Inhibition of Fibronectin Receptor Function by Antibodies Against Baby Hamster Kidney Cell Wheat Germ Agglutinin Receptors

NANCY OPPENHEIMER-MARKS and FREDERICK GRINNELL
Department of Cell Biology, University of Texas Health Science Center, Dallas, Texas 75235

ABSTRACT Previous studies suggest that the baby hamster kidney (BHK) cell fibronectin receptor is also a wheat germ agglutinin receptor (WGA-R). To analyze this possibility further, IgG and Fab fragments of antibodies produced against a BHK cell WGA-R preparation were tested to determine their effects on cell adhesion mediated by fibronectin, wheat germ agglutinin, concanavalin A, and polycationic ferritin. The WGA-R preparation was isolated by octylglucoside extraction of BHK cells followed by chromatography of the extract on WGA-agarose. The antibodies against the WGA-R preparation reacted primarily wih polypeptides of molecular weights 48, 61, 83, 105, 120, 165, 210, and 230 kilodaltons (kdaltons). It was concluded that the antibodies interfered with BHK cell fibronectin receptors on the basis of the ability of anti-WGA-R IgG or Fab fragments to (*a*) inhibit cell spreading on fibronectin-coated substrata; (*b*) cause rounding and detachment of cells previously spread on fibronectin-coated substrata; and (*c*) inhibit binding of fibronectin-coated latex beads to the cells. Antibody activity was blocked by treatment of anti-WGA-R with the WGA-R preparation or by absorption of anti-WGA-R with intact BHK cells. The antibodies also appeared to prevent coupling of ligand-receptor complexes (involving concanavalin A or polycationic ferritin) with the cytoskeleton. Finally, cell rounding and detachment caused by the antibodies were found to require metabolic energy since it did not occur in the presence of azide or at 4°C.

Cell adhesion to extracellular substrata involves ligand-receptor interactions between cell surface receptors and ligand molecules on the substratum (12). A variety of ligands have been shown to function as adhesion factors with fibroblasts, and these include polycationic molecules (16, 32), lectins (1, 6), antibodies to the cell surface (16, 22), and glycosidases (35). The adhesion factor, fibronectin, has been of particular interest because of its ability to promote fibroblast adhesion to physiological substrata such as fibrin (19) and denatured collagen (26, 28), which fibroblasts attach to and migrate on in vivo during wound healing (18).

By analogy to the other ligands mentioned above, it has been assumed that fibronectin bound to the substratum interacts with specific cell surface receptors (11). Direct evidence for the existence of such receptors has been suggested by experiments on the binding of plasma fibronectin to suspended BHK cells at 4°C (17) and by experiments on the binding of plasma fibronectin-coated latex beads to BHK cells (13).

The identity of the fibronectin receptors remains to be clarified. Some experiments have led to the conclusion that cell surface carbohydrate groups are important in fibronectin function (5, 23). Others indicate that glycosaminoglycans are involved on the basis of the accumulation of heparan sulfate in cell-substratum adhesion regions (7) and the ability of heparan sulfate to bind to fibronectin (30). Still others have demonstrated the importance of cell surface glycolipids in fibronectin receptor function based upon the ability of the glycolipids to inhibit fibronectin-mediated cell adhesion phenomena (27, 39). Finally, and of particular interest, a recent study demonstrated that fibronectin on the substratum could be covalently bound to a 48 kdalton cell surface glycopeptide by a photoactivated cross-linking reagent after cell spreading had occurred (2).

The possible role of cell surface carbohydrate groups in fibronectin receptor function mentioned above led us to test whether lectins could inhibit fibronectin-mediated cell adhesion (34). In these studies, evidence was obtained indicating that wheat germ agglutinin (WGA) was able to inhibit the binding of fibronectin-coated latex beads to BHK cells and

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also inhibited cell spreading on fibronectin-coated plastic surfaces. It was concluded that the fibronectin receptor also was a WGA receptor. To extend these observations, wheat germ agglutinin receptors (WGA-R) were prepared from BHK cells and used to obtain anti-WGA-R antiserum. In this report we describe the effects of anti-WGA-R IgG and Fab fragments on cell adhesion mediated by fibronectin and other ligands.

MATERIALS AND METHODS

Cells

Baby Hamster Kidney (BHK) cells modified for growth in suspension culture were a gift from Dr. A. Chappel (Center for Disease Control, Atlanta, GA). The cells were grown as described previously (11). Logarithmically growing cells were harvested by centrifugation at 500 *g* for 4 min (Sorvall HL-4 rotor: Sorvall-DuPont, Newtown, CT), washed and resuspended in adhesion medium (150 mM NaCl, 3 mM KCl, 1 mM $CaCl_2 \cdot 2H_2O$, 0.5 mM $MgCl_2 \cdot 6H_2O$, 6 mM Na_2HPO_4, pH 7.3) or serum-free modified Eagle's medium (MEM) at the designated cell concentrations.

Plasma Fibronectin

Human plasma fibronectin (pFN) was prepared by salt precipitation and ion exchange chromatography as described previously (19). The specific activity of the pFN preparations used in these experiments was ~200–400 U/mg. One unit of activity is defined as the amount of pFN required to promote complete spreading of BHK cells in a standardized assay (11).

Substrata

Falcon 3001 (35-mm) tissue culture dishes (Falcon Labware, Oxnard, CA) were incubated with 1 ml of 5 U/ml pFN in adhesion medium or in 1 ml of Con A (100 μg/ml), WGA (500 μg/ml), or PCF (800 μg/ml) in phosphate buffered saline (PBS) (0.01 M Na Phosphate, 0.15 M NaCl, pH 7.2) for 10 min at 22°C. In some cases the dishes also were treated with 1 ml of 1% heat denatured bovine albumin in PBS to block nonspecific attachment sites (14). The dishes were then rinsed with PBS and used for adhesion experiments. For experiments with 0.3 ml incubation volumes, Falcon 3008 multiwell tissue culture plates were treated as described above. For experiments with 0.1 ml incubation volumes, the central area of each 35-mm tissue culture dish was encircled with a wax crayon and treated as described above.

