Fibroblast Receptor for Cell-Substratum Adhesion: Studies on the Interaction of Baby Hamster Kidney Cells with Latex Beads Coated by Cold Insoluble Globulin (Plasma Fibronectin)

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ABSTRACT   Studies were carried out on the interactions of uncharged latex beads (0.76 μm) with baby hamster kidney cells. Binding of beads to the cells occurred if the beads were coated by cold insoluble globulin (CIG) (plasma fibronectin) but not if the beads were coated by bovine albumin. Bovine albumin-coated beads did not bind to the cells even in the presence of excess CIG in the incubation medium. Binding of beads occurred randomly over the entire surfaces of cells in suspension. However, cell receptors for CIG beads were no longer detectable on the upper surface of cells spread on CIG-coated tissue culture dishes. Binding of CIG beads to cells occurred at all temperatures tested from 4° to 37°C but the rate was lowest at 4°C. At 37°C, binding was accompanied by endocytosis and the beads were found inside vesicles which appeared to be lysosomes. There was also release of radioactivity from radiolabeled CIG beads during incubation with the cells at 37°C. Binding of CIG beads to cells did not require divalent cations. Finally, the cell receptor for CIG beads was lost after cell trypsinization. The data are discussed in terms of current ideas about the basis for cell adhesion.

Cell-substratum adhesion plays a fundamental role in the movements of cells that occur during embryogenesis, wound healing, and metastasis (for a comprehensive review see reference 12). Evidence reported from this and other laboratories suggests that adhesion is mediated by ligand-receptor-like interactions between cell surface components and appropriate substratum sites (15, 22, 23, 37).

There has recently been considerable interest in the role of cold insoluble globulin (CIG) (plasma fibronectin) in cell adhesion to extracellular substrata. This protein has been demonstrated to be involved in fibroblast adhesion to collagen (16, 26, 27) and to fibrin (17) as well as to material surfaces (e.g., plastic) (11, 14). CIG is also involved in the adhesion of cells other than fibroblasts (1, 2, 22, 33) and in the opsonization of particles for phagocytosis by those macrophages that constitute the reticuloendothelial system (30, 36).

Direct analysis of the fibroblast surface receptor for CIG has been problematic. Traditionally, analysis of receptors requires the demonstration of specificity and saturability of binding. This has not been possible because CIG appears to be func-tional with cells only if the CIG is in the solid state, i.e., adsorbed as part of a substratum (12). The binding interaction between soluble CIG and fibroblasts appears to be weak; however, when CIG is adsorbed on a material surface, it acts as if it were multivalent. As a result, the effective strength of the interaction with cells is increased, probably because of the cooperative effect of multiple interactions occurring simultaneously (12, 39). It has recently been estimated that the complete spreading of BHK cells requires ~45,000 CIG molecules beneath each cell (23).

CIG closely resembles the cell surface protein fibronectin (for recent reviews see references 41 and 44). Based upon the ability of "soluble" fibronectin to agglutinate fibroblasts (45), it seems likely that fibronectin interacts with the cell surface. However, fibronectin has a tendency to self aggregate under physiological conditions (unlike CIG). Therefore, it may be the aggregated form of fibronectin that is active in agglutination (21). This form would be multivalent in the sense that there would be more than one fibronectin molecule per aggregate.

In the absence of a direct methodology for studying CIG

interactions with the cell surface, a variety of indirect approaches has been used. Based upon these studies, the following have been implicated in the binding of CIG to its cell surface receptor: glycosaminoglycans (3, 4), ricin receptors (24), actin stress fibers (25, 38), and glycolipids (28). We have recently developed a direct approach for studying CIG receptors on the cell surface. This involves the use of a multivalent form of CIG that has been prepared by adsorbing many CIG molecules to the surfaces of 0.76-μm latex beads. This manuscript describes studies on the interaction of baby hamster kidney (BHK) cells with these CIG-coated latex beads.

MATERIALS AND METHODS

Preparation of CIG

CIG was prepared by salt precipitation and ion-exchange chromatography as described previously (15); however, the heat step was omitted which improved recovery (17). The specific activities of the CIG preparations ranged from 200 to 400 U/mg. 1 U of activity is defined as the amount of CIG required to promote complete spreading of BHK cells in a standardized assay (11, 18).

Radiolabeling Procedure

Radiolabeled CIG (^{3}H-CIG) and bovine serum albumin (BSA) were prepared by the reductive alkylation technique (35). To a 5.0-ml solution of protein (5 mg/ml) in 0.05 M Na borate, 0.089 ml of [^{3}H]formaldehyde (New England Nuclear, Boston, Mass.; 85 mCi/mmol) was added. The reaction was carried out for 30 min at 4°C and stopped by three successive additions of 0.1 ml NaBH$_4$ (12.8 mg/ml in H$_2$O). Subsequently, the reaction mixtures were diluted to 15 ml with 0.15 M NaCl, 0.01 M Tris (pH 7.2), and dialyzed against 2 liters of the Tris-saline buffer. To further purify the ^{3}H-CIG, the dialyzed preparation was repurified on a gelatin-Sepharose column (6), except that the bound ^{3}H-CIG was eluted with 1 M NaBr, 20 mM Na acetate (pH 5) (5). The peak fractions of the eluted ^{3}H-CIG were combined, dialyzed against Tris-saline, and stored at -20°C. The ^{3}H-CIG preparations generally had a sp act of ~4,000 cpm/μg protein and the biological (spreading) activity of the CIG remained the same. The radiolabeled BSA preparation had a sp act of 3,000 cpm/μg.

Polystyrene Beads

Polystyrene latex beads, diameter 0.76 μm, were obtained from Dow Chemical Co. (Indianapolis, Ind.). The concentration of beads was 4.14 × 10^{11} beads/ml according to the manufacturer.

