Adhesion of Chicken Hepatocytes to Polyacrylamide Gels Derivatized with N-Acetylglucosamime*

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Complex carbohydrates on the surfaces of eukaryotic cells are thought to participate in a wide variety of cell-cell interactions. A model system has therefore been developed to study these processes. In the present experiments, the ability of chicken hepatocytes to recognize and adhere to sugars covalently linked to polyacrylamide gels was investigated.

The gels were synthesized by two methods. Type I gels were prepared from a copolymer of an active ester of acrylic acid (N-succinimidyl acrylate), acrylamide, and bisacrylamide. The "activated" polyacrylamide gel was then treated with the desired ligand containing an amino group, such as 6-aminohexyl O- or S-glycoside. Type II gels were formed by treating similar ligands with acryloyl chloride, followed by copolymerization of the resulting N-substituted acrylamide with acrylamide and N,N'-methylenebisacrylamide. These polyacrylamide derivatives offer many advantages for studies with intact cells. They are not toxic to any cell type studied, can be cast in any desired shape, are transparent and stable over a wide range of pH values, and contain no cationic and low to negligible levels of anionic charge (charged groups can be introduced if desired), and the polyacrylamide matrix is stable to common biological agents such as bacteria and enzymes. In addition, type I gels can be synthesized using a broad range of molecules containing amino groups, such as glycopeptides, proteins, etc.

The hepatocytes were prepared by collagenase perfusion of intact chicken livers. The rate and extent of adhesion of the cells to the derivatized gels was determined by measuring lactate dehydrogenase in these cells. This enzyme was also used to assay viability and cell "leakiness." At 37°C, 70 to 100% of the cells adhered within 60 min to gels derivatized with N-acetylglucosamine, i.e. gels derivatized with 6-amino-2-acetamido-2-deoxy-β-D-glucopyranoside (or the corresponding thiglycoside). By contrast, less than 5% of the cells adhered to polyacrylamide or to gels derivatized with 6-aminoheptanol or the 6-amino-6-deoxy glycosides of β-D-glucose, β-D-galactose, α-D-mannose, β-D-maltose, β-D-melibiose, β-D-cellobiose, and (α or β)-D-lactose.

Kinetic studies with the chicken hepatocytes and N-acetylglucosamime gels showed that cell-gel binding was dependent upon Ca²⁺ and was decreased at low temperatures. Binding was inhibited by N-acetylglucosamine or by glycosides of this sugar, the most effective inhibitor being orosomucoid (α1-acid glycoprotein) pretreated with sialidase and β-galactosidase. The cell surface receptor(s) involved in this interaction is not known, but may be related or identical to the chicken liver binding protein described by Lunney and Ashwell (Lunney, J., and Ashwell, G. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 341-343). The present results suggest that this model system should prove useful in delineating cell surface interactions with carbohydrates.

The cell surface clearly plays a major role in the interaction of the cell with other cells and with its environment. The surface appears to regulate cell growth and motility (1, 2), morphogenesis (3-5), and transformation and metastasis (6-8). The original hypothesis of Tyler (9) and Weiss (10) that cells interact specifically through binding of complementary molecules either between or on apposing cell surfaces has been supported by studies with different cell systems (11-15). We proposed (16) a more specific model for cell-cell adhesion in which binding occurs between cell surface glycoconjugates and their respective complex carbohydrate acceptors or between the sugars on the surface of neighboring cells via hydrogen bonding.

That carbohydrates mediate specific intercellular interactions is supported by three observations. (i) In general, cells have a carbohydrate-rich surface coat or glycoconjugate (17, 18). (ii) Carbohydrate-binding proteins such as plant lectins are capable of aggregating many cell types and may cause changes in cellular metabolism and morphology (19). (iii) With a variety of cell types (slime molds, sponges, etc.), cell aggregation is affected by carbohydrate-containing or -binding proteins derived from toxic systems (20-24) or by carbohydrates themselves (25, 26).

In preliminary studies to obtain direct evidence for interaction between cell surface and carbohydrates on apposing surfaces, Chipowsky et al. (27) attached thiglycosides containing an amino group in the aglycon, to Sephadex G-25...
beads activated by cyanogen bromide, and showed specific binding of SV40 virus-transformed BALB/c 3T3 tissue-cultured fibroblasts to beads derivatized with a β-galactoside ligand. Although this was the first evidence that cells could respond to carbohydrates immobilized on surfaces, the use of cyanogen bromide activation led to unavoidable incorporation of cationic charge into the product (28), resulting in nonspecific binding of the polyamonic cells. In addition, the lack of stability between ligand and matrix at physiological pH (29), the difficulty of quantitatively measuring the amount of cell binding, and possible damage to the cells on collision with the beads led us to seek a different method for immobilizing carbohydrates on solid matrices.

The present report describes the synthesis and properties of polyacrylamide gels containing the desired ligands linked to the gel via amide bonds. Two different methods were used. For type I gels (30, 31), an active ester of acrylamide (e.g., N-succinimidyl acrylate) was co-polymerized with acrylamide and N,N'-methylenebisacrylamide (Fig. 1). The activated gel was then treated with ligands containing amino groups, resulting in the displacement of the N-hydroxysuccinimide with the formation of an amide bond between the polyacrylamide matrix and the ligand. Such derivatized gels are designated type I gels.

Subsequently, type II gels were synthesized by a different method (32) in which the amino groups of the ligands were first N-acryloylated with acryloyl chloride, and the resulting N-substituted acrylamides were then co-polymerized with acrylamide and bisacrylamide (Fig. 1).

Gel types I and II have the same basic structure, but differ with respect to anionic charge and ligand distribution within the gel matrix. Thus, the two preparations served as controls for each other. As reported below, chicken hepatocytes interacted similarly with both gel types.

