

Studies on Intercellular Adhesion

INDUCTION OF ADHESION BY MULTIVALENT LIGANDS*

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Multivalent ligands capable of binding to cells facilitate intercellular adhesion by bridging adjoining cells. This process serves as an indirect means of promoting adhesion merely by holding the cells in close proximity. The present paper demonstrates that a different, more specific type of adhesion enhancement results when the cells are induced to adhere by cross-linking specific cell surface receptors.

Antibodies raised against Chinese hamster ovary (CHO) cells (anti-WC IgG) and concanavalin A stimulate intercellular adhesion of CHO cells in suspension to normal monolayer cells and to glutaraldehyde-fixed monolayers. Anti-WC IgG and concanavalin A yield complex, but very similar, stimulation activity curves when the adhesive rate is plotted against the quantity of ligand bound to fixed monolayer cells, suggesting that multiple interactions are necessary to trigger intercellular adhesion. For both ligands an excess of 5×10^5 molecules bound per fixed monolayer cell must be attained for adhesion to be induced.

In contrast, *Ricinus communis* agglutinin-120 stimulates, with a linear dose response, single cell adhesion to normal monolayers, whereas it scarcely stimulates adhesion to fixed monolayers. When suspension and monolayer cells are precoated with monovalent anti-WC Fab' fragments, and anti-Fab antibodies are subsequently added, stimulatory effects resembling those seen with *Ricinus communis* agglutinin-120 are observed.

These results are interpreted to be due to a direct inducement of intercellular adhesion of CHO cells by anti-WC IgG and by concanavalin A, where the formation of specific sets of isotypic intercellular cross-links produced by the ligands is responsible for the enhanced interaction between the cells. Multivalent ligands which do not bind to the proper cell surface components, or heterotypic cross-links, like those produced by anti-Fab bridging Fab-coated cells, give rise only to facilitated adhesion of the cells.

Intercellular adhesion is assumed to govern a number of

cellular events. Specifically, such phenomena as cell motility (1), differentiation (2), morphogenesis (3-5), and growth control (6) are assumed to be regulated at the level of the cell membrane and to be dependent upon intercellular contact. Several attempts to elucidate the molecular mechanism of intercellular adhesion have come to the conclusion that the phenomenon is a multistep, complex system of interactions involving the cell membrane as well as active energy metabolism (7).

A concept of intercellular adhesion held currently by many investigators (8) includes three major stages. The first stage, a charge-dependent, nonspecific juxtapositioning of the cells, makes possible the second stage, a specific acceptor-receptor interaction employing intrinsic glycoproteins. This interaction causes the release of a message leading to the third stage, involving a metabolic activation of the adhering cells that results in the formation of more permanent intercellular bonds, such as desmosomes and tight junctions.

The data presented herein suggest that antibodies directed to cell surface components are capable of inducing intercellular adhesion of single cells in suspension to normal, as well as to fixed, monolayer cells, through sets of specific intercellular cross-links of antigens. The observation that Con A¹ showed the same properties suggests the involvement of membrane glycoproteins in the observed induction of intercellular adhesion. Careful quantitation of ligand binding to fixed monolayers versus the stimulation of adhesion demonstrated the requirement for the formation of a threshold level of cross-links. Specificity requirements were indicated by the fact that cross-linking of these cells by other lectins, or of rabbit Fab' coated cells by goat anti-rabbit-Fab, simply gave rise to facilitated adhesion by merely linking cells together. Preliminary results have been presented (9).

EXPERIMENTAL PROCEDURES

Cultures

Maintenance of proline-requiring Chinese hamster ovary cells (CHO-K1 American Type Culture Collection) has been described (10). Cultures were free of mycoplasma as tested by the criteria of Schneider *et al.* (11).

Materials

The following materials were purchased from commercial sources as indicated: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, D-glucose, α -methyl-D-mannoside, β -lactose, and amino acids, Sigma Chemical Co.; pepsin A, Worthington; bovine serum albumin ($5 \times$ crystallized), Calbiochem; glutaraldehyde (8% aqueous), Polysciences, Inc.; Sephadex G-100 and Protein A-Sepharose CL-4B, Pharmacia Fine Chemicals; Aqueous Counting Scintillant, Amersham Corp.; [3 H]-leucine (40-60 Ci/mmol) and NaB³H₄ (100 mCi/mmol), New England Nuclear; concanavalin A ($3 \times$ crystallized), all other lectins,

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¹ The abbreviations used are: CHO, Chinese hamster ovary; Con A, concanavalin A; RCA, *Ricinus communis* agglutinin-120.

and goat antisera to rabbit Fab fragments (lyophilized) were obtained from Miles Laboratories, Inc. The corresponding IgG was purified from the antisera as described under "Methods."