Preparation of CIG/BSA and BSA/CIG Bead Mixtures

To obtain CIG/BSA beads, 0.005 ml of beads was incubated with CIG (~0.15 mg) in 0.15 ml phosphate saline (0.15 M NaCl, 0.001 M Na phosphate, pH 7.2) for 10 min at 22°C. Subsequently, 0.4 ml of BSA (20 mg/ml in phosphate saline) was added to the incubation which was continued for an additional 10 min. The preparation was sonicated for 5 s at 60 W with a microprobe (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.) and the mixture was used immediately in cell-bead binding experiments. BSA/CIG bead mixtures were prepared as described above, except that the beads were treated with BSA and then with CIG.

Conditions for Binding CIG/BSA and BSA/CIG Beads with BHK Cells

The incubation mixtures generally contained 2.5 × 10^6 BHK cells in 0.5–1.0 ml of adhesion medium (150 mM NaCl, 3 mM KCl, 1 mM CaCl$_2$·2 H$_2$O, 0.5 mM MgCl$_2$· 6H$_2$O, 6 mM Na$_2$HPO$_4$, pH 7.3) to which were added ~10^9 CIG/BSA beads/ml (or BSA/CIG beads). Shaking at 150 rpm was carried out during the incubation on a reciprocating shaker (New Brunswick Scientific Co., Edison, N. J.) (1-inch stroke). At the end of the incubations, the cell bead mixtures were centrifuged for 2 min at 2,000 rpm (Sorvall HL 4-OC rotor; DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.). The cells were then washed with 5 ml of phosphate saline, recentrifuged, and resuspended in a minimal volumn of 3% formaldehyde or 3% glutaraldehyde in 0.1 M Na phosphate (pH 7.2). Then, the fixed cells were processed for fluorescence microscopy or electron microscopy.

Preparation of ^{3}H-CIG Beads

To obtain ^{3}H-CIG beads, 0.1 ml of beads was mixed with ^{3}H-CIG (~1 mg) in 1.5 ml of Tris-saline buffer. After incubation at 22°C for 10 min, 4.0 ml of BSA (20 mg/ml) was added. The incubation mixture was diluted to 40 ml with phosphate saline and centrifuged at 10,000 rpm for 20 min (Sorvall HB4 rotor). The beads were resuspended in 2.5 ml of phosphate saline and sonicated for 5 s at 60 W. The dispersed bead suspension was again diluted to 40 ml, centrifuged, resuspended in 2.5 ml of phosphate saline, and stored at 4°C. Before cell-binding experiments, aliquots of the beads were sonicated. To determine the percent of radioactivity released from the beads, aliquots were centrifuged (microfuge; Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 4 min. Less than 1% of the radioactivity remained in the supernate. The amount of radioactivity associated with the beads varied with the specific radioactivity of the CIG preparations. The average was 1.56 ± 0.65 (SD) μg of ^{3}H-CIG bound per 10^8 beads.

Conditions for Binding ^{3}H-CIG Beads with Cells

The incubation mixtures generally contained 2.5 × 10^6 cells in 0.75 ml of adhesion medium to which were added ~10^9 ^{3}H-CIG beads. Shaking was carried out as with CIG/BSA bead mixtures. At the end of the incubations, 0.05 ml of each incubation mixture was sampled to determine total ^{3}H-CIG in the experiments. Another aliquot (0.125 ml) of each incubation mixture was centrifuged for 4 min in a Beckman microfuge, and 0.1 ml of this supernate was sampled to determine the amount of nonsedimentable radioactivity released from the ^{3}H-CIG beads. A final aliquot (0.6 ml) of each incubation mixture was layered onto 1.0 ml of BSA (50 mg/ml in phosphate saline) and centrifuged for 2 min at 2,000 rpm (Sorvall HL4-OC). Unbound beads remained in the supernate whereas cells and cells with bound beads sedimented. The pellets were resuspended in 1.0 ml of H$_2$O and the radioactivity was determined. Samples for radioactive determination were mixed with 10 ml of Aquasol (New England Nuclear) and counted in a Nuclear-Chicago Mark II scintillation spectrophotometer (Nuclear-Chicago Corp., Des Plaines, Ill.).

Cells

BHK cells (subline BHK-21-13s) were a gift from Dr. Adrian Chappel (Center for Disease Control, Atlanta, Ga.). The cells were grown in suspension culture in Eagle's mimimum essential medium (spinner modified; Grand Island Biological Co. [GIBCO], Grand Island, N. Y.) with double the concentration of amino acids (except 1 × glutamine) and vitamins, and supplemented with HEPES buffer (20 mM) (Sigma Chemical Co., St. Louis, Mo.), 0.1 g/liter ferric nitrate, 2 g/liter dextrose, 10% tryptose phosphate, and 10% fetal calf serum (GIBCO). The final sodium bicarbonate concentration was adjusted to 0.5 g/liter. Cells were harvested from suspension cultures by centrifugation at 500 g for 2 min (Sorvall HL-4 rotor). They were then washed with 5 ml of adhesion medium, recentrifuged, and used as described. BHK cells grown in suspension do not have cell surface fibronectin as determined by immunofluorescence analysis (13).

RESULTS

Preparation of a Multivalent Form of CIG

Initial experiments were carried out to prepare a multivalent form of CIG by chemical cross-linking of CIG molecules using glutaraldehyde or a soluble carbodiimide. These treatments resulted in biological inactivation of the CIG molecules.

A subsequent approach to preparing multivalent CIG was to adsorb the CIG onto the surfaces of small latex beads. Preliminary experiments indicated that CIG adsorbed well to both negatively charged beads and uncharged beads. However, in the absence of CIG the negatively charged beads were able to bind nonspecifically to the surfaces of BHK cells even with excess BSA in the medium. On the other hand, the nonspecific binding of uncharged beads was prevented by BSA. Therefore, attention was focused on the interaction of cells with uncharged latex beads.