The new systems have the following advantages. 1) Linking the ligand to the matrix through an amide bond results in an uncharged species stable over a wide range of pH. 2) After coupling is complete, there is no extraneous cationic charge, and anionic charge is either low (type I gels) or essentially absent (type II gels). 3) The form of the polyacrylamide matrix can be varied in shape and porosity to fit the desired application. For cell interaction studies, for example, flat derivatized surfaces were used. 4) The polyacrylamide gel itself is hydrophilic, unreactive, and stable to both chemical and microbial attack. 5) The derivatized surfaces are transparent, thereby permitting observation of cell morphology as cells interact with the immobilized components.

The use of these gels, described in this report, shows that chicken hepatocytes specifically bind to polyacrylamide gels derivatized with N-acetylglucosamine. This interaction is not only sugar-specific, but is also cell-specific in that other cell types tested did not adhere to N-acetylglucosamine gels. The results indicate that the polyacrylamide gel systems described here are useful for the study of cell surface interactions with immobilized biomolecules. Preliminary reports of these results have been presented (33, 34). We have also recently reported (35) that rat hepatocytes bind specifically to β-galactoside gels.

**Experimental Procedures**1,2

**Properties of Cell Suspensions**

The cells in the final preparations were homogeneous in size, averaging 13 μm in diameter, were 85 to 95% viable as judged by trypan blue exclusion, and contained 70 to 90% single cells; the remainder consisted of aggregates containing two to three cells each. The cells could be stored in Medium A for up to 5 h on ice with no decrease in either viability or adhesive properties. The final cell suspensions consisted of >95% hepatocytes.

The following cell types, prepared as previously described (33), were also tested with the derivatized gels: CHO-W5 tissue culture cells, BALB/c 3T3 tissue-cultured fibroblasts, chick embryo neural retina cells, and rat hepatocytes.

**Measurement of Cell Adhesion**

**Type I Gels**

For the standard cell adhesion assay, type I gels were equilibrated by incubation at 4°C with Medium A (3 ml/gel, 16 h, two changes of medium). The gels were then placed with the derivatized surfaces upward in Linbro multiple well tissue culture dishes (2 cm²/well, Catalogue No. FB-16-24-1C, Linbro Chem. Co., New Haven, Conn.) and incubated with medium (1 ml) for approximately 20 min in a 37°C water bath prior to addition of cells.

The incubation medium was removed from each of the gels by aspiration and immediately replaced with a 1-ml portion of a chicken hepatocyte preparation which had been diluted to 5 x 10⁶ cells/ml with Medium A (37°C) immediately before addition to the gels. Cells were allowed to settle on the gel surface at 37°C without agitation. After the suspension was not agitated, only those cells contained in a volume above the gel surface in the well could make physical contact with the derivatized surface. It was calculated that these comprised about 52% of the cells added to the wells. The numbers of cells adhering to the gels are corrected for this value, and are expressed as the percentage of cells in the suspensions which make contact with the cells (62% of total).

**Abbreviations** (Footnote 1) and most of the methods are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full-size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 78M-388, cite author(s), and include a check or money order for $1.00 per set of photocopies.

1 M. Kuhlenschmidt, unpublished observations.

2 The term “adhesion” is purely operational and is not meant to suggest specific molecular mechanisms or a relationship to intercellular adhesion. As used here, adhesion between cells and gels means that bonds have formed which are stable to shear forces generated during washing of the gels, as described in the text. Although all of the results in the text are based on this definition of “adhesion,” similar results were obtained when milder shear forces were used. When cell-gel interactions were viewed microscopically after gentle agitation of the mixture to determine whether the cells were actually anchored or were simply lying on top of the gel, qualitatively similar results were obtained.

3 The derivatized surface could be detected in either of two ways. The gels were slightly convex at the derivatized surface, and were also translucent at the underivatized surface.

**Fig. 1. Synthesis of type I- and type II-derivatized gels (schematic).** Type I, N-succinimidyl acrylate was co-polymerized with acrylamide and bisacrylamide (Reaction 1), resulting in an activated gel. The gel was treated with ligands (R) containing amino groups (Reaction 2), and a stable amide linkage between ligand and matrix was formed with concomitant release of N-hydroxysuccinimide. Type II, ligands containing amino groups were treated with acryloyl chloride (Reaction 3) to yield the N-acryloylated derivatives. The derivatives were then co-polymerized with acrylamide and bisacrylamide (Reaction 4), resulting in the incorporation of the ligand (R) into the gel matrix. The abbreviations for ligands are given in Footnote 1 (miniprint).
After the indicated incubation times, nonadhering cells were removed with a Pasteur pipette by aspiration (water aspirator) and the gels were washed with Medium A (37°C). The wash medium (1 ml) was carefully pipetted against the side wall of the well, and removed by aspiration. The gels were again overlaid with medium (1 ml), impaled on 0.8-mm (outside diameter) glass capillary tubes, and removed from the well. They were then gently immersed in Medium A at 37°C to remove cells carried on the bottom of gel. The gels, with adherent cells, were finally transferred to lysis buffer for quantitation of cell number as described below.

In some experiments, before removal of the gels from the wells, they were viewed with an inverted phase contract microscope (Wild).

Type II Gels

Assays of cell binding to derivatized gels were performed by either of two methods, which gave identical results. In each case, gels were washed before use at room temperature, twice in 0.15 M NaCl, and then twice in the medium in which the cells were suspended (usually Medium A). For each wash, the gels were placed in 16-ml polystyrene tubes, filled with the wash medium and rotated on a revolving cylinder for 30 min. Procedure A was designed for assaying many gels at a single time point, while Procedure B was used for assaying samples at different incubation times.

**Procedure A—**Gel pieces (as many as 10 gels, 0.36 cm²/gel) were placed in a circular area in a 60-mm diameter polystyrene Petri dish (not tissue culture type). Gels were first blotted on No. 50 Whatman filter paper and, using a narrow bladed spatula, placed on the dry plate at room temperature, care being taken that air bubbles were not trapped. A drop of medium was placed on each gel to prevent dehydration while the other pieces were transferred to the dish. The bottom of the dish was marked to identify each gel piece. Gels placed on polystyrene dishes in this way will remain in place on the plastic during swirling or even inversion of the dish. The drop of medium was removed from the gel and replaced with a 45-μl drop of an ice cold cell suspension with the use of a Pipetman (Rainin Instruments) with a wide bore tip. The dish was then placed in water (approximately 0.5 cm deep) in a 37°C water bath, and covered. At the indicated times, the gels were removed from the water and placed in 1 ml of lysis buffer on ice.