Isolation of BHK Cell WGA-Receptors

Approximately 10^9 BHK cells were harvested by centrifugation at 650 *g* for 5 min (Sorvall GSA rotor: Sorvall-DuPont) and the cells were washed twice using 100 ml and then 50 ml of adhesion medium. Subsequently, the cells were resuspended in 20 ml of 40 mM octylglucoside (OG) solution (10 mM $CaCl_2$, 8 mM HEPES, 0.2 mM phenylmethylsulfonyl fluoride [PMSF], pH 6.2), vortexed for 10 s and incubated for 30 min at 4°C (Table I, *Starting Material*). This mixture was then centrifuged at 100,000 *g* for 60 min (Beckman 42.1 rotor: Beckman Instruments, Fullerton, CA). The supernatant (Table I, *OG Extract*) was diluted 10-fold with 10 mM HEPES (pH 6.2). A subsequent centrifugation at 100,000 *g* for 60 min sedimented the extracted material. This pellet was dissolved in 10 ml WGA-agarose column buffer (0.01 M Tris, 0.5 M NaCl, 0.5% Nonidet P-40 [NP-40], pH 7.0) and centrifuged for 10 min at 12,000 *g* (Sorvall SS-34 rotor). The supernatant (Table I, *Column Load*) was applied to a washed WGA-agarose column (Vector Laboratories, Inc. Burlingame, CA.) (0.8 × 2.8 cm). After the unbound material had been collected (Table I, *Unbound*), the column was washed with 100 ml of column buffer. The WGA-R fraction was eluted with 15 ml of 1 M *N*-acetyl-D-glucosamine (Sigma Chemical Co., St. Louis, MO), dialyzed against 0.01 M NaPi, pH 7.0, and lyophilized. The lyophilized material was dissolved in a small volume of water. (Table I, *WGA-Receptors*).

In some cases, WGA-R were isolated from cells which had been grown for 16 h in the presence of ^{3}H[1,6]-D-glucosamine (sp act 32.5 Ci/mmol: New England Nuclear, Boston, MA) or [^{35}S]L-methionine (sp act 1225 Ci/mmol at time of receipt: Amersham Radiochemicals, Arlington Heights, IL). The harvested cells were washed three times. The WGA-R fraction was isolated according to the above protocol except that the volume of detergent solutions and buffers was decreased one-fourth.

Protein Analysis

Protein was analyzed by the method of Lowry et al. (31) using bovine albumin as the standard. With samples that contained detergent, aliquots to be tested were diluted to 1.0 ml with H_2O, mixed with 100 μl of 1 mg/ml sodium deoxycholate, and then incubated for 10 min at 22°C with trichloroacetic acid (final concentration, 9%). The samples were microfuged and the pellets were dissolved in 1 ml of the Lowry reagent and incubated for 5 min at 22°C. Subsequently, 100 μl of phenol reagent (diluted 1:1 with water) were added and the solutions were incubated for 45 min at 22°C in the dark. To remove any detergent precipitate that formed, the samples were centrifuged for 4 min at 500 *g*.

TABLE I

Wheat Germ Agglutinin Receptor Preparation

Fraction*	Protein or radioactivity		
	Protein‡	[^{35}S]Methionine§	[^{3}H]Glucosamine‖
	mg	*cpm*	*cpm*
Starting material	154.38 (±35.54)	2.12×10^7	1.70×10^5 (±0.33)
OG extract	108.65 (±28.0)	7.82×10^6	1.41×10^5 (±0.05)
WGA-agarose column load	13.07 (±3.75)	1.45×10^6	4.98×10^4 (±1.89)
Unbound	5.78 (±0.49)	8.28×10^5	3.39×10^3 (±2.09)
WGA receptors	0.68 (±0.07)	5.94×10^4	1.22×10^4 (±0.33)
Percent of total receptors¶	0.44	0.28	7.18

* See Materials and Methods.

‡ Average (±SD) from four preparations.

§ 6.0×10^6 BHK cells in 30 ml of growth medium were grown for 16 h in the presence of 300 μCi [^{35}S]methionine before isolation of WGA-R.

‖ 12.5×10^6 BHK cells in 50 ml of growth medium were grown for 16 h in the presence of 10–12.5 μCi [^{3}H]glucosamine before isolation of WGA-R. Average (±SD) from two preparations.

¶ Percent of total in receptors equals protein or cpm in WGA receptors/Starting material.

Production of WGA-R Antibodies

Antiserum against BHK cell WGA-R was produced in New Zealand White (NZW) rabbits by three injections at 2-wk intervals of ~100 μg WGA-R mixed 1:1 with Freund's complete adjuvant on the first immunization and Freund's incomplete adjuvant on subsequent immunizations. The injections were at multiple interdermal sites. Blood was drawn from an ear vein 1 wk after the last injection and serum was obtained by allowing the blood to clot overnight at 4°C. The IgG fraction was isolated by two sodium sulfate precipitations (18%, 16%) followed by chromatography on DEAE-cellulose (Whatman) (9).

Fab fragments were prepared according to standard methods (10). Immune IgG (8–10 mg/ml) was dialyzed against 0.1 M NaPi, pH 7.0 and digested for 8–12 h at 37°C with papain (1 mg/100 mg IgG) in the presence of 2 mM EDTA and 0.1 M cysteine. At the end of the incubations, the papain digests were dialyzed against 0.02 M Na acetate at 4°C. The pH of the dialyzate was adjusted to 5.5 with glacial acetic acid and was diluted with H_2O to a final concentration of 0.01 M acetate. This material was then applied to a CM-52 column (1.25 × 20 cm), which was previously equilibrated with 0.01 M Na Acetate, pH 5.5, and eluted by the stepwise addition of two column volumes of acetate buffer (pH 5.5) at 0.05 M, 0.1 M, 0.225 M, and 0.45 M. Protein in each fraction was detected spectrophotometrically (absorbance at 280 nm) and peak fractions were pooled. Detection of Fab fragments in the six major peaks was by immunodiffusion analysis against goat anti-rabbit IgG, goat anti-rabbit Fab'$_2$, and goat anti-rabbit Fc (Cappel Laboratories, Cochraneville, PA). Peaks which contained only the Fab region of the immunoglobulin molecule, were pooled, concentrated (Millipore Corp., Bedford, MA) to greater than 6 mg/ml, and stored at −75°C. The Fab preparations contained no intact IgG molecules as determined by SDS PAGE under reducing and nonreducing conditions.

Electrophoresis

SDS PAGE was performed on slab gels according to the method of Jarvik and Rosenbaum (24). The resolving gel (14 × 8.5 × 0.1 cm) was a linear 4–16% acrylamide gradient gel containing a gradient of 3–8 M urea. The stacking gel was 3% acrylamide. After electrophoresis, gels either were fixed and silver-stained

by the method of Merril et al. (33), or were electroblotted onto nitrocellulose paper for immunological analysis by the method of Burnette (4).

Indirect Immunofluorescence

BHK cells in 1 ml adhesion medium were incubated with pFN-coated substrata for 60 min at 37°C. The cells were fixed with 3% formaldehyde and indirect immunofluorescence was carried out on nonpermeabilized or Triton-permeabilized cells as described previously (14, 15). In the incubation with the primary antibody, the concentration of anti-WGA-R IgG was 1 mg/ml. Fluorescent observations were made using a Zeiss Photomicroscope III equipped with phase contrast and epifluorescence.