Adsorption of CIG to Latex Particles

The conditions under which CIG was adsorbed to the latex beads are described in Materials and Methods. CIG adsorbed

to the bead surfaces within minutes, which was similar to our previous findings with CIG on tissue culture surfaces (18). In most experiments, the ratio of CIG concentration to bead surface area was chosen such that CIG was in excess of the concentration required to saturate the bead surfaces. Once the beads were saturated with CIG they were unable to adsorb BSA as determined by indirect immunofluorescence analysis with anti-BSA and by direct binding measurements with radio-labeled BSA. Conversely, it was possible to prepare beads whose surfaces were saturated with BSA and unable to subsequently bind CIG.

In experiments using radiolabeled CIG, it was determined that at saturation there was ~870 ng of CIG adsorbed per cm^2 of bead surface area, which is in reasonable agreement with the recently published figure of 698 ng/cm^2 on tissue culture plastic (23). Based on a molecular weight of 440,000, a density of 870 ng/cm^2 calculates to be ~21,000 CIG molecules per bead.

Light Microscope Analysis

In the first experiments, latex beads were incubated with varying concentrations of CIG followed by excess BSA. Then the CIG/BSA bead mixtures were incubated with the cells. The addition of CIG to the beads resulted in some bead aggregation and therefore, the bead mixtures were briefly sonicated immediately before addition to the cells.

No binding of beads to cells occurred with beads that had been incubated with 2% BSA alone. As the CIG concentration to which 2×10^9 beads were exposed before BSA treatment was increased from 8 to 32 U in a volume of 0.03 ml, the ability of the beads to interact with the cells was markedly enhanced. If the order of pretreatment with CIG and BSA was reversed, i.e., BSA before CIG (BSA/CIG beads), then no binding of the beads to the cells occurred even though excess CIG was present in the incubation medium. These results demonstrate the specificity of the bead-cell interaction and are consistent with the previous finding that adhesion of cells to CIG-coated culture dishes requires the presence of CIG on the culture dish surface, not just in the medium (12, 18).

Incubation of CIG/BSA bead mixtures with cells resulted in binding of beads at 4, 22, or 37°C. On the other hand, binding of BSA/CIG beads to the cells did not occur at any temperature. Maximum coverage of the cells by CIG/BSA beads in a 10-min, 22°C incubation required a bead concentration of ~3 $\times 10^9$ beads/ml. This was a bead to cell ratio of 1,000:1. Similar coverage of cells at 4°C required a 10-fold higher bead concentration.

In Fig. 1, results are presented from an experiment in which cells were treated with mixtures of CIG/BSA beads for 10 min at 22°C after which a portion of the cells was fixed and the remainder washed and incubated for an additional 50 min at 37°C. Subsequently, the cells were fixed and stained for indirect immunofluorescence with anti-CIG. After 10 min the CIG/BSA beads were distributed around the entire cell surface as observed by phase-contrast (Fig. 1 A), dark-field (Fig. 1 B), and fluorescence (Fig. 1 C) microscopy. However, after the second incubation, the beads appeared to be inside the cells and not on the cell surfaces (Fig. 1 D and E) and were no longer accessible to anti-CIG (Fig. 1 F). These results suggested that the latex beads were being endocytosed. This was later confirmed by electron microscope studies (below). In other
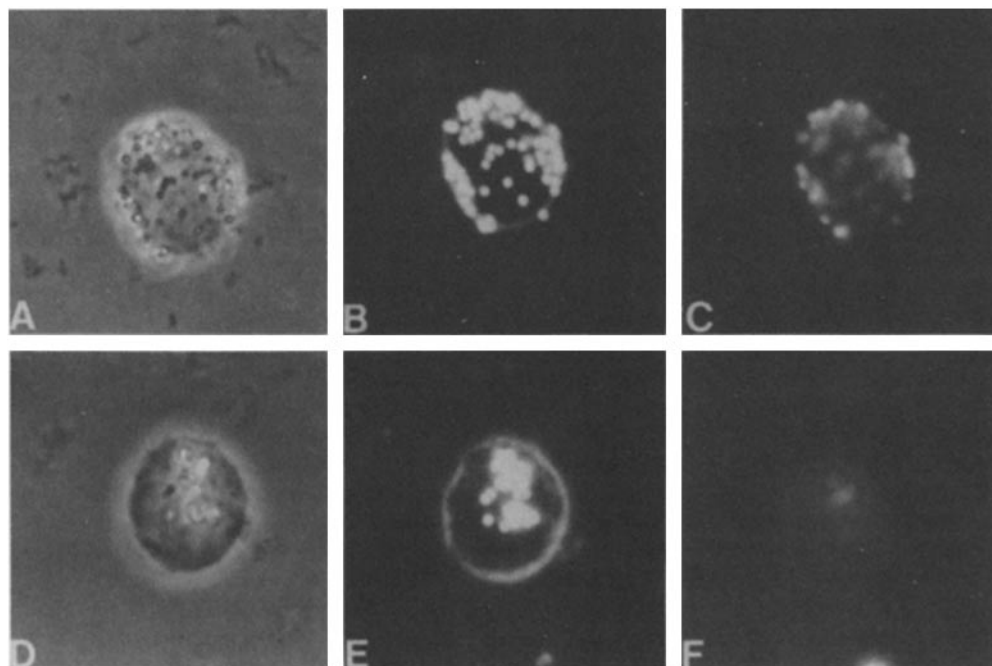

FIGURE 1   Binding and endocytosis of CIG/BSA beads by BHK cells. Cells were incubated with CIG/BSA beads for 10 min at 22°C (A–C) after which a portion of the cells was washed and fixed with formaldehyde while the remainder was resuspended in fresh adhesion medium and permitted to incubate an additional 50 min at 37°C (D–F). At the end of the incubations, cells were washed, fixed with formaldehyde, and attached to dishes for immunofluorescence as follows: dishes were incubated with polycationic ferritin (1 mg/ml, Miles Laboratories Inc., Elkhart, Ind.) for 10 min at 22°C, rinsed, and incubated with cells for 60 min at 37°C in a humidified incubator. Subsequently, samples were processed for indirect immunofluorescence with the immunoglobulin fraction of a monospecific anti-CIG antiserum as described previously (13). (A and D: phase contrast; B and E: dark field; C and F: fluorescence). Other details are in Materials and Methods and Results. X 1,100.

experiments it was found that little endocytosis of CIG/BSA beads occurred at 22° or 4°C and endocytosis of BSA/CIG beads was not observed under any incubation conditions.