**Procedure B—**Each equilibrated gel (usually 0.64 cm²) was blotted and placed in a Lidob well (2 cm²/well Catalogue No. FC 10-24-1C) in a 37°C water bath. Then, 60 μl of cell suspension were quickly placed on the gel, and the well was covered with a glass marble. At the indicated times, 0.5 ml of medium (37°C) was added gently along the side of the well. The media and any nonadherent cells were then aspirated from the well using a Pasteur pipette attached to a water aspirator maintained at 25 cm Hg. The washing operation was repeated and each gel piece was removed; the bottom was blotted on Whatman No. 50 filter paper, and then placed in 1 ml of lysis buffer on ice.

**Measurement of Cell Number**

The concentrations of single cells in the stock suspensions used in each experiment were determined by direct counting with a hemocytometer. However, a more sensitive assay for cell number, and one which could be readily used to assay a large number of samples, was required for assaying cells bound to the gels. For this purpose, cell samples were lysed by adding detergent, and soluble lactate dehydrogenase activity was determined. A standard curve was constructed using appropriate dilutions of the stock suspension. When these samples were lysed and lactate dehydrogenase activity measured, it was found that enzyme activity varied linearly with cell number.

The standard curve was obtained as follows (Fig. 2). To 0.50 ml of cell suspension in Medium A (5 × 10⁶ cells) were added 4.5 ml of lysis buffer. After 15 min at room temperature, portions of the lysate (0.01 to 0.5 ml) were placed in a 1-ml quartz cuvette and brought to 0.5 ml with lysis buffer, and 0.45 ml of lactate dehydrogenase assay buffer was added. The samples were mixed and the decrease of absorbance at 340 nm was immediately and continuously recorded using a Beckman DU spectrophotometer and a Gilford multiple sample absorbance recorder. The absorbance change at 340 nm was about 0.31/10,000 cells/10 min. This rate varied linearly with cell number over the range 1,000 to 50,000 cells, and was eliminated by omitting pyruvate from the reaction mixture, or by heating the lysate at 100°C for 3 min.

**Measurement of cells adhering to gels** was performed as follows. Washed gels with adherent cells were placed in 1 ml of lysis buffer and incubated for 15 to 60 min at room temperature to ensure cell lysis. Portions of the mixed lysate containing up to 25,000 disrupted cells (0.01 to 0.5 ml) were added to 1-ml quartz cuvettes (1-cm pathlength) and adjusted to 0.5 ml with 0.1 M potassium phosphate buffer, pH 7.0. Each sample was treated with 0.45 ml of lactate dehydrogenase assay buffer, and the lactate dehydrogenase activity was determined as described above.

Lactate dehydrogenase activity can be used to measure cell viabilty as well as cell number. For determining cell number, total enzyme activity was determined after cell lysis with detergent, whereas viability or cell leakiness was determined by measuring extracellular lactate dehydrogenase in the absence of detergent (42). For these analyses, aliquots of the solution above the gels were assayed as follows. Just before washing of nonadherent cells from the gels, an aliquot (0.10 ml, type I gels; 15 μl, type II gels) of the incubation medium was removed from as far above the gel surface as possible to avoid transfer of any intact cells. Each aliquot was placed in a 1-ml cuvette, the volume was adjusted to 0.5 ml with 0.1 M potassium phosphate, pH 7.0, and lactate dehydrogenase activity was measured as described above. The degree of lysis was determined by comparing this activity with the standard curve described above. Lactate dehydrogenase activity was stable in Medium A, with or without Triton X-100, for at least 90 min. The lactate dehydrogenase assay for cell leakiness appeared to be slightly more sensitive than did the trypan blue procedure. With three cell preparations, the following values were obtained for cell viability: 92, 95, and 94%, respectively, by the trypan blue method; 90, 91, and 90% by the lactate dehydrogenase procedure.

**RESULTS**

**Analysis of Derivatized Polyacrylamide Gels—**Typical analyses of type I and type II gels derivatized with glycodies or control ligands are presented in Table I. The data are expressed as micromoles of ligand (or charge) per cm² of gel surface area. Type II gels, which were polymerized from a homogeneous solution of acryloylated ligand and other gel components, contain the ligand distributed throughout the 250-μm thickness of the gel. In contrast, type I gels contain ligands localized at the surface of the 2- to 3-mm thick discs. The thickness of the derivatized layer was assessed by using

**Fig. 2.** Determination of chicken hepatocyte number by the lactate dehydrogenase assay. Chicken hepatocytes were prepared as described under "Experimental Procedures," and counted in a hemocytometer. A 0.50-ml aliquot containing 5 × 10⁶ cells was diluted to 5.0 ml with 0.5% Triton X-100 in 0.1 M potassium phosphate buffer, pH 7.0. Aliquots of the lysate (0.01 to 0.5 ml) were transferred to 1-ml quartz cuvettes, the volumes adjusted to 0.50 ml with lysis buffer, and lactate dehydrogenase activity was assayed as described in the text. **Cell number** refers to the number of cells (lysed) added to the cuvette.
were tested for their ability to bind to gels (either type I or type II) containing 6-aminohexyl O- or, in some cases, S-glycosides of the following d-pyranosides: β-glucose, α-mannose, β-galactose, β-N-acetylglucosamine, β-maltose, β-cellobiose, β-melibiose, and lactose. Chicken hepatocytes specifically adhere only to those gels containing the N-acetylglucosamine (Figs. 3 to 5). The two independently developed systems showed essentially identical results: cellular adhesion to a specific carbohydrate. Microscopic examination (Fig. 3)

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Specific Cell Adhesion to Immobilized Carbohydrates

Fig. 5. Kinetics of cell adhesion to derivatized type II gels. Type II gels were derivatized with O-glycosides or aminohexanol as described under "Experimental Procedures." A 45-μl drop, containing 3.7 × 10^6 cells, was placed over each gel. Adhesion of chicken hepatocytes to the gel pieces (0.36-cm² discs) was measured at the indicated times as described under Procedure A. The number of adhered cells was determined by the lactate dehydrogenase assay described in the text, and the data are expressed as a percentage of cells added to the gel.

revealed that the cells adhered uniformly over the surface of GlcNAc-gels while very few cells adhere to other glycosides or control gels. Cell binding to GlcNAc-gels was tenacious in that cell adhesion was resistant to washing by aspiration (see below).

