glycoproteins (20). Cell recognition must be an integral part of more complex processes such as specific intercellular adhesion (21) and "sorting out" (22), but whether the same or different functional groups are involved in cell recognition and adhesion remains to be determined.

The results reported here show that SV40/3T3 cells can specifically distinguish β-galactosyl from β-glucosyl and *N*-acetyl-β-glucosaminyl end groups, and that they adhere to beads containing covalently linked β-galactosyl groups. In addition, cells that adhere to the beads act as a nucleus for cell–cell adhesion, leading to the formation of large mixed aggregates containing both cells and Gal–beads. The nucleation phenomenon implies that Gal–beads induce increased adhesive affinity of the cells for each other.

The carbohydrates that coat cell surfaces are of complex structure, and it is of interest to find that simple monosaccharide analogues can evoke such profound physiological responses. These observations, therefore, lead to the possibility that similar, insoluble analogues of cell-surface components may prove useful in selecting mutants and in studying even more complex physiological phenomena, such as cell motility and contact inhibition of growth.

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