Electron Microscope Analysis

The appearance of CIG/BSA beads on the cell surfaces was readily observed by scanning electron microscopy after a 10-min incubation at 22°C (Fig. 2A). At high magnification the beads appeared relatively round and smooth and were interspersed among the cell microvilli (Fig. 2B). However, when cells were first incubated with CIG/BSA beads for 10 min, washed, and then incubated for an additional 50 min at 37°C, there were no beads observed on the surfaces (Fig. 2C).

Similar results were found by transmission electron microscopy. After 10 min at 22°C the beads were on the cell surface (Fig. 3A, arrows) and in most cases were associated with microvilli (Fig. 3C, arrow) or appeared to be in newly forming endocytotic vesicles (Fig. 3D, arrow). After an additional 50 min at 37°C, the CIG/BSA particles were inside the cells (Fig. 3B) and often observed fusing with multivesicular bodies (Fig. 3E, arrow) or in structures which had the morphological appearance of secondary lysosomes (Fig. 3F, asterisk).

Aggregation of BHK Cells

During the 37°C experiments described above, substantial cell aggregation was observed by the end of the incubations. When cells were incubated in the presence of CIG/BSA beads at 37°C, no aggregation was apparent after 1 min of incubation (Fig. 4A). Some aggregation of cells was seen after 10 min and aggregation was extensive after 1 h (Fig. 4B). Similar aggregation did not occur with beads alone or with the CIG/BSA mixture in the absence of beads. Unlike fibronectin, CIG does not cause cell aggregation (43).

Binding of Beads to Cells Previously Spread on CIG-Coated Substrata

Experiments were carried out in which cells were permitted to attach and spread for various time periods on culture dishes pretreated with 20 U/ml CIG and then the attached cells were incubated with CIG/BSA beads. Cells incubated with the substratum for only 5 min did not spread and beads bound all over the cell surfaces. Cells incubated on a CIG substratum for 60 min were mostly spread and these cells did not bind CIG/BSA beads.

Experiments were also carried out in which cells were permitted to attach and spread for 45 min on culture dishes coated with different amounts of CIG after which the attached cells were incubated with CIG/BSA bead mixtures. Cells attached to uncoated dishes did not spread and beads bound all over the surfaces of the cells (Fig. 5A). Cells attached to culture dishes pretreated with CIG at 0.5 U/ml were partially spread and beads were able to bind to these cells also (Fig. 5B). Cells were completely spread on dishes coated with CIG at 4 U/ml and in this case the ability of beads to bind to the cells was much reduced but not absent (Fig. 5C). With cells spread on dishes coated with CIG at 16 U/ml, beads were no longer able to bind to the cells (Fig. 5D).

In other experiments, cells were pretreated with CIG/BSA beads (2.65 × 10⁹ beads/ml for 10 min at 22°C) and then plated out on culture dishes coated with 2 U/ml of CIG. There was a marked inhibition in cell spreading of the bead-coated cells compared to untreated controls.
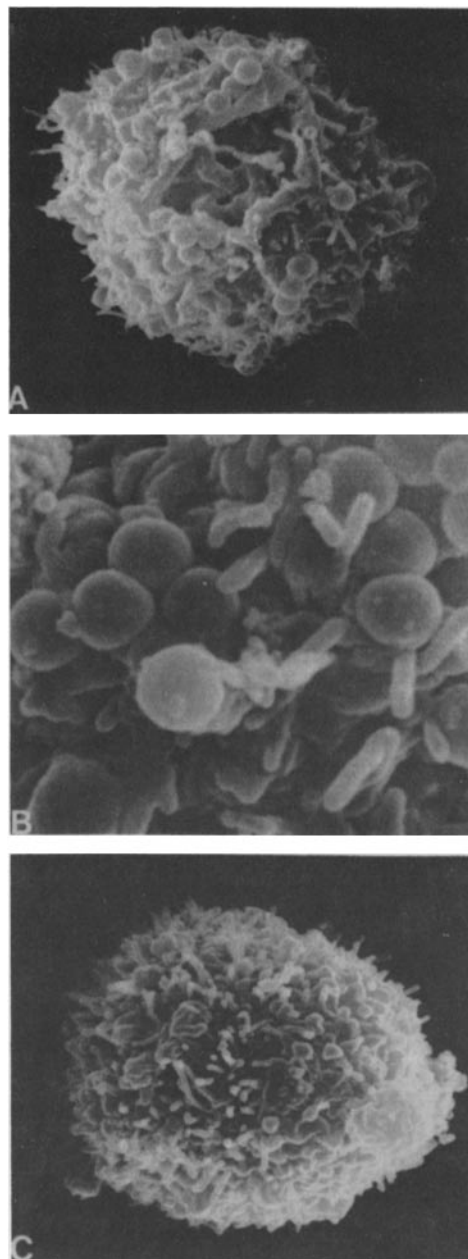

Figure 2 SEM of BHK cells incubated with CIG/BSA beads. Cells were incubated with CIG/BSA beads for 10 min at 22°C (A and B) after which a portion of the cells was washed and fixed with glutaraldehyde while the remainder was resuspended in fresh adhesion medium and permitted to incubate for an additional 50 min at 37°C (C). Fixed cells were attached to glass coverslips coated with polycationic ferritin and processed for SEM as described previously (13). Other details are in Materials and Methods and Results. (A) × 4,300; (B) × 14,000; (C) × 4,300.