A more accurate assessment of cell adhesion to the gel was performed by quantitating the cells attached to the gels by the lactate dehydrogenase method. The number of cells that adhered to a gel (from 10^4 to >2 × 10^6 adherent cells) was readily and reproducibly measured in this way.

In some experiments, nearly all cells adhered that came into contact with the GlcNAc-gel surface, while less than 5% of the cells adhered to any of the other glycosides or control gels (Figs. 4 and 5). The maximal adhesion to GlcNAc-gels varied in individual experiments (50 to 100% of added cells, type I gels; 70 to 100% of added cells, type II gels). However, in over 40 experiments with 5 different type I gel preparations and 4 different type II gel preparations, the marked specificity for N-acetylgalactosamine-derivatized gels was always observed.

In these experiments adhesion is defined by the shear forces applied during washing of the nonadherent cells from the gel surfaces. The geometry of type I gels in a Linbro well resulted in the generation of greater shear forces during washing than those generated during washing of type II gels. For this reason, the kinetics of cell-gel binding to type II gels (Fig. 5, 50% complete in 0 to 8 min) was more rapid than to type I gels (Fig. 4, 50% complete in about 25 min). However, when type II GlcNAc-gels were placed on top of underivatized type I discs, incubated with chicken hepatocytes, and then washed using the type I gel washing procedure, the rate of adhesion was slower and resembled the kinetics obtained with type I gels (results not shown). Thus, the measured rate of adhesion appeared to be a function of the wash procedure and the particular geometry of the system. Both gel systems were used to ensure that sugar-specific adhesion was a property of the cells and was not simply due to some property of the gel system such as physical structure, ionic charge, or shear forces produced during washing. While the quantitative rates of chicken hepatocyte adhesion to the gels varied in the two systems (Figs. 4 and 5), identical conclusions such as specificity for N-acetylgalactosamine were obtained in all experiments with the two procedures.

To assess whether the adherent cells remained intact and viable during the experimental manipulations, viability was monitored throughout the incubations. Initial cell viability was always greater than 85% as measured by trypan blue exclusion. The viability (as measured by the amount of spontaneously released lactate dehydrogenase activity) did not change during the course of these experiments (5 to 90 min). Furthermore, when type II gels (GlcNAc) with adherent cells were incubated with 0.4% trypan blue in Medium A, greater than 95% of the cells excluded the dye, indicating that adhered cells remained intact.

The adhesion to GlcNAc-gels appeared to reflect a specific property of chicken hepatocytes in that other cell types (such as CHO-W5 tissue culture cells, chick embryo neural retina cells, and BALB/c 3T3 tissue-cultured fibroblasts), showed low levels of binding to the gels, and no sugar specificity under these conditions (33). In experiments reported previously (35), rat hepatocytes, prepared in a similar manner to the chicken hepatocytes, showed preferential adhesion to galactose-derivatized gels.

As noted above, types I and II gels differ significantly in anionic charge. However, a more important parameter, concentration of sugar on the gel surface available to the cells, has not yet been measured, so that a direct comparison between the two gel types is not yet possible. The two gel types behaved similarly in a qualitative sense with the chicken hepatocytes, and, as already discussed, any quantitative differences appear to be explicable in terms of the geometry of the assay systems. However, marked quantitative differences were observed with the rat hepatocyte system where a very high galactose content in the type I gels was required for adhesion compared to type II gels. These and other quantitative differences between the two gel types may be explained if the sugar distribution and/or accessibility on the surfaces of the two types of gels differ from each other.

Inhibition of Cell Binding to Gels—The binding of chicken hepatocytes to GlcNAc-derivatized gels (both types) was inhibited by free N-acetylgalactosamine, N-acetylgalactosaminide, or by molecules containing N-acetylgalactosamine at the non-reducing terminus of an oligosaccharide chain, but was not inhibited by substances lacking N-acetylgalactosamine, or containing N-acetylgalactosamine at internal or reducing terminal positions (Table II). The inhibition curves using simple saccharides are shown in Fig. 6. In each case, a 1.5- to 2-fold increase in inhibitor concentration effected a change from no inhibition to nearly complete inhibition. This sharp threshold for inhibition is similar to the sharp threshold obtained when cell-gel binding was measured as a function of N-acetylgalactosamine concentration in the gel³ (34). The requirement of terminal N-acetylgalactosamine residues for inhibition is illustrated by experiments with the glycoprotein orosomucoid (α-acid glycoprotein). Orosomucoid is 44,100 daltons and contains at least five branched oligosaccharide chains (averaging approximately 16 monosaccharide residues each). Three or four branches have L-fucose at the nonreducing terminus. The following tetrasaccharide sequence is found at the other non-reducing termini of the oligosaccharide units (43): sialyl→β-galactosyl→β-N-acetylgalactosaminyl→α-mannosyl→oligosaccharide→protein. Sequential treatment of the native glycoprotein with purified sialidase and β-galactosidase (44) yields the series of modified glycoproteins shown in Table II, terminated, respectively, by sialic acid and L-fu-

TABLE II

Inhibition of adhesion of chicken hepatocytes to GlcNAc-derivatized gels by carbohydrates

AH-S-GlcNAc-derivatized type I gels were prepared and preincubated as described in the text. Chicken hepatocytes (5 x 10^6 cells/ml) were kept at 0°C in Medium A until used. The medium on the gels was replaced with a 1:10 dilution of the cells in Medium A at 37°C, containing inhibitors at concentrations ranging from 1 pM to 100 mM (monosaccharides and oligosaccharides) or 0.1 μg/ml to 1.2 μg/ml (glycoproteins). Cells were diluted into inhibitor-containing medium immediately before addition to the gel. After 30 min, the gels were analyzed for cell adhesion as described in the text. It should be noted that Medium A contains 5.5 mM glucose.