Adhesion Assays

To assay for the effects of WGA-R antibodies on cell spreading, BHK cells in adhesion medium were treated with antibodies for 10 min at 4°C. Subsequently, the cell/antibody mixture was incubated on the designated substrata for 45 min at 37°C in a humidified incubator at which time cell spreading was determined microscopically (11).

To assay for the effects of WGA-R antibody on already spread cells, BHK cells in adhesion medium or MEM were incubated on the designated ligand-coated substrata for 60 min at 37°C in a humidified incubator. At this time, >90% of the plated cells spread into the typical fibroblast morphology. The medium was removed from the dishes and fresh medium with antibodies was added. The incubations then were continued at 37°C for the designated time periods. Cell detachment was quantitated using cells that were grown for 16 h in the presence of 10 μCi/ml methyl-^{3}H-thymidine (sp act 5 mCi/mol, Amersham Radiochemicals). The harvested cells were washed three times with MEM and then incubated on the ligand-coated substrata. After treatment with anti-WGA-R, the detached cells and the cells remaining attached to the dishes were dissolved in 1% SDS. These samples then were mixed with 5 ml of Aquasol solution and radioactivity was determined in a Nuclear Chicago Mark II liquid scintillation spectrophotometer. The percent release of cells was calculated by taking the total counts recovered in the experiment minus the counts released, divided by the total counts, and this value was normalized to take into consideration the percent counts released in control experiments (2% at 1 h and 5% at 2 h).

Preparation of pFN and Lectin-coated Latex Beads

Polystyrene latex beads (0.76 μm, Dow Diagnostics, Indianapolis, IN.) were noncovalently coated with pFN using the method described previously (13). 8.3 × 10^9 beads (0.02 ml) were incubated with 0.6 ml pFN (120 U) for 10 min at 22°C. Subsequently, 32 mg of BSA in 1.6 ml PBS were added and the incubations continued an additional 10 min at 22°C. The pFN/BSA bead mixture then was sonicated for 5 s at 60 Watts (Heat Systems-Ultrasonics, Inc., Plainview, NY: microprobe) and used immediately. As shown previously (13), pFN-coated beads interact with cells similarly with or without removal of unbound pFN and BSA. Latex beads were coated with lectins as described previously (34). The concentration of lectin used in the incubations with the beads was 5.0 mg/ml of Con A or WGA. Since free lectin might have competed with the lectin-coated beads for cell binding sites, the lectin-coated beads were washed free of unbound lectin by centrifugation at 12,000 *g* for 10 min (Sorvall SS-34 rotor). No leakage of pFN or lectins from the beads occurred under the conditions used in these studies.

Antibody Blocking Assay

NP-40 was removed from the WGA-R preparation or an equivalent amount of WGA-agarose column buffer (dialyzed and lyophilized) as follows. An aliquot (0.25 ml) was mixed with 0.25 ml of 1% heat-denatured BSA (to prevent nonspecific interactions) and then incubated with 0.5 ml of packed SM-2 Bio-Beads (Bio-Rad Laboratories, Richmond, CA) for 15 min at 22°C on a New Brunswick R-2 shaker at 350 rpm. The SM-2 beads had been previously washed according to the method of Holloway (20). The receptor bead mixture was placed in a centrifugal microfilter (Bioanalytical Systems, Inc., West Lafayette, IN) and centrifuged for 4 min at 500 *g* (HL-4 rotor). Filtrates from the centrifugations were mixed with anti-WGA-R as described below.

Detergent extracted WGA-R or WGA-agarose column buffer was incubated with or without anti-WGA-R for 15 min at 22°C. Subsequently, the volume was brought to 0.3 ml by the addition of adhesion medium. BHK cells were resuspended in the various solutions and then incubated on pFN-coated substrata for 45 min at 37°C. It should be pointed out that failure to remove detergent from the preparation resulted in lysis of the cells in the incubations.

RESULTS

Isolation of WGA-R and Their Reactions with Antibodies

Wheat germ agglutinin receptors were isolated from octylglucoside extracts of BHK cells by affinity chromatography on WGA-agarose (see Materials and Methods). Some properties of the fractions obtained by the isolation procedure are shown in Table I. The final preparation of receptors contained slightly less than 0.5% of the starting Lowry protein and [^{35}S]L-methionine label. The final preparation also contained ~7% of the carbohydrate in the starting material based upon incorporation of [^{3}H]D-glucosamine. The higher level of carbohydrate was expected since the lectin column selects for carbohydrate-containing components.

A typical SDS PAGE profile of the glycopeptides in the WGA-R preparation is shown in Fig. 1*A*. A mixture of components was observed with molecular weights ranging from