F12 medium was prepared from a powdered nutrient mixture (Gibco, Inc.) and supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Inc.). This growth medium for cell cultures is denoted as Medium A. Medium B (pH 7.2) consisted of 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered (0.01 M) Hank's balanced salt solution lacking phenol red but containing D-glucose, and amino acids at the concentrations listed for minimum essential amino acids (MEM, Gibco, Inc.). PBS (pH 7.2) was comprised of (in grams/liter) NaCl, 8.00; KCl, 0.02; Na_2HPO_4 , 1.15; KH_2PO_4 , 0.20.

Methods

Immunization of Rabbits—CHO cells were grown in 500-ml spinner bottles (1960-00500, Bellco Glass, Inc.) in Medium A to a density no greater than 8×10^5 cells/ml within a 2-day period. They were then harvested from the medium by centrifugation at $200 \times g$, after which the cell pellet was washed twice with 40 ml of PBS. The cells were resuspended in PBS at a density of approximately 3×10^6 cells/ml and 0.3 ml of this suspension ($5\text{--}10 \times 10^7$ cells) was injected into the ear vein of adult male New Zealand rabbits. Initially, four weekly injections were administered, followed by injections at monthly intervals.

IgG and Fab' Preparation—Antisera obtained from rabbits were subjected to precipitation in 35% saturated ammonium sulfate. The precipitate was dissolved in and dialyzed against 0.01 M potassium phosphate, pH 8.0, and applied to a Protein A-Sepharose CL-4B column (1.6×5 cm) equilibrated in the same buffer. The column was thoroughly washed with the phosphate buffer at a flow rate of 20 ml/h. Elution of protein was monitored by collecting 2-ml fractions and measuring their absorbance at 280 nm. When the absorbance returned to baseline, the IgG fraction was obtained by pumping 0.1 M glycine-HCl, pH 3.0, through the column. These fractions were then dialyzed against 0.05 M ammonium bicarbonate, lyophilized, and finally dissolved in PBS.

Preparation of monovalent Fab' fragments, requiring pepsin digestion of IgG, was performed according to Brackenbury *et al.* (12). The resulting material was dialyzed against 0.05 M ammonium bicarbonate and passed through a Sephadex G-100 column (60×2.6 cm). 3-ml fractions were collected and their A_{280} was measured. Fractions of the major peak, corresponding to a $M_r = 50,000$ protein, were pooled and lyophilized. Determinations of the final concentrations of IgG and Fab' preparations dissolved in PBS were performed according to the procedure of Lowry *et al.* (13) using bovine serum albumin as a protein standard.

Assay for Intercellular Adhesion—The assay devised by Walther *et al.* (14) for measuring the adhesion of labeled single cells in suspension to confluent monolayers was employed, with revisions developed for CHO cells (10).

Twenty-four h prior to the assay, CHO cells were released from tissue culture dishes (No. 3025 Falcon Labware) by a 4-min trypsin treatment as described (10) and washed once with Medium A to remove cellular debris. 1.0-ml aliquots of a cell suspension in Medium A, diluted to a density of 350,000 cells/ml, were added to individual wells of a multiple-well Linbro dish (No. 76-033-05 Flow Laboratories, Inc.). Observation of the monolayers after 24 h verified that they were confluent.

Single cells were plated at a density of 1×10^5 cells/ml and grown in suspension for 22 h in Medium A with the addition of L-[4,5- ^3H] leucine (1 $\mu\text{Ci}/\text{ml}$). The containment of these cultures in Falcon Petri dishes (No. 1001) obviated a need for trypsin treatment. Two h before the assay, the single cells were harvested and centrifuged, and the isotope was then chased by a 2-h incubation in fresh Medium A.

Before the assay, these single cells were centrifuged, washed twice with Medium B, diluted to a density of 1.5×10^5 cells/ml, and maintained at 37°C . Monolayers were washed twice with Medium B and preincubated for 20 min at 37°C in 0.4 ml of a Medium B plus 0.1 ml of PBS containing the multivalent ligand at the designated concentration.