<table>
<thead>
<tr>
<th>Compound tested</th>
<th>Concentration resulting in 50% inhibition of binding in mM</th>
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<tbody>
<tr>
<td>N-Acetyl-d-glucosamine</td>
<td>2.5</td>
</tr>
<tr>
<td>Methyl α-N-acetyl-d-glucosaminide</td>
<td>2.0</td>
</tr>
<tr>
<td>Methyl β-N-acetyl-d-glucosaminide</td>
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<tr>
<td>Phenyl β-N-acetyl-d-glucosaminide</td>
<td>1.5</td>
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<tr>
<td>N-Acetyl-l-mannosamine</td>
<td>&gt;100</td>
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<tr>
<td>N-Acetyl-d-galactosamine</td>
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</tr>
<tr>
<td>Glucose, mannose, galactose</td>
<td>&gt;100 (each)</td>
</tr>
<tr>
<td>D-N-acetylgalactohexoside</td>
<td>4.0</td>
</tr>
<tr>
<td>Cellulose</td>
<td>&gt;50</td>
</tr>
<tr>
<td>Hyaluronic acid tetrasaccharide</td>
<td>&gt;10</td>
</tr>
<tr>
<td>Orosomucoid</td>
<td>&gt;0.02</td>
</tr>
<tr>
<td>Agalacto-orosomucoid</td>
<td>&gt;0.03</td>
</tr>
<tr>
<td>Asialo-agalacto-orosomucoid</td>
<td>7 x 10^-7</td>
</tr>
</tbody>
</table>

* Samples of N-acetylmannosamine and N-acetylgalactosamine were analyzed for containing N-acetylglucosamine by hydrolysis (4 N HCl, 4 h, 100°C) and separation on an automated amino sugar analyzer (39). The N-acetylmannosamine (Sigma) contained a small amount (less than 1.5%) of N-acetylglucosamine, while the N-acetylglucosamine (Sigma) contained no detectable N-acetylglucosaminogalactosamine (less than 0.5%).

* The values for these inactive compounds are the highest concentrations tested. No inhibition was detected at these concentrations.

* The tetrasaccharide is glucuronosyl-(β, 1→3)-N-acetylgalactosaminyl-(β, 1→4)-N-acetylgalactosaminyl-(β, 1→3)-glucuronosyl-(β, 1→4)-N-acetylgalactosaminyl.

* The concentrations of the glycoproteins were: orosomucoid, 0.02 mM = 1 mg/ml; agalacto-orosomucoid, 0.03 mM = 1.1 mg/ml; asialo-agalacto-orosomucoid, 7 x 10^-5 mM = 2.5 μg/ml.

Fig. 6. Inhibition of cell binding of GlcNAc-derivatized gels by N-acetylglucosamine and N-acetylgalactosaminides. Chicken hepatocytes were incubated with type I gels as described under "Experimental Procedures." An aliquot of ice cold cell suspension (10^6 cells/ml) was diluted 1:10 with Medium A (37°C) containing the inhibitors immediately before addition to the gels. After 60 min at 37°C, nonadhering cells were removed and cell adhesion was determined by the lactate dehydrogenase assay as described in the text. Results are expressed as the percentage of cells bound to GlcNAc-gels in the absence of inhibitors; 100% represents 67% of the added cells bound. A, GlcNAc (○), di-N-acetyltrehalose (●), B, phenyl α-N-acetylgalactosaminide (○), phenyl β-N-acetylgalactosaminide (●), methyl α-N-acetylgalactosaminide (△), methyl β-N-acetylgalactosaminide (●), and methyl β-N-acetylgalactosaminide (●).
fraction of the cells was released from the gels by N-acetylglucosamine or EGTA, or both (Fig. 8C). At 60 min, for example, these reagents removed only 30% of the adherent cells from the gels. Thus, when chicken hepatocytes are incubated with GlnAc-gels for more than 20 to 40 min, changes in the cells or gels, or both, apparently occur, which prevent cell de-adhesion. Current studies are aimed at determining the basis for these results.

Properties of Cells Released from N-Acetylglucosamine derivatized Gels—Besides the study of adhesion, the polyacrylamide gel systems could also be used to fractionate heterogeneous populations of cells, provided that cells which adhere to the gels can be recovered in viable form. Some properties of the chicken hepatocytes released from the GlnAc-gels by free N-acetylglucosamine were therefore studied. The cells, which were released from the gels over a 60-min second incubation period in the presence of N-acetylglucosamine (Fig. 8B), were pooled, harvested by centrifugation, washed, and resuspended in Medium A. Of the cells in the suspension, 91% were viable by trypan blue exclusion, and consisted of 84% single cells, values which compared favorably with the original untreated cell suspension, 91% viable and 91% singles. The cells released from the gels also retained their adhesive properties, both with respect to the rate of adhesion to GlnAc-gels and to each other (Fig. 9).

Effect of Temperature—The effect of temperature in the range 0°C–37°C on cell-gel binding was studied and typical results are presented in Fig. 10. Using type I gels, both the rate and extent of cell binding to GlnAc-gels were decreased at low temperature. At 0°C, for example, the "initial rate" (10 min) and "extent" (60 min) were about 50% of the values obtained at 37°C. Even greater inhibitions at low temperatures were observed with the assay system used for type II gels (as much as 5-fold decrease in the initial rates, data not shown). In addition, nonspecific cell binding to both gel types increased significantly at low temperatures. If the values obtained with the control gels at 0°C and 4°C are subtracted from those obtained with the GlnAc-gels in Fig. 10, then so-called "specific binding" to the latter is substantially reduced.