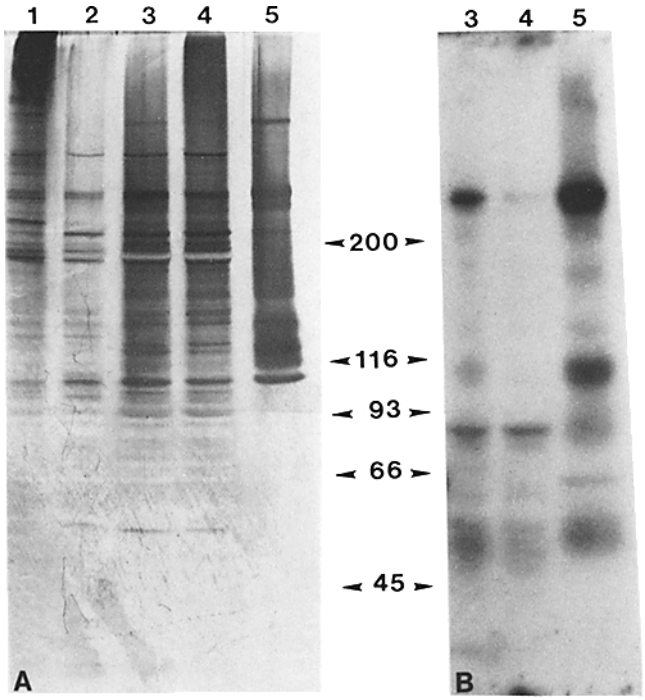

FIGURE 1 (*A*) SDS PAGE of the major fractions of a WGA-R preparation. Lanes: (*1*) Starting material, (*2*) Octylglucoside extract, (*3*) WGA-agarose column load, (*4*) WGA-agarose unbound material, and (*5*) WGA receptors. 25 μg of protein were loaded on each lane. (*B*) Autoradiograph of immune electroblot of WGA-agarose column load, unbound material, and receptor fractions shown in *A*. 25 μg of protein were loaded on each lane. After electrophoretic transfer of proteins (in 20 mM Tris-base, 150 mM glycine, 20% methanol electrode solution, 150 mA 1 h then 300 mA 1 h, 4°C) to nitrocellulose paper (Schleicher and Schuell), the paper was washed for 30 min at 37°C in 0.9% NaCl, 0.01 M Tris-HCl, 0.2% NP-40, 5% BSA, pH 7.4 (Tris-saline-BSA) and then incubated for 2 h at 22°C with 30 ml of Tris-saline-BSA containing 50 μg/ml anti-WGA-R IgG. The paper was rinsed briefly in 0.9% NaCl, 0.01 M Tris-HCl, pH 7.4 (Buffer A), washed for 20 min with two changes of 200 ml 0.9% NaCl, 0.01 M Tris-HCl, 0.2% NP-40, 0.25% sodium deoxycholate, 0.1% SDS, pH 7.4, rinsed with buffer A, and then incubated for 45 min at 22°C with 30 ml of Tris-saline-BSA containing 1 μg/ml ^{125}I-protein A (sp act 1,200 cpm/ng). The paper then was washed again as described above, dried and exposed to Kodax XAR-5 film on an intensifying screen for 16 h. See Materials and Methods for other details.

about 40 kdaltons to greater than 300 kdaltons (lane 5). Several components were considerably enriched compared to the starting material. There was no way of knowing which if any of the glycopeptides might be involved in fibronectin receptor function. Therefore, the entire mixture was used to immunize rabbits to prepare antibodies.

To determine which of the polypeptide components in the WGA-R preparation were bound by the antibodies that were produced, the electroblotting technique was used. This involved electrophoretic transfer of polypeptides from the SDS PAGE gels to nitrocellulose paper and incubation of the paper with anti-WGA-R followed by ^{125}I-protein A (Fig. 1 *B*). It should be noted that almost complete transfer of polypeptides from the gels to the nitrocellulose paper occurred as determined by silver staining (data not shown). The antibodies were found to react with eight major components of the WGA-R preparation of molecular weights approximately 48, 61, 83, 105, 120, 165, 210, and 230 kdaltons (Fig. 1 *B*, lane 5). No binding of nonimmune IgG to the components in the fractions of the WGA-R preparation was detected (data not shown).

Immunofluorescent Studies of Anti-WGA-R Binding to BHK Cells

Binding of anti-WGA-R to BHK cells was analyzed by indirect immunofluorescence staining. On intact cells, the antibody bound uniformly to the lower cell surfaces (i.e., focused at the plane of the substratum) (Fig. 2 *B*). On the upper cell surface, binding was most prominent in discrete regions that appeared to be microvilli (Fig. 2 *C*). When the cells were permeabilized with 0.1% Triton before staining, strong binding of the antibodies was observed in a juxtanuclear position,

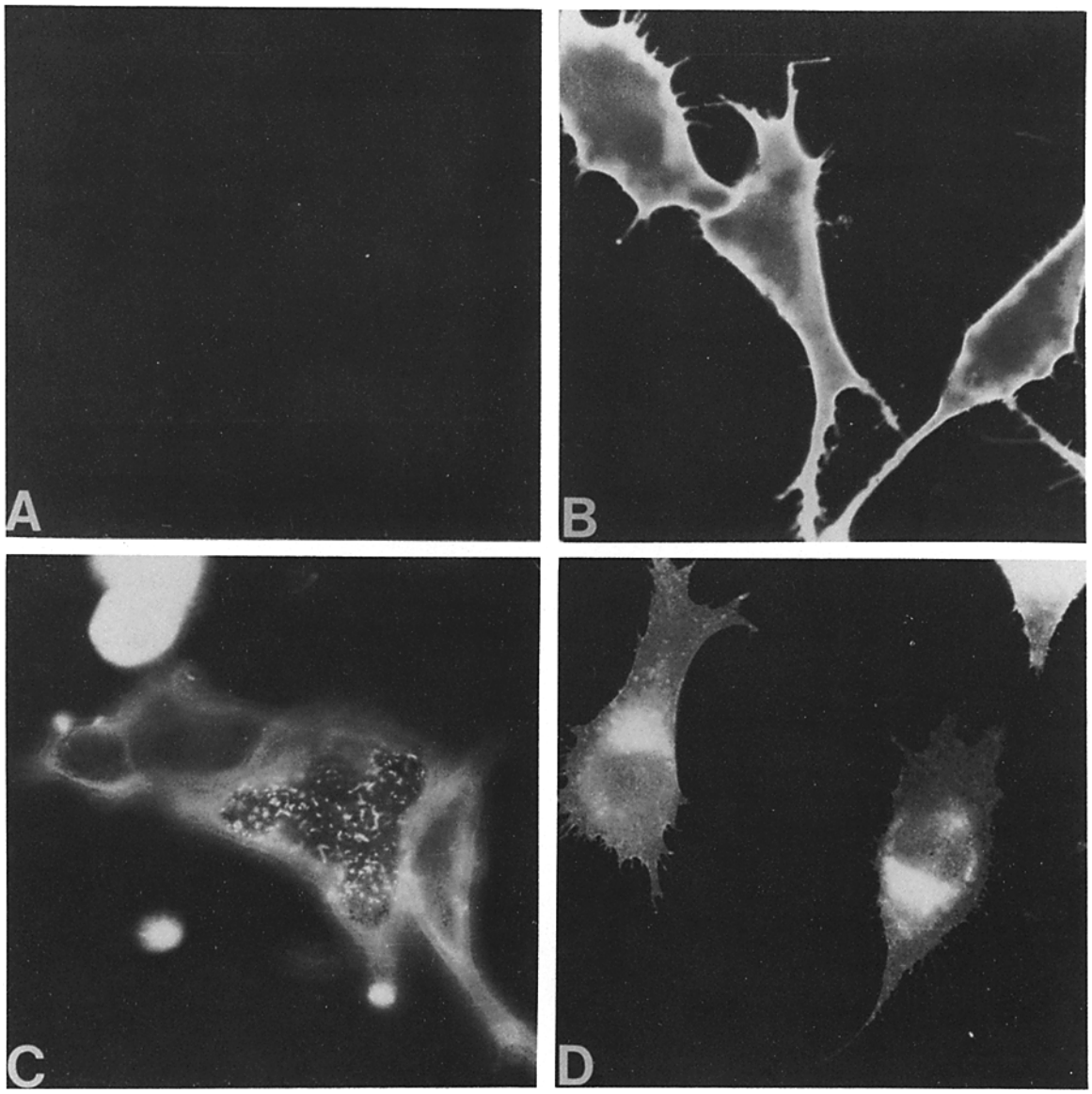

FIGURE 2 Distribution of anti-WGA-R binding sites on BHK cells. 2.5 × 10^5 BHK cells in 1 ml adhesion medium were allowed to spread on pFN-coated substrata. Subsequently, the cells were fixed and anti-WGA-R IgG binding was determined by indirect immunofluorescence. (*A*) There was no primary antibody. (*B*) Focus is at the level of the substratum. (*C*) Focus is at the upper cell surface. (*D*) The cells were permeabilized before antibody staining and focus is intracellular. Other details are in Materials and Methods. × 750.

which presumably is the location of the Golgi (Fig. 2 *D*). No fluorescent staining of the cells was observed with preimmune serum or when primary antiserum was omitted (Fig. 2 *A*).