Effect of Trypsin on Binding of CIG/BSA Beads to BHK Cells

We have previously presented indirect evidence that trypsin removes the adhesion receptor from BHK cells (19). Therefore, it was of interest to determine if the receptor for CIG/BSA bead binding was trypsin sensitive. Treatment of cells with trypsin at 0.1 mg/ml for 10 min at 37°C completely prevented subsequent binding of beads (Fig. 6B compared to 6A). Reduced binding of beads was observed if cells were first treated
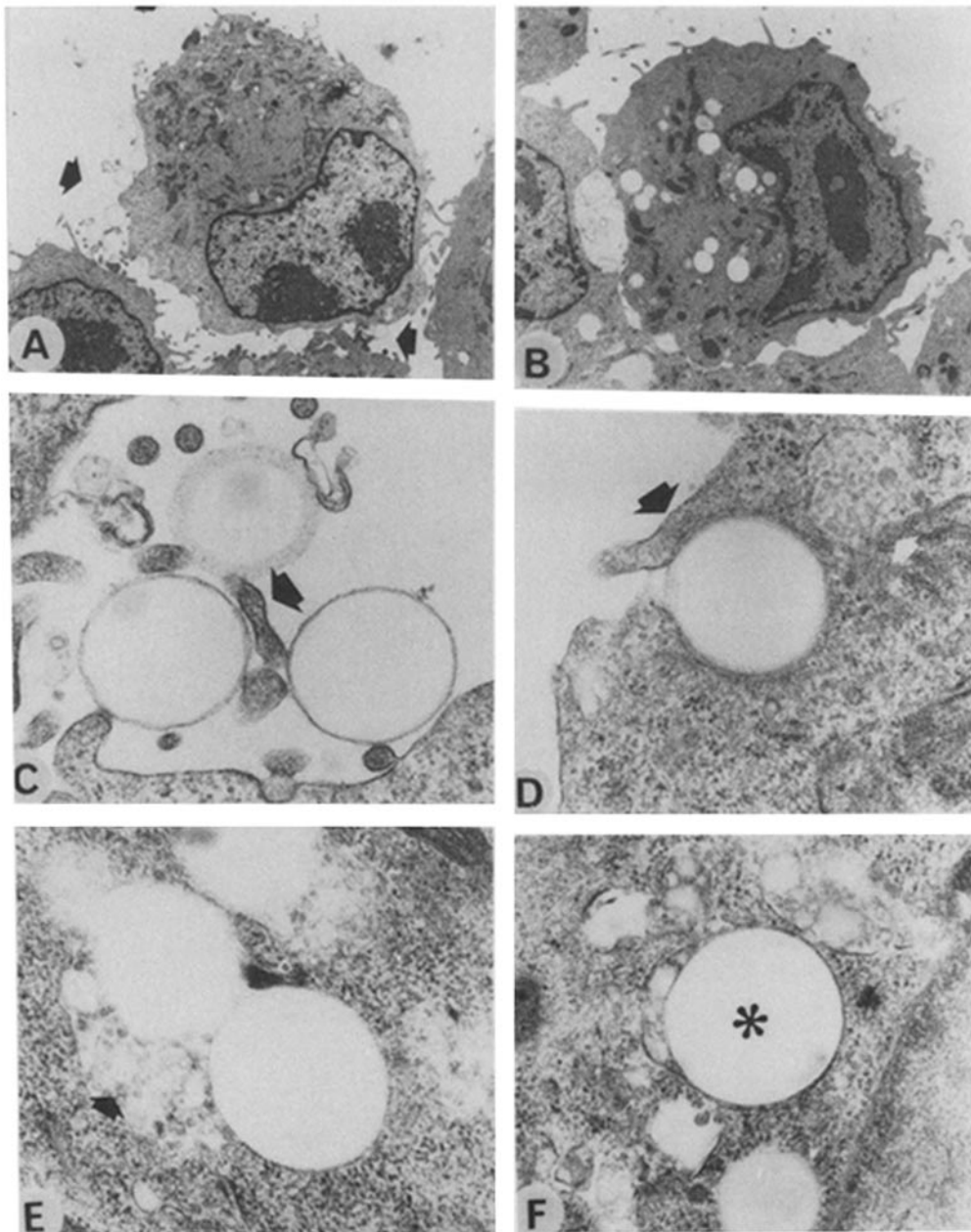
FIGURE 3   TEM of BHK cells incubated with CIG/BSA beads. Cells were incubated with CIG/BSA beads for 10 min at 22°C (A, C, and D) after which a portion of the cells was washed and fixed with glutaraldehyde while the remainder was resuspended in fresh adhesion medium and permitted to incubate for an additional 50 min at 37°C (B, E, and F). Fixed cells were processed for TEM as described previously (20). After 10 min at 22°C the beads were on the cell surface (Fig. 3 A, arrows) and in most cases were associated with microvilli (Fig. 3 C, arrow) or appeared to be in newly forming endocytic vesicles (Fig. 3 D, arrow). After an additional 50 min at 37°C, the CIG/BSA particles were inside the cells (Fig. 3 B) and often observed fusing with multivesicular bodies (Fig. 3 E, arrow) or in structures that had the morphological appearance of secondary lysosomes (Fig. 3 F, asterisk). Other details are in Materials and Methods and Results. (A and B) × 4,250; (C–F) × 31,000.

with trypsin at 0.01 mg/ml. These results were somewhat surprising because our previous findings indicated that cells had to be treated with a high level (5 mg/ml) of trypsin to prevent subsequent attachment to tissue culture dishes in a 60-min, 37°C assay. However, the bead-binding experiments were carried out for only 10 min at 22°C. When the time-course of cell attachment was measured at 22°C, pretreatment of cells with trypsin was found to result in almost complete inhibition at a trypsin concentration of 0.1 mg/ml and partial inhibition at 0.01 mg/ml (Fig. 7).