Fig. 7. Effect of divalent cations on chicken hepatocyte binding to GlnAc-derivatized gels (type I). Hepatocytes were prepared as described under "Experimental Procedures" and were divided into two portions immediately after the filtration through nylon. One portion was further purified as outlined under "Experimental Procedures," and was finally suspended in Medium A. The other portion was treated in an identical manner except that the cells were washed twice (by resuspension) with Buffer 1 containing 1.5% bovine serum albumin and were finally suspended in Buffer 1, a salts solution. Cell concentrations were adjusted to 10⁶ cells/ml in each buffer, and cells were stored at 0°C until used. Immediately before incubation with the AH-S-GlcNAc-derivatized gels (equilibrated with Buffer 1 rather than Medium A), the cells were diluted 1:10 with Buffer 1 alone or Buffer 1 supplemented with the following salts (at 37°C): ■, calcium chloride, 2.22 mM; ●, magnesium chloride, 2.22 mM; ○, calcium and magnesium chloride, 2.22 mM each; □, no additions. Medium A-suspended cells were diluted with Medium A (□). Portions of each suspension (1 ml) were placed on the AH-S-GlcNAc-gels, and cell adhesion assayed at the indicated times by methods described in the text. No detectable cell lysis occurred during the course of the incubations as measured by release of lactate dehydrogenase.

Fig. 8. Effect of N-acetylglucosamine on reversibility of binding of chicken hepatocytes to GlnAc-derivatized gels. A, kinetics of binding of hepatocytes to gels. Cells (6 x 10⁵/ml) and AH S GlnAc derivatized type I gels were incubated in Medium A at 37°C. At the indicated times, a gel was washed and the number of adherent cells was determined by the lactate dehydrogenase assay. C, effect of first incubation time on reversibility of binding. Cells (10⁵/ml) were allowed to bind to gels for various periods of time as described in A (first incubation). At the indicated times, nonadherent cells were removed by washing, and replicate gels were treated with 1 ml of Medium A lacking Ca²⁺ and Mg²⁺ and supplemented with 25 mM N-acetylglucosamine and 2 mM EGTA. (Either compound alone gave essentially the results shown with the mixture.) After 40 min at 37°C (second incubation), the gels were washed and the number of adherent cells was determined. These represent cells bound irreversibly to the GlnAc-gels. The results are presented as the fraction (as percentage) of adherent cells that were irreversibly bound at each time point (i.e., number of irreversibly bound cells/total cells bound x 100).
The results of these experiments therefore indicate that chicken hepatocytes bind more slowly and to a lesser degree to N-acetylglucosamine-derivatized gels at reduced temperatures. Whether this type of inhibition results from a decrease in metabolic rates, a change in cell surface geometry (or conformation), a decreased mobility of the relevant cell surface receptors because membrane lipids are below their transition temperatures, or for other reasons, remains to be determined.

**FIG. 9. Adhesive properties of cells released from GlcNAc-derivatized gels by N-acetylglucosamine.** Chicken hepatocytes (10⁶ cells/ml) and AH-S-GlcNAc-derivatized gels (type I, 34 gels) were incubated in Medium A for 25 min at 37°C as described in the text. The gels were then impaled with microcapillary tubes (0.8 mm outside diameter), washed free of nonadhering cells by immersion in Medium A, and incubated in this solution for 60 min at 0°C with gentle swirling for 10 s at 10-min intervals. More than 90% of the cells were released during this incubation. The cell suspension was centrifuged at room temperature for 3 min at 190 g, the cells were resuspended in cold Medium A (30 ml), centrifuged (190 x g, 3 min), resuspended in 4 ml of cold Medium A, and finally filtered through a 15-μm Nitex filter to remove small aggregates (<5% of total cells). The final suspension was stored at 0°C until used (approximately 1 h). An aliquot was diluted 1:1 with trypsin blue solution and examined by phase microscopy. The recovered cells were 91% viable, and 84% single cells, the remaining 16% consisting of two-cell aggregates. •—•, control cells (not preincubated with gels). ○—○, cells incubated with AH-S-GlcNAc-gels, released by N-acetylglucosamine, and recovered as described above. Left, control cells or cells harvested as described above were diluted to 4 x 10⁶ cells/ml and binding to AH-S-GlcNAc-gels (1 ml/gel) was determined as described in the text. Right, control cells or cells harvested as described above were diluted to 2.2 x 10⁶ cells/ml and cell-cell adhesion was measured by the Coulter counter assay at 37°C in Medium A as previously described (13).

**Fig. 10. Effect of temperature on adhesion of chicken hepatocytes to derivatized gels.** Derivatized gels were incubated in Medium A for 16 h at 4°C, and for an additional hour in Linbro wells at the indicated temperatures. Chicken hepatocytes were prepared and suspended at 4°C in Medium A at a concentration of 10⁶ cells/ml. Adhesion experiments were initiated by replacing the medium on the gels with 1 ml of a 1:10 dilution of the hepatocyte suspension (Medium A) at the indicated temperature, and the incubation was continued at that temperature. At the indicated times, the gels were washed, and cell adhesion was measured as described in the text.

**Fig. 11. Effect of cell concentration on chicken hepatocyte adhesion to AH-S-GlcNAc-derivatized gels (type I).** AH-S-GlcNAc-derivatized gels were prepared and incubated at 37°C in Medium A as described in the text. Chicken hepatocytes were diluted at 0°C with Medium A to densities ranging from 10⁶ to 18 x 10⁶ cells/ml. Adhesion was initiated by replacing the medium on the gels with 1-ml portions of the cold cell suspension. After 60 min at 37°C, the nonadherent cells were removed and the gels were assayed for bound cells as described in the text. The abscissa represents the total number of cells added to each well. Duplicate determinations within a single experiment are presented. In four other experiments with different cell preparations, similar results were obtained up to 20 x 10⁶ cells added/gel. At higher cell densities, variable results were obtained. In some cases, for example, at cell densities of 30 x 10⁶ cells added per gel, the number of cells which remained attached to the gel was about 25% less than observed at 20 x 10⁶ cells per gel (data not shown). This decrease may result from increased intercellular adhesion in the suspension, making fewer single cells available to the gels, or from the fact that cell aggregates adhering to the gels are unable to resist the shear forces generated during washing.
Specific Cell Adhesion to Immobilized Carbohydrates

In experiments, an apparent maximum binding of 1.2 to 1.9 x 10^6 cells/cm² gel surface was observed, even when cell density in the suspension was increased to 18 x 10^6. Above this density, variable results were sometimes obtained (see legend to Fig. 11). Based on an average measured diameter of 13 µm/cell, it was calculated that the gel surface would be covered with a monolayer of cells at a cell concentration of 1 to 2 million cells/cm². Thus it appeared that either (a) the adhesion of a cell to the GlcNAc-gel rendered its upper surface unadhesive towards other cells, or (b) cell to cell adhesion is less stable to the washing procedure than cell to gel adhesion.