Effect of anti-WGA-R on Cell Spreading

A series of experiments were then carried out to determine the effects of anti-WGA-R on cell spreading. BHK cells were treated with anti-WGA-R for 10 min at 4°C and then incubated on plasma fibronectin-coated substrata in the continued presence of the antibody. As shown in Fig. 3, the whole antiserum and the IgG fraction of the antiserum inhibited cell spreading (see also Fig. 7).

With IgG molecules it is possible to get cross-linking of cell surface components. This cross-linking might result in modulation of the cytoskeleton as has been reported with Con A (8, 36). This would not occur with Fab fragments. Therefore, Fab fragments of anti-WGA-R were prepared, isolated, and characterized as described in Materials and Methods. As shown in Fig. 3, Fab fragments also inhibited cell spreading, which demonstrates that inhibition by the antibodies is a result of blocking individual sites. It should be pointed out that cells treated with the antibodies were viable as determined by trypan blue exclusion and retention of lactate dehydrogenase.

In other experiments, the possibility was tested that any antibodies capable of binding to and covering any cell surface sites would inhibit cell spreading. To analyze this point, cells were incubated with the IgG fraction of an antibody prepared against lithium diiodosalicylate extracts of BHK cell membranes (anti-Lis). (This antiserum was generously provided by Dr. Clayton Buck, Wistar Institute Philadelphia, PA.) At concentrations similar to those used with anti-WGA-R, anti-Lis IgG bound uniformly to BHK cell surfaces as determined by indirect immunofluorescence, but had no effect on cell spreading on fibronectin-coated substrata (data not shown).

Experiments on cell spreading also were carried out to determine whether the effect of the antibodies was specific for the fibronectin receptor. As indicated earlier, BHK cells can use a variety of ligands other than fibronectin as cell spreading factors. Several of these ligands, concanavalin A (Con A), and polycationic ferritin (PCF), have been shown to promote cell spreading by interacting with cell surface receptors that are different from the fibronectin receptor based upon their proteolytic sensitivities (16, 34). If anti-WGA-R Fab fragments were inhibiting only fibronectin receptor function, then it was anticipated that the antibodies would not prevent cell spreading on substrata coated by other ligands. It was found, however, that anti-WGA-R Fab fragments inhibited cell spreading on Con A, PCF, or WGA-coated substrata at concentrations similar to those that inhibited cell spreading on fibronectin-coated substrata (data not shown).

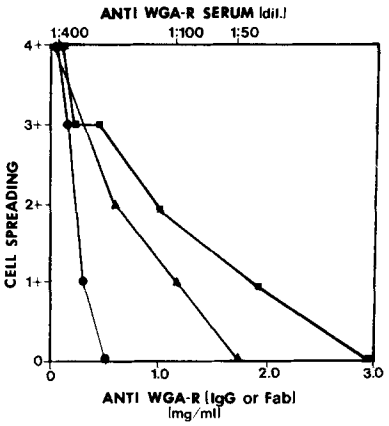

FIGURE 3 Effect of anti-WGA-R serum (▲), IgG (●), or Fab (■) on BHK cell spreading on pFN-coated substrata. 2.5 × 10^4 BHK cells in 0.1 ml adhesion medium were incubated for 10 min at 4°C with the designated concentrations of anti-WGA-R serum, IgG, or Fab and then the solutions were incubated on pFN-coated substrata. Other details are in Materials and Methods.

Effect of Anti-WGA-R on Cell Rounding and Detachment

Several other laboratories have described antibodies that cause rounding, and in some cases detachment, of previously spread cells (21, 38). Therefore, it was of interest to make a similar determination for anti-WGA-R. When anti-WGA-R IgG or Fab fragments were added to BHK cells that were previously spread on fibronectin-coated surfaces, complete cell rounding occurred within 2 h (see Fig. 4 *A* and *C*). Nonimmune IgG, on the other hand, had very little effect. Quantitative studies showed that anti-WGA-R IgG caused cell detachment faster and to a greater extent than anti-WGA-R Fab (Table II). Nevertheless, in the presence of the Fab fragments, a large percentage of the cells did detach from the substratum by 2 h of incubation.

The cells detached from the fibronectin-coated substrata by anti-WGA-R IgG were intact as determined by trypan blue exclusion and retention of lactate dehydrogenase. When these cells were washed and replated on fibronectin-coated substrata, they were found to attach by 24 h of incubation, but were only partially spread (data not shown).

It was curious that cell rounding and detachment required several hours. One explanation for this finding is that cell rounding requires metabolic activity. This suggestion has been made regarding the rounding and detachment effects of trypsin or divalent cation chelating reagents on spread cells (3, 36). When cells were treated with anti-WGA-R at 4°C, no rounding of cells occurred. If, however, the cells were then washed and transferred to 37°C, rounding was observed (data not shown). This observation is consistent with a requirement for metabolic energy in cell rounding but may also have been a result of decreasing cell surface receptor mobility because the lipid phase of the cells was frozen (25, 37).

To directly test the requirement for metabolic energy in cell rounding, experiments were carried out using the inhibitor, sodium azide. If azide was included in the incubations with the antibodies, the ability of the antibodies to cause cell rounding was markedly decreased (Fig. 4, compare *E* with *C*). When the azide and IgG-containing medium subsequently was removed and replaced with fresh medium, the cells rounded up (Fig. 4 *F*). Addition of azide alone had no effect on already spread cells (Fig. 4, compare *B* with *A*). Also, anti-WGA-R IgG was able to cause rounding of spread cells previously treated with azide and then washed (Fig. 4, compare *D* with *C*). These results, therefore, strongly suggest that rounding of spread cells caused by anti-WGA-R requires metabolic activity.

Experiments also were done to determine the effects of anti-WGA-R IgG and Fab fragments on cells that had been allowed to spread on other ligand-coated substrata. Concentrations of IgG or Fab similar to those that were effective with cells spread on fibronectin-coated substrata also caused rounding of cells spread on Con A-coated substrata (Fig. 5). Similar results were obtained with cells that were spread on polycationic ferritin or WGA-coated substrata (data not shown). It should be pointed out, however, that although the cells rounded up on these various ligand-coated substrata, no cell detachment was observed.