The assay was initiated by the addition of 1.0 ml of single cell suspension to each well. The dishes were kept stationary in a 37°C water bath for the specified time, 30 min for most experiments, after which the monolayers were washed twice with Medium B and prepared for liquid scintillation counting (10). Results are represented as the percentage of labeled single cells originally administered which remain attached to the monolayers after washing.

Measurement of the Degree of Intercellular Bridging—Intercel-

lular bridging caused by the multivalent ligands was measured by incubating the monolayer and single cells with Medium B containing 1.6 mM KCN and 1.0 mM iodoacetate for 10 min before initiating the assay. The percentage of labeled single cells remaining bound to the monolayers after the washing procedure is defined as the degree of intercellular bridging.

Fixation of Monolayers—Fixed monolayers were obtained by washing normal monolayers twice with PBS and then incubating them for 30 min at 37°C with 0.5% glutaraldehyde in PBS. The fixed monolayers were washed with PBS and incubated for 20 min in 0.15 M glycine-PBS, pH 7.2.

Tritium Labeling of Proteins—IgG, Fab' fragments, and lectins were labeled by reductive methylation utilizing NaBH_4 after the procedure of Ascoli and Puett (15). The specific activity achieved by this method was approximately 20,000–30,000 dpm/ μg of protein as determined by the method of Lowry (13).

Competition binding experiments with the unmodified proteins revealed that reductive methylation of IgG and RCA had no effect on their binding to monolayers; however, modification of Con A reduced its binding by 15 per cent. This impairment was corrected for when determining the quantity of unmodified Con A which bound to the monolayers.

Binding Assays—Washed monolayers were incubated at 37°C in 200 μl of Medium B containing tritium-labeled multivalent ligands. At the specified times, monolayers were washed twice to remove unbound ligand. Normal monolayers were prepared for liquid scintillation counting as described previously, but the fixed monolayers required an initial treatment with 200 μl of 0.1% trypsin in PBS.

Nonspecific binding of IgG was measured by determining the binding of tritium-labeled IgG prepared from preimmune rabbit serum. Nonspecific binding of Con A and RCA was measured in the presence of 200 mM α -methyl mannoside and β -lactose, respectively. Specific binding of the ligands was calculated as the total binding minus the nonspecific binding. For all ligands, nonspecific binding was less than 10% of the total binding. Counts obtained from binding experiments were corrected for quenching by the channels ratio method and converted to disintegrations per min, from which the mass of ligand bound to monolayers was calculated using the predetermined values of specific activity of each ligand. The average number of ligand molecules bound per cell was then computed for 7×10^5 cells present per monolayer and using the following molecular weights for each ligand: RCA, 120,000; Con A, 110,000; IgG, 150,000.

RESULTS

Measurement of Intercellular Adhesion by the Monolayer Assay

Adhesion was identified by the number of cells which had formed junctions sufficiently stable to resist the washing procedure. Since CHO cells displayed linear adhesion kinetics for at least 60 min in the monolayer assay, with an apparent lag period of 4–5 min (Fig. 1), the duration of most experiments given in this paper was 30 min. The rate of adhesion, as measured by this assay, can be increased by the introduction of multivalent ligands able to bind to the cell surface (16). Under such conditions, the maximum percentage of cells which adhered to the monolayer in 30 min was extended from 15% to approximately 35%, regardless of which multivalent ligand was administered (Table I). To test the possibility that only a specific population of the single cells adhered, the nonadhering single cells which had been incubated with monolayers were transferred to a second set of monolayers and incubated for an additional 30 min (Table II). The data demonstrate that these cells adhered to the second monolayer at the same rate as in the first incubation, both in the controls and in the presence of a multivalent ligand. Therefore, the assay is not selective for a specific population of single cells; rather, the major determinants of the adhesive rate are the rate at which single cells settle upon the monolayer and the adhesiveness of the adjoining monolayer and single cells (14). A constant rate of descent can account for the linear kinetics observed for at least 60 min.