The second possibility (case b) is strengthened by experiments using type II gels. Under normal washing conditions, no saturation of the gels was observed even when the adherent cell density was greater than 10^6 cells/cm² (data not shown). However, when the type II gels were subjected to the more vigorous washing procedure used for type I gels by placing them on type I gels in Limbro dishes, saturation of the type illustrated in Fig. 11 was observed at a concentration of approximately 2 x 10^6 cells/cm². Therefore, cell-cell adhesion can occur in the presence of cell-gel adhesion, but we infer that the cell-cell bonds are more sensitive to shear forces than the cell-gel bonds.

**DISCUSSION**

The molecular events underlying cell-cell recognition and adhesion are not known, primarily because of the complexity of these phenomena. We have, for example, reported (45) that the formation of stable intercellular bonds involves at least two (possibly three) steps within the first few minutes of cell-cell contact. Based on the hypothesis that cell surface carbohydrates are involved in one or more steps in the over-all process, we have designed specific probes to be used in an attempt to analyze these steps. The probes (type I and type II gels), synthesized by two distinct chemical methods, consist of simple mono- and disaccharides covalently linked to a polycrlylamide matrix; the cells used in this study do not adhere to the matrix itself. The method for making type I gels can also be used for linking other ligands to the gel, such as proteins, peptides, glycopeptides, etc. The numerous advantages of the polycrlylamide gels for this kind of study are listed above and in our preliminary communications (30-35).

In other experiments now in progress, in which we are attempting to determine the effects of ligands (such as carbohydrates) on cell motility, growth, etc., it is important that the cells adhere to the matrix, and in this case polystyrene is used in place of polycrlylamide.

Chicken hepatocytes, prepared as previously described (15), showed marked specificity for polycrlylamide gels containing covalently linked N-acetylglucosamine. With this sugar, from 60 to 100% of the cells formed remarkably stable adhesive bonds to both gel types in 10 to 60 min at 37°C. Less than 5% of the cells adhered to any other derivative tested. Other cell types were tested, and did not bind to the N-acetylg glucosamin gels. As previously reported, however, rat hepatocytes adhered to galactose-derivatized gels (35). Marked sugar specificity has, therefore, been demonstrated with both these cell types, with the use of many different carbohydrate gels in each case.

The attachment of chicken hepatocytes to N-acetylglucosamine-derivatized gels could be prevented by two different types of inhibitors. Not surprisingly, chelators such as EDTA prevented adhesion by forming complexes with calcium ions, which are required for the process. Adhesion was also inhibited by N-acetylglucosamine and its glycosides, although it was not inhibited by any other sugars tested. Inhibition of cell-gel binding required that the N-acetylglucosamine residue be at the nonreducing terminus of the molecule; moreover, high molecular weight substances such as suitably modified orosomucoid were much more effective inhibitors than low molecular weight compounds, and α- and β-glycosides were equally effective inhibitors.

In studying the phenomenon further, by measuring the effects of a range of concentrations of inhibitors on cell-gel binding, it was found that the degree of inhibition was sharply dependent on the concentration of the inhibitor. Thus, a small increase in inhibitor concentration resulted in a change from maximal adhesion to complete inhibition (Fig. 6). This effect is similar to that observed on the binding of cells to gels containing increasing amounts of sugar. Chicken hepatocytes require a critical concentration of N-acetylglucosamine in the gel before they can bind (34); at this concentration, essentially maximal binding occurs, whereas at concentrations 10 to 15% below this value, no cells are bound. Similar results are obtained with rat hepatocytes and galactose-derivatized gels. The molecular basis underlying these threshold phenomena is not known.

The reversibility of cell-gel binding was also studied with chicken hepatocytes and the N-acetylglucosamin gels. In these experiments, the cells were permitted to attach to the gels for different binding periods and were then treated with N-acetylglucosamine or EGTA, or both, at concentrations far in excess over those which would inhibit attachment of cells to the gels. It was found that the ability of the N-acetylglucosamin to release cells depended on the time that the cells had originally been in contact with the gel. When the initial binding time was 20 min or less, all of the cells that adhered to the gels were released, and these cells adhered to gels or to themselves in a manner identical with the original cell population. However, when the binding period exceeded 20 min, a fraction of the adherent cells was not removed by N-acetylglucosamin or EGTA, or both (Fig. 8). When the binding period was 60 min, for example, 70% of the bound cells remained attached to the gels after incubation with the inhibitors (25 mm N-acetylglucosamin or 2 mm EGTA, or both) for 40 min at 37°C. This “irreversibility” is under study, but preliminary results suggest that the cells modify the gels when they are permitted to bind to the gels for longer than 20 to 30 min. For these studies we used the β-thioglycoside of N-acetylglucosamin, and we emphasize that the thioglycosidic linkage is very resistant to the action of known glycosidases (46). Further, hexanol linked to the polycrlylamide, the expected product of hydrolysis, did not support the binding of cells to gels.

Ashwell and Morell (47) showed that mammalian species contain carbohydrate-binding proteins in liver cells that specifically recognize galactosides. Subsequently, Lunney and Ashwell (48) and Kawasaki and Ashwell (49) demonstrated a similar receptor in avian species that binds N-acetylglucosamin-terminated glycoproteins. The cell preparations described in this report contain this binding activity (data not shown). The possible relationship between the latter binding protein and the receptor involved in cell-gel binding is under study.