Effect of Anti-WGA-R on Ligand-coated Bead Binding to Cells

The results presented above are consistent with the view that anti-WGA-R antibodies interfere with fibronectin receptor function, but do not rule out the possibility that a cell surface site involved in coupling ligand-receptor complexes to the cell cytoskeleton is being blocked. This possibility could explain why the antibodies also interfered with the adhesion reactions involving ligands other than fibronectin. To analyze more directly the effect of anti-WGA-R on ligand receptors, experiments were done to determine whether the antibodies inhibited binding of ligand-coated latex beads to the cells. As described previously (13), this assay provides a measure of cell-surface-receptor activity without the complicating requirement for cytoskeletal activity.

As shown in Fig. 6, addition of anti-WGA-R IgG or Fab fragments inhibited the binding of fibronectin-coated beads to the cells. A large percentage of the cells bound 35 or more beads in the presence of nonimmune IgG or Fab fragments whereas no cells bound this many beads in the presence of the anti-WGA-R antibodies and most of the cells bound less than 6 beads. The results with WGA-coated beads were somewhat different. In this case, addition of anti-WGA-R Fab fragments caused an inhibition of bead binding, but addition of anti-WGA-R IgG did not. The ability of the Fab fragments to inhibit WGA-coated bead binding to cells was expected since the antibodies were prepared against WGA receptors. That

TABLE II

Effect of Anti-WGA-R IgG or Fab on BHK Cells Spread on pFN-coated Substrata *

Additions	Cells released‡	
	1 h	2 h
	%	
Anti-WGA-R IgG	71.5 ± 5.8	96.7 ± 0.6
Anti-WGA-R Fab	4.3 ± 0.4	51.5 ± 11.8
Nonimmune IgG	1.3 ± 0.8	10.2 ± 1.8

* 2.5×10^4 [^{3}H]thymidine-labeled BHK cells in 0.3 ml MEM were allowed to spread on pFN-coated substrata. Subsequently, the medium was removed and replaced with 0.3 ml of medium containing 1 mg/ml anti-WGA-R IgG, 3.0 mg/ml anti-WGA-R Fab, or 1 mg/ml nonimmune IgG. After additional incubations of 60 or 120 min, nonattached cells were resuspended by shaking the dishes for 10 s at 50 rpm (New Brunswick R-2 shaker, 1-inch stroke). Other details are in Materials and Methods.

‡ Average ± SD from duplicate experiments.

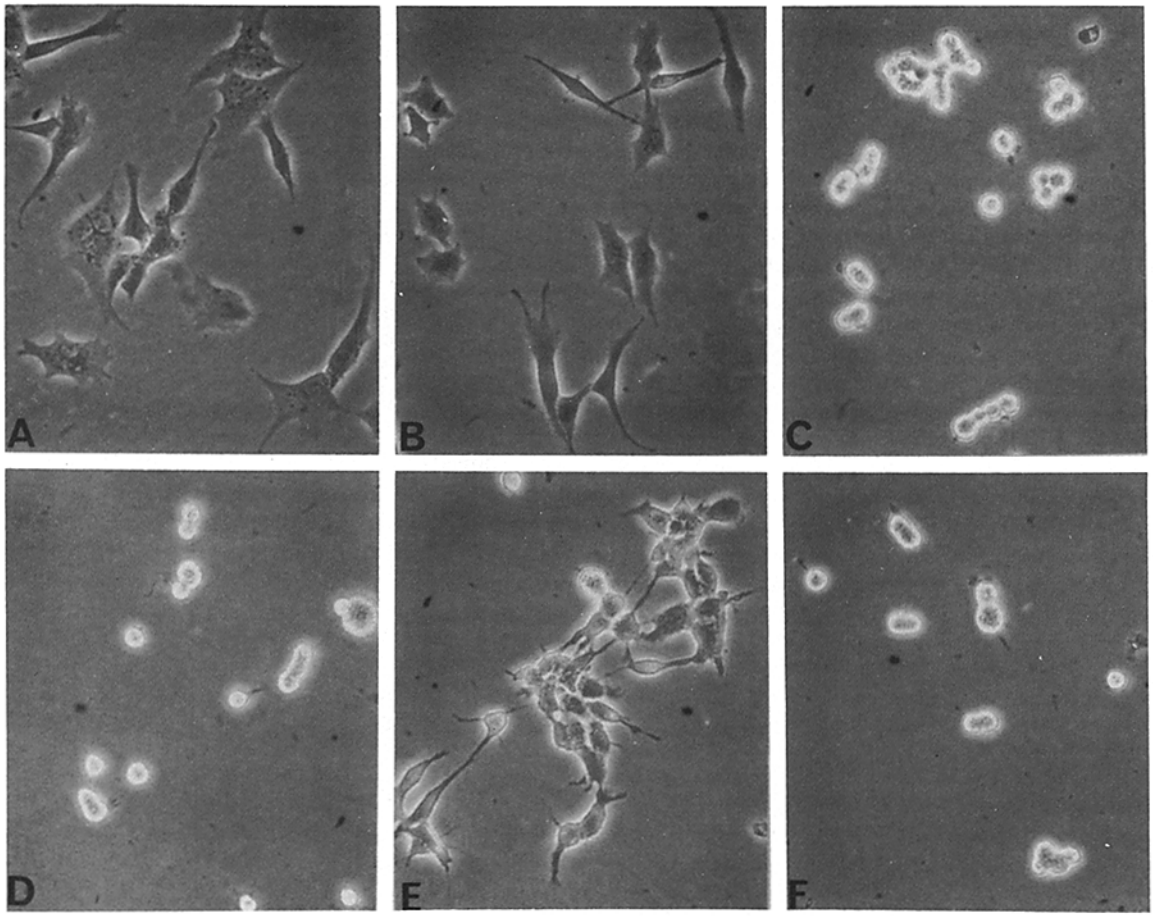

FIGURE 4 Effect of Na azide on anti-WGA-R induced rounding and detachment of BHK cells. 2.5×10^5 BHK cells in 1 ml of adhesion medium were allowed to spread on pFN-coated substrata. Subsequently, the medium was removed and replaced with 1 ml of adhesion medium containing no additions (*A*); 3 mM Na azide (*B*, *D*); 1 mg/ml anti-WGA-R IgG (*C*); or 3 mM Na azide plus 1 mg/ml anti-WGA-R IgG (*E*, *F*). These incubations were carried out for 60 min at 37°C in a humidified incubator. In *D*, the Na azide-containing medium was removed, the dish was rinsed with PS, and 1 ml of adhesion medium containing 1 mg/ml anti-WGA-R IgG was added. In *F*, the Na azide/IgG-containing medium was removed, the dish was rinsed with PS, and 1 ml of adhesion medium was added. These two incubations were then continued for an additional 60 min at 37°C. Other details are in Materials and Methods. × 200.