Induction of Adhesion by Anti-WC IgG—Antibodies directed against entire CHO cells exhibited a hyperbolic dose response with respect to their ability to stimulate intercellular adhesion (Fig. 2). IgG isolated from preimmunized rabbit

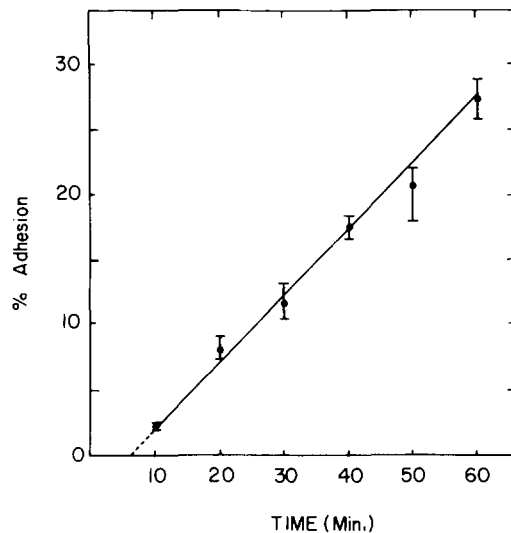


FIG. 1. Kinetics of single cell adhesion to monolayer cells. Single cell and monolayer cultures were prepared as described under "Methods." At zero time, each monolayer was inoculated with 1.0 ml of the single cell suspension. At the indicated times, nonadherent cells were removed from wells by aspiration and the monolayers were subsequently washed twice with Medium B and processed for liquid scintillation counting. The average and range of four identical assays at each time point is displayed.

TABLE I

Maximum adhesion in 30 min stimulated by anti-WC IgG and various lectins

Adhesion was measured as described under "Methods." The minimum concentration of each multivalent ligand needed to elicit the maximum stimulatory response was determined in a separate experiment. Concentrations above this minimum requirement were used and are listed as follows: RCA, 250 $\mu\text{g}/\text{ml}$; wheat germ agglutinin, 50 $\mu\text{g}/\text{ml}$; Con A, 50 $\mu\text{g}/\text{ml}$; anti-WC IgG, 200 $\mu\text{g}/\text{ml}$. Data are expressed as the compiled results of four determinations. It should be stated that some variation in the control and maximum rates of adhesion are seen on different days, but the relative rates remain the same.

Multivalent ligand	Maximum per cent adhesion (mean \pm S.D.)
None	13.9 \pm 0.4
<i>Ricinus communis</i> agglutinin-120	32.8 \pm 0.9
Wheat germ agglutinin	33.6 \pm 3.2
Concanavalin A	32.1 \pm 1.9
Anti-WC IgG	34.0 \pm 2.1

TABLE II

Incubation of nonadherent single cells to a second monolayer

Adhesion of single cells to the first monolayer was performed as described. Nonadherent cells were retrieved from individual wells and resuspended by vortexing. 0.5 ml of the resulting suspension from each well was added to a second monolayer prepared identically as the first monolayer, but containing 1.0 ml of Medium B per well. An additional 0.5 ml of each suspension was processed for liquid scintillation counting so that the per cent adhesion could be calculated in the second incubation. The mean and standard deviation of four individual assays are represented.

	% adhesion \pm S.D.	
	1st incubation	2nd incubation
Control	16.4 \pm 0.8	18.1 \pm 1.4
+600 $\mu\text{g}/\text{ml}$ anti-WC IgG	31.6 \pm 3.0	29.1 \pm 3.5
+50 $\mu\text{g}/\text{ml}$ concanavalin A	31.5 \pm 1.5	28.8 \pm 2.4

serum had no stimulating effect. Furthermore, monovalent Fab' fragments prepared from anti-WC IgG did not alter the rate of adhesion unlike Fab' fragments obtained by other