We have previously reported the kinetics of chicken (and rat) hepatocyte cell-cell adhesion (15). Although N-acetylglucosamin and asialo-agalacto-orosomucoid inhibit the binding of these cells to N-acetylglucosamin gels, they have no effect on cell-cell binding (data not shown). These results suggest that cell-cell and cell gel adhesion involve different cell surface receptors. However, a clear decision on this important point cannot yet be made for two reasons. First, cell-cell adhesion is a multistep event so that inhibition at one of the later steps may not be recognized by present techniques. Second, the cell...
surface receptors may have far higher binding constants for the natural surface ligands than for the synthetic derivatives tested in the cell-gel adhesion assay.

Whatever the nature of the cell surface receptor involved in cell-gel adhesion, or whether it is related to cell-cell adhesion, it is clear that the techniques described in this report offer a novel approach for probing the cell surface, and may, in addition, provide a new and mild technique for fractionating heterogeneous cell populations.

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Specific Cell Adhesion to Immobilized Carbohydrates

**Materials**

The following were purchased from commercial sources and further purified, as indicated: avidin-conjugated goat anti-rabbit IgG (Bio-Rad), avidin-coated polystyrene microplates (Microtest, Dénmark), ethanolamine (Fisher Chemical Co.), N-acetylglucosamine (Sigma Chemical Co.), bovine serum albumin (Fraction V, Ficol), poly-L-lysine (Calbiochem-Behring), rhodaminyl-agarose beads, biotinylated-agarose beads, blue native electrophoresis buffer (Amersham, France), cellulose nitrate (Fisher Scientific), ethylene glycol bis(aminepropyl)glycine, 6-aminohexyl (4-O-α-D-glucopyranosyl)-α-D-glucopyranoside (1-0.2 mg/ml), 6-aminohexyl (4-O-α-D-galactopyranosyl)-α-D-glucopyranoside (1.0 mg/ml), and 6-aminohexyl (4-O-α-D-glucopyranosyl)-α-D-galactopyranoside (0.1 mg/ml).

**Methods**

1. **Cell Adhesion**
   - Avidin-conjugated goat anti-rabbit IgG (1 mg/ml) was immobilized on 3-aminopropyltriethoxysilane-coated polystyrene microplates. The microplates were incubated with a saturating concentration of avidin-conjugated goat anti-rabbit IgG for 2 h and then washed with PBS. The microplates were then incubated with 3% BSA (bovine serum albumin) in PBS for 1 h to block non-specific binding sites.
   - Avidin-coated polystyrene microplates were incubated with a saturating concentration of avidin for 2 h and then washed with PBS. The microplates were then incubated with 3% BSA in PBS for 1 h to block non-specific binding sites.

2. **Avidin-Binding Capacity**
   - The avidin-binding capacity of the microplates was determined by measuring the amount of avidin that could be reversibly adsorbed. Avidin-coated polystyrene microplates were incubated with a saturating concentration of avidin for 2 h and then washed with PBS. The microplates were then incubated with 3% BSA in PBS for 1 h to block non-specific binding sites.

3. **Cell Adhesion**
   - Cell adhesion experiments were performed by incubating the microplates with the test cells at 37°C for 1 h. The microplates were then washed with PBS to remove non-adherent cells. The adherent cells were then fixed with 4% paraformaldehyde and stained with 1% crystal violet.

4. **Data Analysis**
   - The data were analyzed by calculating the percentage of cells that adhered to the microplates. The experiment was repeated at least three times, and the results were expressed as the mean ± standard deviation.

**Results**

The results obtained from the cell adhesion experiments are summarized in Table 1. The data show that the avidin-coated polystyrene microplates have a higher avidin-binding capacity compared to the avidin-conjugated goat anti-rabbit IgG microplates. Moreover, the avidin-conjugated goat anti-rabbit IgG microplates have a higher cell adhesion capacity compared to the avidin-coated polystyrene microplates.

**Conclusion**

In conclusion, the results of this study demonstrate that the avidin-coated polystyrene microplates have a higher avidin-binding capacity compared to the avidin-conjugated goat anti-rabbit IgG microplates. Moreover, the avidin-conjugated goat anti-rabbit IgG microplates have a higher cell adhesion capacity compared to the avidin-coated polystyrene microplates. These findings suggest that avidin-coated polystyrene microplates can be used as a versatile tool for the development of cell adhesion assays.
Specific Cell Adhesion to Immobilized Carbohydrates

Materials and buffers

Modified Eagle’s medium consisted of Gibco C-21 (Gibco Grand Island Biological Company), adjusted to 1 liter and pH 7.4 after adding the following components: 2.5 g of NaHCO3, 210 mg of NaCl, 30 mg of pyruvate, 150 mg of glucose, 2 mg of ascorbic acid, 23.5 mg of thiamine, 974.6 mg of CaCl2, 2.5 g of KCl, and 0.5 g of CaCl2 and 2 mg of MgSO4, which was the concentrations of 1.0 M NaOH. Buffer 1 consisted of 0.1 M NaCl, 0.5 g of KCl, and 0.5 g of Tris and was adjusted to pH 7.4 by the addition of 1 M NaOH (0.5 ml).

Buffer 1 was prepared by dissolving collagenase 9891 (GIBCO) in a solution containing 0.001 M NaCl, 0.5 g of KCl, 0.5 g of CaCl2, 0.2 g of CaCl2, 0.2 g of MgSO4, 0.2 g of mannitol, and 0.2 g of trypsin inhibitor solution adjusted to pH 7.4 by the addition of 1 M NaOH (9.5 ml). Just prior to use, the collagenase was dissolved in the solution to the desired values. The mixture was filtered on the basis of 5 mg/ml and was used to obtain the desired values.

Cell preparation

Chick liver hepatocytes were prepared by a collagenase perfusion method as previously described (38). Briefly, the liver was placed in a single chamber perfused with 0.5% of peritoneal protease and 0.45 M NaCl in 0.1 M potassium phosphate buffer, pH 7.4.

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