IgG did not inhibit was unexpected. One possibility is that there is binding of the WGA-coated beads to the carbohydrate portions of the IgG molecules bound to the cells. With the Con A-coated beads, neither anti-WGA-R IgG or Fab fragments inhibited bead binding. In contrast, addition of α-methyl mannoside, for which Con A has a high affinity, caused a marked reduction in binding of Con A-coated beads to the cells. The bead binding results indicate in a more direct way that the antibodies are interfering with fibronectin receptor function and WGA receptor function but not Con A receptor function. Similar experiments also were carried out with polycationic ferritin-coated beads but extensive nonspecific binding of the beads to the cells occurred under all conditions tested.

Blocking Anti-WGA-R Activity with WGA-R or by Absorption with Intact BHK Cells

To insure that the effects of anti-WGA-R were specific for components of WGA-R, a blocking assay was developed similar to that described by others (29). It was found that the ability of anti-WGA-R IgG to inhibit spreading of BHK cells (Fig. 7, compare *B* with *A*) was blocked if anti-WGA-R IgG was treated with the WGA-R preparation (Fig. 7 *C*). It should be noted that excess detergent was removed from the WGA-R preparation with SM-2 beads (see Materials and Methods). As a control, anti-WGA-R IgG was treated with detergent-containing buffer that had been extracted with SM-2 beads. This had no effect on the inhibitory activity of anti-WGA-R IgG (Fig. 7 *D*).

Since the WGA-R preparation was prepared from whole cell extracts, it was of interest to determine whether the relevant antibodies were directed at cell surface antigens. To make this determination, anti-WGA-R IgG (1 mg in 0.1 ml of adhesion medium) was absorbed with 10^7 BHK cells that had been fixed for 20 min at 22°C with 3.5% formaldehyde. The absorbed antibodies were no longer able to inhibit cell spreading on pFN-coated substrata or cause rounding and detachment of cells previously spread on pFN-coated substrata (data not shown).

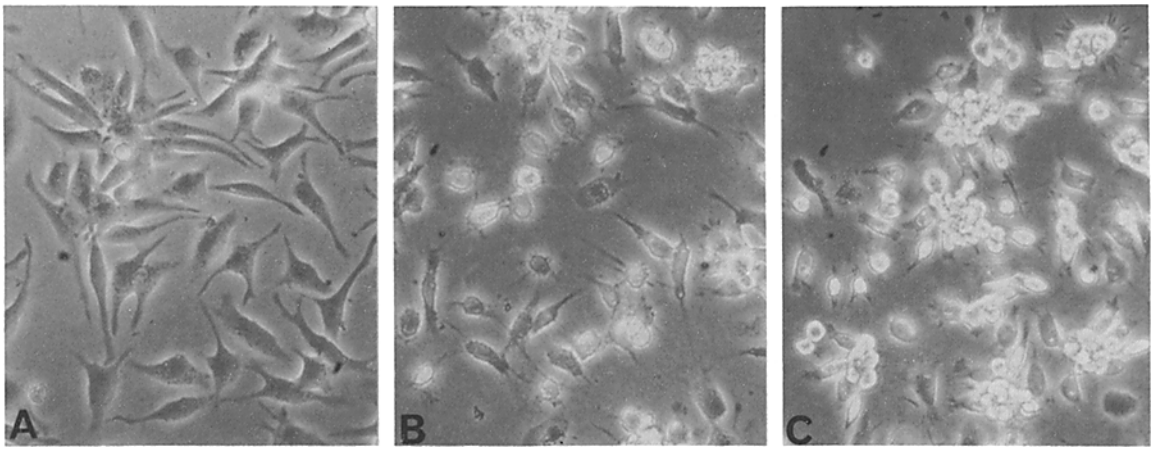

FIGURE 5 Effect of anti-WGA-R IgG or Fab on BHK cells spread on Con A-coated substrata. 2.5×10^4 BHK cells in 0.1 ml adhesion medium were allowed to spread on Con A-coated substrata (*A*). Subsequently, the medium was removed and replaced with 0.1 ml adhesion medium containing 0.5 mg/ml anti-WGA-R IgG (*B*) or 3 mg/ml anti-WGA-R Fab (*C*). These incubations were carried out for 60 min at 37°C. Other details are in Materials and Methods. × 225.

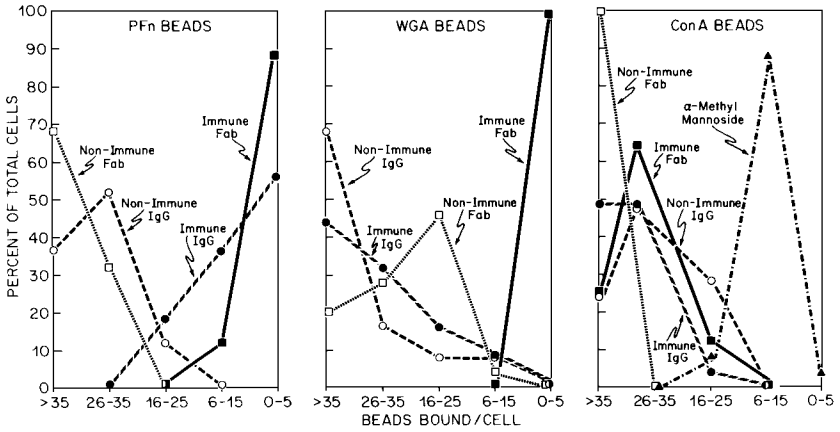

FIGURE 6 Binding of ligand-coated beads to anti-WGA-R IgG or Fab treated cells. 2.5×10^4 BHK cells in 0.1 ml adhesion medium were allowed to attach to pFN-coated substrata for 5 min at 37°C. Subsequently, the medium was removed and replaced with 0.1 ml adhesion medium containing 1 mg/ml immune IgG, 3 mg/ml immune Fab, 1 mg/ml nonimmune IgG, or 3 mg/ml nonimmune Fab, as designated. For experiments to be carried out with Con A-coated beads an additional incubation with 25 mM α-methyl mannoside was included. Incubations of the cells with antibodies or α-methyl mannoside were done for 10 min at 4°C. At this time the medium from each dish was removed and mixed with 0.1 ml of ligand-coated beads. A 0.1-ml aliquot of this antibody/ligand-coated bead mixture was added back to the respective dishes and the incubations were continued for 10 min at 22°C while shaking at 50 rpm (New Brunswick R-2 shaker, 1-inch stroke). Subsequently, the cells were rinsed well with PS, fixed with 3% formaldehyde, coated with one drop of 0.1 M Tris, 90% glycerol (pH 9.4) and coverslipped. Beads bound to cells were counted under dark field optics. The researcher who made the counts was unaware of the experimental design. Other details are in Materials and Methods.