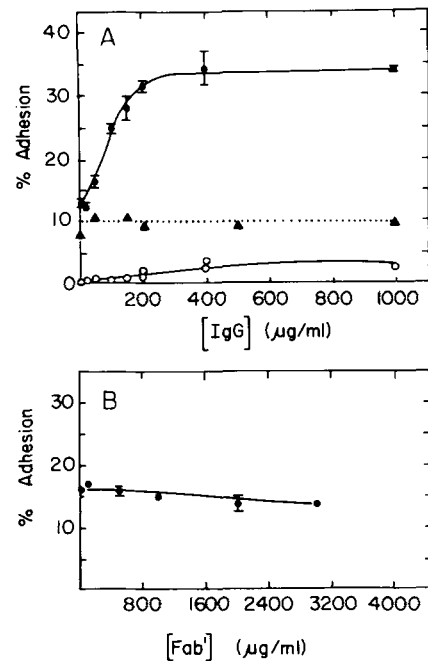


FIG. 2. Effects of IgG and monovalent Fab' fragments on cell adhesion. A, monolayers were preincubated for 20 min at the designated IgG concentrations in a Medium B mixture prepared as described under "Methods." The preincubation medium was left in the wells upon addition of 1.0 ml of labeled single cell suspension; the assay was terminated after 30 min. Effects of IgG purified from preimmunized serum (\blacktriangle) and of serum from immunized rabbits (\bullet , \circ) are depicted. Open circles represent the percentage of single cells attached in the presence of 1.6 mM KCN and 1.0 mM iodoacetate. B, conditions identical with those in A were used except monovalent Fab' fragments prepared from anti-WC IgG were utilized. Bars represent the range of duplicate determinations.

groups (12, 17, 18). Clearly, the divalent nature of the antibodies is required to produce the stimulation. The observed increase in the number of cells bound to the monolayers is a consequence of an energy-dependent adhesion process and is not due to the formation of intercellular bridges, as evidenced by KCN-iodoacetate poisoning of the cells which completely abolishes intercellular adhesion in the presence of the antibodies (Fig. 2A). Microscopic observations showed that cells induced to adhere by anti-WC IgG were anchored individually to the monolayer. There was a 4-min lag period before the cellular junctions formed could withstand the shear forces of the washing procedure. This transient lag period was also encountered when adhesion was induced by anti-WC IgG (Fig. 3).

Effect on Adhesion of Various Lectins—Con A demonstrated a hyperbolic dose response, as was seen with anti-WC IgG, whereas RCA displayed a linear dose response (Fig. 4). Wheat germ agglutinin also stimulated adhesion in a hyperbolic manner, but soybean agglutinin had no effect on adhesion (data not shown).

Comparison of Intercellular Bridging and Induction of Adhesion—Any multivalent ligand able to bind cells would be expected to facilitate adhesion between neighboring cells simply by holding the cells in juxtaposition, thereby increasing the probability of them forming adhesive contacts (16). The tetravalent lectins Con A and RCA, which bind different subsets of cell surface carbohydrates, were able to stimulate adhesion to the maximum rate (Table I) and also demonstrated equal capabilities to bridge cells as measured in the presence of KCN and iodoacetate (Fig. 4). Con A, however, stimulated adhesion to the maximum at a much lower concentration than RCA. This was due to qualitative, not quan-

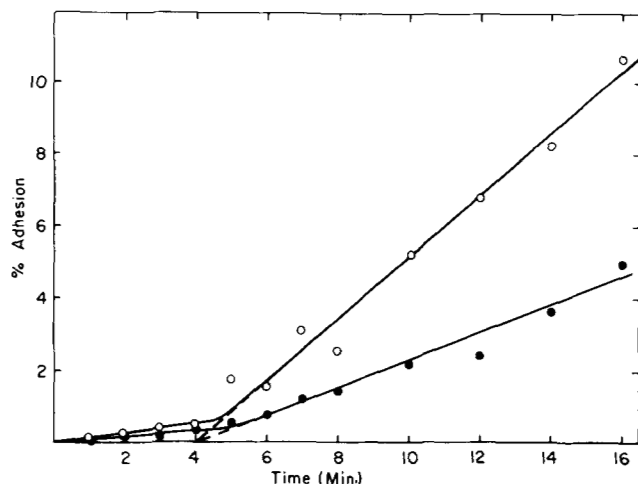


FIG. 3. The influence of anti-WC IgG on the lag period. The procedure listed under Fig. 2 was used with the assays terminated at the indicated times following addition of the single cells. Typical kinetics of adhesion in duplicates containing (○) or lacking (●) 200 µg/ml of anti-WC IgG in the medium are shown.