Quantitation of CIG/BSA Bead Binding to BHK Cells

A quantitative assay for CIG/BSA bead binding to cells was accomplished using ^{3}H-CIG; however, there were several complications. CIG beads tend to self aggregate and are difficult to resuspend after centrifugation. This difficulty was resolved by using brief sonication in between washing steps. Also bead aggregates that formed during incubation with the cells sedimented with cells under usual centrifugation conditions. This
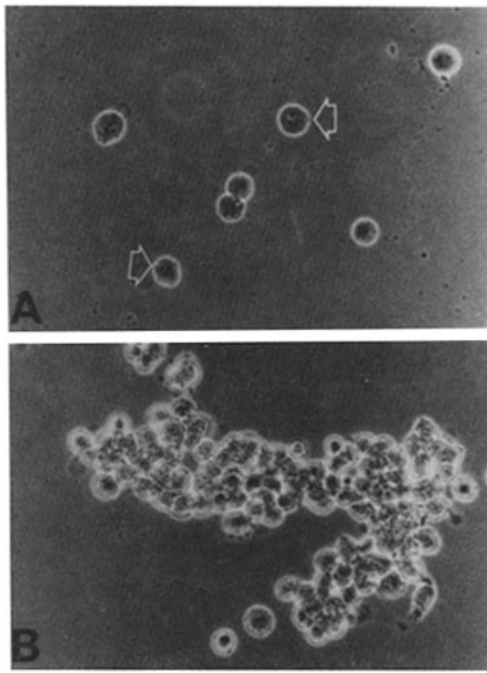
FIGURE 4 Aggregation of BHK cells with CIG/BSA beads. Cells were incubated with CIG/BSA beads at 37°C for various time intervals ranging from 1 to 60 min. At the end of incubation periods, cells were washed and fixed with formaldehyde. Photographs are shown for cells incubated with beads for 1 min (*A*) and 60 min (*B*). Some beads were observed to have bound to the cells even after 1 min (arrows) and aggregation was extensive after 60 min. Other details are in Materials and Methods. × 350.

difficulty was resolved by layering the reaction mixtures of cells incubated with ^{3}H-CIG beads on concentrated (50 mg/ml) BSA solutions and carrying out centrifugation. Under these conditions, the beads remained on top of the BSA layer regardless of their self aggregation.

Typical kinetics of binding of ^{3}H-CIG beads to BHK cells are shown in Fig. 8. Cells were mixed with ^{3}H-CIG beads for various time periods at 4°, 22°, or 37°C. At the end of the incubations, samples were taken and analyzed to determine the total amount of ^{3}H-CIG in the incubations (all of which was originally adsorbed to beads); the percent of the total ^{3}H-CIG bound to the cells; and the percent of the total ^{3}H-CIG which was released from the beads into the medium in a nonsedimentable form. The initial rate of binding was similar at 22° and 37°C and in both cases much more rapid than the rate of binding at 4°C. In addition, the unexpected observation was made that at 37°C, a substantial portion of the radioactivity was released from the ^{3}H-CIG beads during the incubations. Release of radioactivity from the ^{3}H-CIG beads was not observed in the absence of cells at 37°C, indicating that ^{3}H-CIG was not leaking off the beads. In addition, release was not observed in the presence of cells at 4°C and was much less at 22° than at 37°C.

The radioactive material released from the beads at 37°C was precipitable with trichloroacetic acid, indicating that it might be a limited proteolytic cleavage product of CIG. In other experiments it has been found that radioactivity is also released from ^{3}H-CIG-coated culture dishes during initial cell attachment and spreading (F. Grinnell and M. Feld, manuscript in preparation).
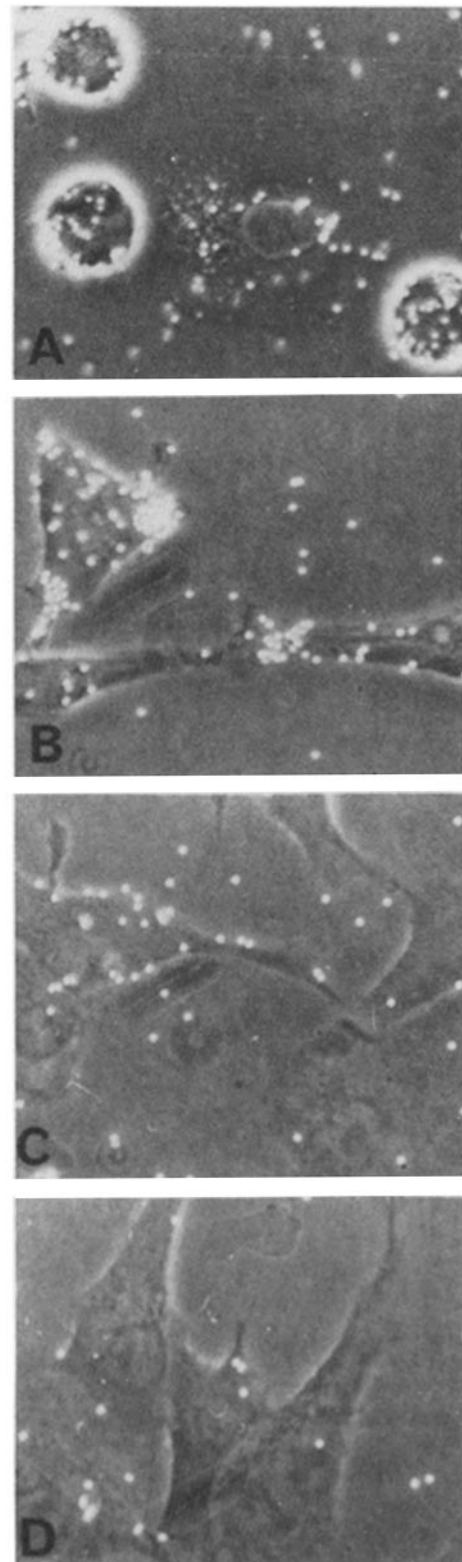