DISCUSSION

The studies reported in this paper were done to investigate further our previous observation that WGA inhibited fibronectin receptor function (34). The major results are summarized in Table III. Based upon the ability of anti-WGA-R Fab fragments to inhibit cell spreading on fibronectin-coated substrata, to cause rounding and detachment of cells previously spread on fibronectin-coated substrata, and to inhibit binding of fibronectin-coated beads to cells, it seems likely that the antibodies are directed against the fibronectin-receptor sites. Nevertheless, the possibility cannot be excluded that the antibodies are binding to cell surface components adjacent to the fibronectin receptor and causing inhibition by steric hindrance.

The WGA-R preparation is heterogeneous and the antibodies bind to more than one component. Therefore, the specific component(s) that is important cannot be identified as yet. It is significant, however, that addition of the WGA-R preparation to anti-WGA-R IgG blocked the ability of the antibodies to inhibit cell spreading. In addition, absorption of anti-WGA IgG with intact fixed cells also removed the inhibitory activity of the antibodies. These results indicate the specificity of the anti-WGA-R for WGA-R exposed on the cell surface.

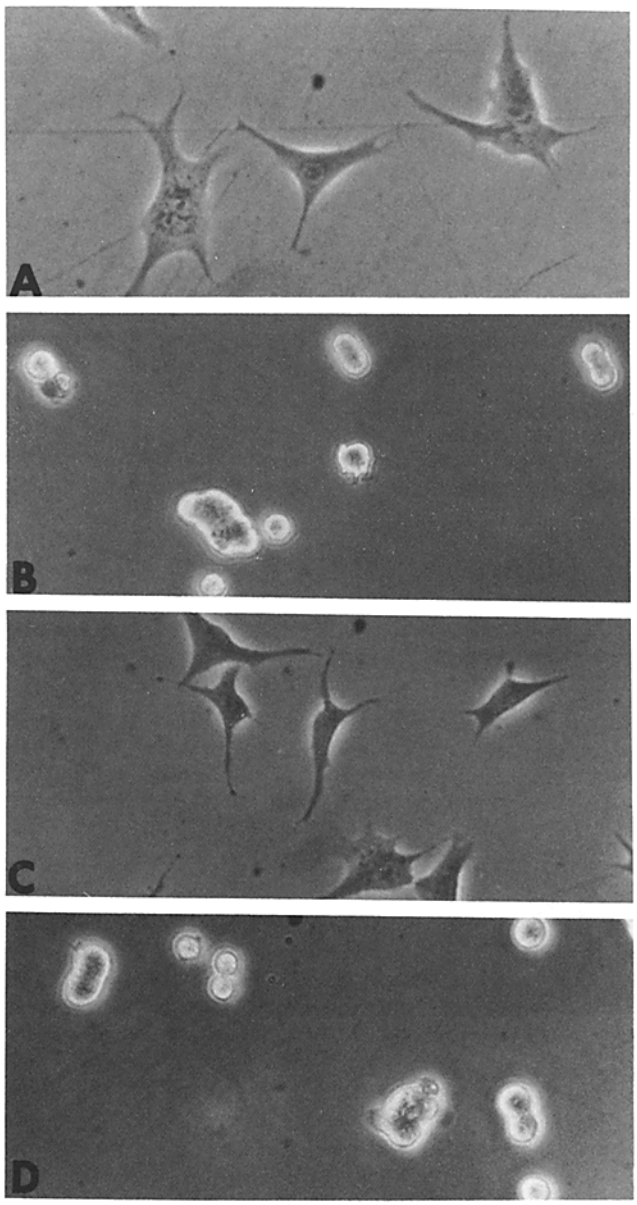

FIGURE 7 Effect of WGA-R on anti WGA-R activity. BHK cells (7.5×10^4) were incubated on pFN-coated substrata in 0.3 ml of adhesion medium containing: (*A*) WGA-agarose column buffer; (*B*) anti WGA-R (0.15 mg); (*C*) WGA-R (0.057 mg) plus anti WGA-R (0.15 mg); (*D*) WGA-agarose column buffer plus anti WGA-R (0.15 mg). Other details are in Materials and Methods.

TABLE III

Effect of Anti-WGA-R Fab on Various Adhesion Interactions *

Ligand on substratum or beads	Inhibition of cell spreading	Rounding (R) or detachment (D) of spread cells	Inhibition of bead binding
pFN	+	D	+
WGA	+	R	+
ConA	+	R	−
PFC	+	R	

* See text for details.

One of the components of the WGA-R preparation to which the antibodies are directed is a 48-kdalton glycopeptide. Several other laboratories have presented evidence indicating that glycopeptides in this molecular weight range on the undersurfaces of spread cells can be selectively labeled by either lactoperoxidase treatment (6) or by covalent binding to fibronectin or other ligands to which a cross-linking reagent has been attached (2). In addition, recent antibody absorption experiments, done with trypsin- and chymotrypsin-treated cells have shown that the low molecular weight components of WGA-R (<120 kdaltons) appear to be important in mediating cell adhesion to fibronectin-coated substrata (Oppenheimer-Marks and Grinnell, Manuscript in preparation).

The requirement for metabolic activity in cell rounding induced by anti-WGA-R is noteworthy. One explanation is that rounding requires cell motility. It may be that the antibodies prevent the formation of new cell-substratum attachments so that as old ones are broken, the cells eventually are released. Consistent with this view, preliminary analysis of antibody-induced cell rounding by time lapse microscopy indicates that cells become detached segmentally beginning at the tips of lamellipodia, and retraction of the lamellipodia occurs at one end of the cell before the other.

An important question remains as to why the antibodies inhibit cell spreading and cause cell rounding on Con A and PCF-coated substrata if the antibodies do not block the cell surface receptors for these ligands. Perhaps related to this is the question why the antibodies cause cell detachment from fibronectin-coated substrata but only cause cell rounding on the other ligand-coated substrata. The explanation for these findings may be that the antibodies are also directed against a common ligand-receptor/cytoskeletal coupling site. That such a site functions in cell-substratum adhesion has been inferred previously (16). Binding of this site would prevent ligand-receptor complexes from becoming linked to the cytoskeleton. One possibility is that the fibronectin receptor site is also the common ligand-receptor coupling site. The present data neither support nor contradict this idea. Previous studies, however, have shown that fibronectin receptors are no longer detectable on the surfaces of spread cells regardless of the ligand on which they are spread (34).

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