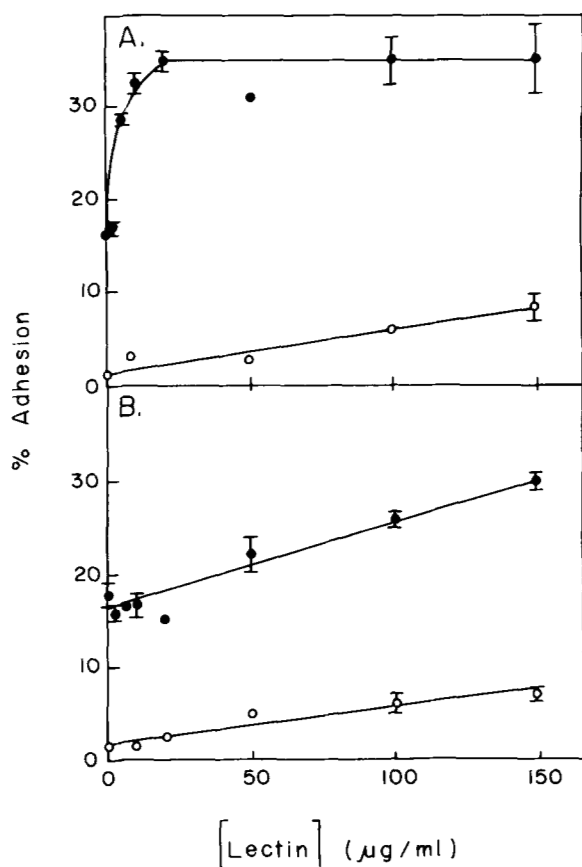


FIG. 4. Stimulation of adhesion by lectins. Adhesion of single cells to monolayers during a 30-min incubation is depicted in the presence of Con A (A) and RCA (B). Open symbols display cell attachment when 1.6 mM KCN and 1.0 mM iodoacetate were included in the medium. Final concentrations of lectin after addition of single cells (1.5 ml/well) are indicated. Bars represent the range of duplicate experiments.

titative, differences in binding of the lectins to the monolayers since more RCA bound at 50 µg/ml than Con A or anti-WC IgG, at concentrations exceeding those required to stimulate adhesion to the maximum rate (Fig. 5A). To determine if the impotency of the RCA preparation was accounted for by a contamination with the $M_r = 65,000$ toxin ricin, the effect of

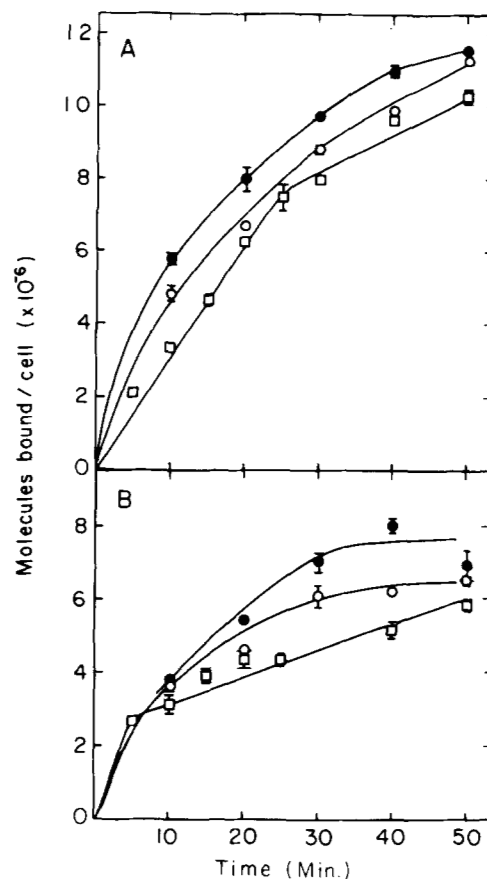


FIG. 5. Binding of multivalent ligands to monolayers. Specific binding in duplicates of multivalent ligands to normal monolayers (A) and fixed monolayers (B) in the presence of RCA (●), Con A (○), and anti-WC IgG (□) was measured as explained under "Methods." Concentrations of ligands used are: 50 µg/ml of RCA, 50 µg/ml of Con A, 300 µg/ml of anti-WC IgG.

ricin on intercellular adhesion was tested. Even at 15 µg/ml, the highest concentration tested, purified ricin had no influence on intercellular adhesion (data not shown). Moreover, the stimulation by anti-WC IgG was not affected by RCA (Table III).