FIGURE 5 Binding of CIG/BSA beads to BHK cells attached to CIG-coated culture dishes. Falcon 35-mm tissue culture dishes (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, Calif.) were uncoated (*A*) or coated with CIG at 0.5 U/ml (*B*), 4 U/ml (*C*), or 16 U/ml (*D*) for 10 min at 22°C. BHK cells (0.75 × 10^6) in 1.0 ml adhesion medium were incubated on the dishes for 45 min. Subsequently, the dishes were rinsed and incubated for 10 min at 22°C while shaking at 50 rpm with CIG/BSA beads. Other details are in Materials and Methods and Results. × 880.
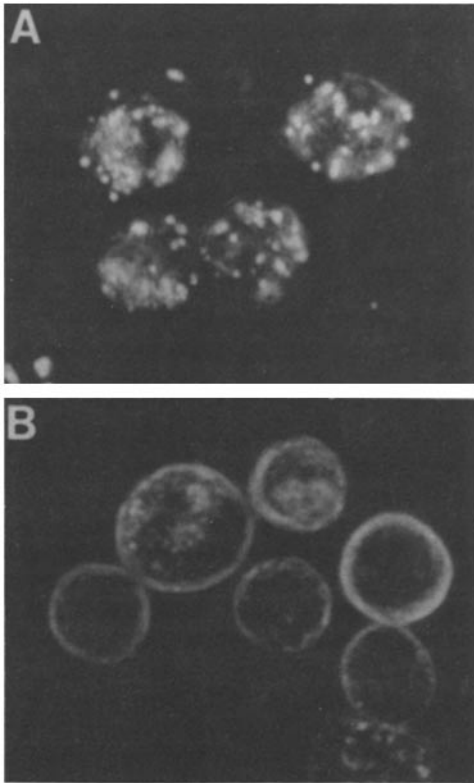
FIGURE 6 Effect of trypsin on CIG/BSA bead binding to BHK cells. BHK cells (3 × 10⁶) were incubated in 2.0 ml of phosphate saline for 10 min at 37°C without trypsin (A) or with trypsin (Sigma type XI) at 0.1 mg/ml (B). The incubations were stopped by the addition of 1.0 ml of trypsin inhibitor (5 mg/ml, Sigma type 1-S) and the cells were then centrifuged, washed once with adhesion medium, and resuspended in 0.75 ml of adhesion medium. The treated cells were then incubated with CIG/BSA beads for 10 min at 22°C. Other details are in Materials and Methods. × 880.

Binding of ³H-CIG Beads to BHK Cells in the Presence and Absence of Divalent Cations

Preliminary experiments using microscope analysis indicated that in the absence of divalent cations, CIG/BSA beads bound to BHK cells. In fact, beads bound to cells even in the presence of excess EGTA and EDTA. This observation was confirmed by directly measuring bead binding as shown in Fig. 9. The kinetics of bead binding and the appearance of released CIG were measured at 22°C with the usual incubation medium (i.e., phosphate saline supplemented with Ca^{2+} and Mg^{2+}) or with phosphate saline alone. The percent of the total ³H-CIG beads in the incubations that bound to the cells was similar in either medium. The percent of total ³H-CIG in the incubations that was released from the beads in a nonsedimentable form was also similar.

DISCUSSION

A new approach to studying the cell receptor involved in cell adhesion has been carried out using latex beads coated with CIG. Before discussing the data in detail, it should be pointed out that the binding of CIG beads to the cells and of cells to CIG-coated culture dishes are very similar and therefore probably represent two manifestations of the same cellular activity. Both phenomena require the presence of CIG on the material surface. With both phenomena, the formation of bonds of

attachment per se is temperature independent and does not require divalent cations but is inhibited by prior trypsinization of cells. Both phenomena demonstrate a secondary reorganization event: initial binding of CIG beads to cells is followed by endocytosis of the beads; initial attachment of cells to CIG culture dishes is followed by cell spreading.

Binding of Latex Beads to Cells Requires CIG and CIG Beads Act as Multivalent CIG Complexes

Binding of latex beads to BHK cells occurred if the beads were precoated with CIG and then incubated with BSA. However, if the beads were precoated with BSA and then incubated with CIG, no binding to cells was observed. Thus, for beads to bind to cells, the CIG must be on the beads. The presence of CIG in the medium is not sufficient once the beads are coated with BSA. These results are identical with our previous findings on BHK cell adhesion to tissue culture plastic. That is, CIG is required for adhesion and it is both necessary and sufficient that CIG be on the substratum for adhesion to occur (12). Consistent with the notion that the CIG beads are acting as
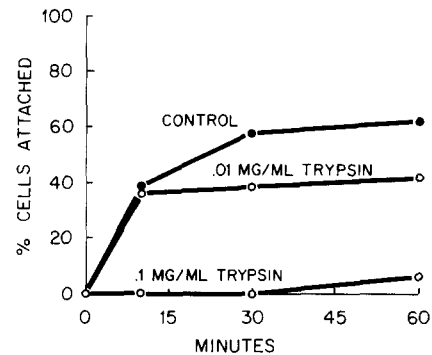

FIGURE 7 Adhesion of trypsin-treated BHK cells to CIG substrata. Falcon 35-mm tissue culture dishes were coated with CIG (10 U/ml in adhesion medium) for 10 min at 22°C. BHK cells were treated with or without trypsin as designated using the protocol described in the legend to Fig. 8. Subsequently, treated cells were resuspended in 1.0 ml of adhesion medium and incubated in the dishes at 22°C for the time periods shown. Cell attachment was measured by the turbidometric method described previously (16). No cell spreading occurred. Other details are described in Materials and Methods.
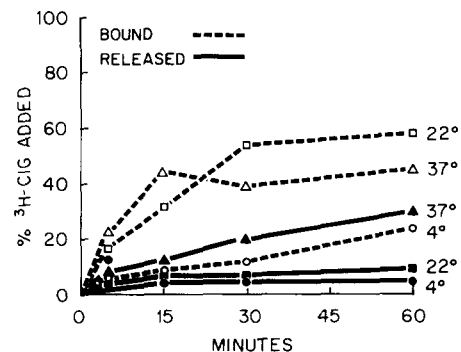

FIGURE 8 Quantitation of ³H-CIG bead binding to BHK cells. Cells were incubated with ³H-CIG beads for the time periods shown at the temperatures indicated. Then, the percent of the total ³H-CIG beads that bound to the cells and the percent of the total ³H-CIG that was released from the beads in a nonsedimentable form were determined. Other details are in Materials and Methods and Results.
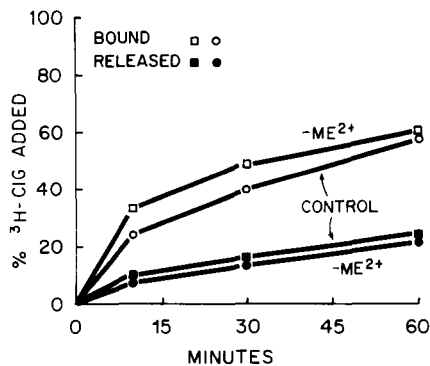
FIGURE 9 Quantitation of ^{3}H-CIG bead binding to BHK cells with and without divalent cations. Cells were incubated with ^{3}H-CIG beads in adhesion medium (control) or in phosphate saline and no divalent cations ($-Me^{2+}$) at 22°C for the time periods shown. The percent of the total ^{3}H-CIG beads that bound to the cells and the percent of the total ^{3}H-CIG that was released from the beads in a nonsedimentable form then were determined. Other details are in Materials and Methods and Results.

multivalent CIG complexes, the CIG beads were able to cause fibroblast aggregation (Fig. 4), which does not occur with individual CIG molecules in solution.

Receptors for CIG Are Located over the Entire Cell Surface but Are No Longer Detectable when Cells Spread on CIG-coated Culture Dishes

Incubations of CIG-coated beads with cells at 4° or 22°C appeared to result in the binding of beads over the entire cell surface. There was an indication that initial binding was in association with microvilli (Figs 2 B, 3 C, and 3 D). However, this may simply have been a result of beads cross-linking between the surfaces of microvilli and the cell body (c.f., Fig. 5 in reference 20). Random binding of beads was also observed on round cells attached to uncoated culture dishes (Fig. 5 A); but with cells that were spread on CIG-coated dishes, binding was diminished or absent depending upon the concentration of CIG used to coat the dishes (Fig. 5 B–D).

One possible interpretation of this finding is that CIG receptors migrate to the cell-dish contact region where they bind to the CIG on the surface of the dish. A similar interpretation has recently been put forth to explain the disappearance of Fc receptors from the upper surfaces of macrophages which occurred after the macrophages spread on dishes coated by immune complexes, but not on dishes coated by complement (29).

An alternative explanation for the disappearance of the CIG receptors is that a minimum number of receptors must simultaneously interact with each bead for stable cell-bead binding to occur. If the effective receptor-to-receptor spacing is much greater with spread cells than with cells in suspension, then the likelihood of the necessary number of interactions occurring would be decreased. Even with cells in suspension some redistribution of CIG receptors might favor binding of CIG beads to cells which would explain why the rate of bead binding was much less at 4° than at 22°C (Fig. 8). The idea that a minimum number of bonds is required to stabilize cell-bead interactions is consistent with the previous suggestions that local reorganization of receptors occurs during cell-substratum attachment (8, 34, 40) and that a threshold minimum number of interac-

tions are required (23, 42). It should be pointed out that a change in receptor-to-receptor spacing cannot be accounted for simply by a change in total cell surface area because the total surface area is the same for round cells and spread cells (7). Nevertheless, there may be specific topographical restrictions which are different for round and spread cells.

Receptors for CIG Are Available for Binding at 4 °C and Binding Occurs in the Absence of Divalent Cations

Previous evidence based upon studies in which cells were centrifuged against serum-coated culture dishes led to the proposal that low temperature and absence of divalent cations did not interfere with formation of bonds of attachment per se between cells and the dishes (8, 9). Nevertheless, inhibition by low temperature and a requirement for divalent cations are features of the cell-dish contact process and of cell spreading (see reference 12 for a review). The present findings indicate that CIG beads bind to cells at 4°C and in the absence of divalent cations (Fig. 9). These observations suggest that the interaction of CIG beads with the cells is detecting the formation of bonds of adhesion per se, and is independent of any contact process or spreading process. The lack of a divalent cation requirement for CIG bead binding tends to rule out the proposed role of divalent cations as mediators of adhesion bonds, as a cofactor for an enzyme involved in the formation of adhesion bonds, or in the stability of the cell receptor for CIG (see reference 12).

Receptors for CIG Are Trypsin Sensitive

The results indicate that brief pretreatment of cells with 0.1 mg/ml of trypsin interferes with subsequent binding of beads to the cells (Fig. 6). Similarly treated cells were also unable to attach to CIG-coated culture dishes at 22°C (Fig. 7). These data suggest that the CIG receptor has a protein component. A similar conclusion was reached in earlier studies that demonstrated an inhibition of cell adhesion in serum-containing medium by prior trypsinization of the cells (19). It was also shown that regain of adhesiveness after trypsinization occurred in 2–4 h and was prevented by cycloheximide.

Cell Spreading on Tissue Culture Dishes Probably Reflects an Attempt by the Cells to Phagocytize a Particle of Infinite Diameter

Finally, the suggestion was previously made that spreading on culture dishes by macrophages is an attempt on the part of the macrophages to phagocytose the dishes (31, 32). The present findings support this notion with fibroblasts. There is little question that the beads which are first bound to the cell surfaces can be endocytosed at 37°C (Figs. 1–3) and subsequently end up in structures which appear to be lysosomes (Fig. 3 E and F). Therefore, the same receptors that are capable of causing cell spreading on CIG-coated dishes are also capable of causing endocytosis of CIG-coated beads. The expression of the different phenomena depends upon the sizes of the surfaces involved.

It is possible that the release of radioactivity from ^{3}H-CIG beads at 37°C was in some way related to the endocytosis of the beads. Alternatively, release might have been caused by cellular proteolytic activity. The latter possibility would be consistent with the observation that the released radioactivity

was still precipitable with trichloroacetic acid. Also, radioactivity was released from ³H-CIG-coated culture dishes. This observation is interesting in light of the previous finding that cellular proteases may be required for cell adhesion (10).

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