Adhesion to Fixed Monolayers—Aggregation of hormone receptors, either by the hormone, or by anti-hormone receptor IgG on the cell surface, is reported to be required to elicit the hormone response (19–21). Aggregation of some specific cell surface antigen(s) by the multivalent ligands could possibly account for the anomalous behavior of these ligands to stimulate adhesion; that is, anti-WC IgG and Con A could cause an aggregation of cell surface antigens not recognized by RCA, and thereby signal a cellular change, which renders the cells more adhesive (22). In such a mechanism, lateral mobility of cell surface components in the plane of the plasma membrane must be important (23). Such movement would be minimized if the monolayer cells were fixed prior to the addition of ligands. Furthermore, the probability that a bound multivalent ligand would have a free binding site available for bridging an adjacent single cell would be increased. These characteristics of a fixed cell were used to test whether the induction of adhesion is a result of intracellular receptor clustering or bridging of juxtapositioned cells. Preliminary experiments demonstrated that single cells adhered to monolayers fixed with 0.5% glutaraldehyde for 30 min, although the control rate was diminished from 15% to 5% adhesion in 30 min. KCN-iodoacetate poisoning, or fixation of the single cells in conjunction with the monolayers, lowered the adhesive rate to

TABLE III
Effect of RCA on Anti-WC IgG Induced Adhesion

Monolayer cultures were preincubated for 20 min in 0.4 ml of Medium B containing RCA. 1.0 ml of labeled single cell suspension and 0.1 ml of anti-WC IgG dissolved in Medium B were added simultaneously following the preincubation, and adhesion was measured after 30 min. Final concentrations, which are suboptimal, of the multivalent ligands are shown in the Table. Results are presented from duplicate assays.

Incubations	% adhesion (mean \pm S.D.)
0 μ g/ml RCA	9.4 \pm 1.1
25 μ g/ml RCA	10.0 \pm 0.3
50 μ g/ml RCA	15.7 \pm 0.6
0 μ g/ml RCA, 130 μ g/ml anti-WC IgG	23.3 \pm 2.1
25 μ g/ml RCA, 130 μ g/ml anti-WC IgG	24.2 \pm 1.2
50 μ g/ml RCA, 130 μ g/ml anti-WC IgG	30.0 \pm 2.8

less than 1% (data not shown). Fig. 5B demonstrates that ligand binding to fixed monolayer is only slightly reduced.

Effect of Ligands on Adhesion to Fixed Monolayers—Fixed monolayers were preincubated with different concentrations of ligand for 20 min and washed free of excess ligand prior to adding single cell suspensions. Anti-WC IgG and Con A, but not RCA, were potent stimulators when using fixed monolayers (Fig. 6), inasmuch as the maximal rate of adhesion could still be attained. Essentially the same stimulation was observed when the single cells, and not the monolayers, were fixed and preincubated with ligand. If, however, the unfixed rather than the fixed cells in the above described experiments were preincubated with anti-WC IgG or Con A, no stimulation in adhesion was observed in either case (Fig. 6). These experiments suggest that the stimulation of adhesion is most likely due to intercellular cross-links rather than intracellular clustering of sites on the unfixed cells.

This conclusion was further corroborated by data obtained when the rates of adhesion were measured in the presence of increasing concentrations of ligands. Fig. 7 shows that when excess ligand was left in the wells during the assay, optimal concentrations of anti-WC IgG and Con A are seen, with higher concentrations yielding a lesser stimulation. It is reasonable to assume that the reduced stimulation at higher concentrations was due to excess ligand binding to the single cells, thereby hampering the formation of intercellular cross-links. When RCA was present during the assay with fixed monolayers, a minor stimulation results plateauing at one-third the maximal rate, apparently the consequence of a more efficient anchoring of single cells to the monolayer.

Requirement for Isotypic Cross-links—Both binding domains of an IgG molecule possess identical antigenic specificity. Thus, the observed induction of intercellular adhesion by anti-WC IgG was the result of cross-linking sets of isotypic cell surface antigens. To verify that heterotypic intercellular cross-linking is insufficient to induce adhesion, the following experiment was performed. Single cells and normal and fixed monolayers were preincubated with monovalent anti-WC Fab' fragments; free, unbound Fab fragments were removed by washing. The rate of adhesion of these Fab-coated cells was determined in the presence of increasing concentrations of goat anti-rabbit-Fab IgG (anti-Fab). Since the anti-WC Fab' preparation should recognize a host of cell surface antigens, it was anticipated that the intercellular cross-linking pattern produced by anti-Fab in this experiment was predominantly of the heterotypic form.

With respect to the concentration of anti-Fab used, a linear dose response was observed, regardless of whether the monolayers were fixed or not (Fig. 8), and the stimulation seen with unfixed monolayers in this experiment resembled that pro-

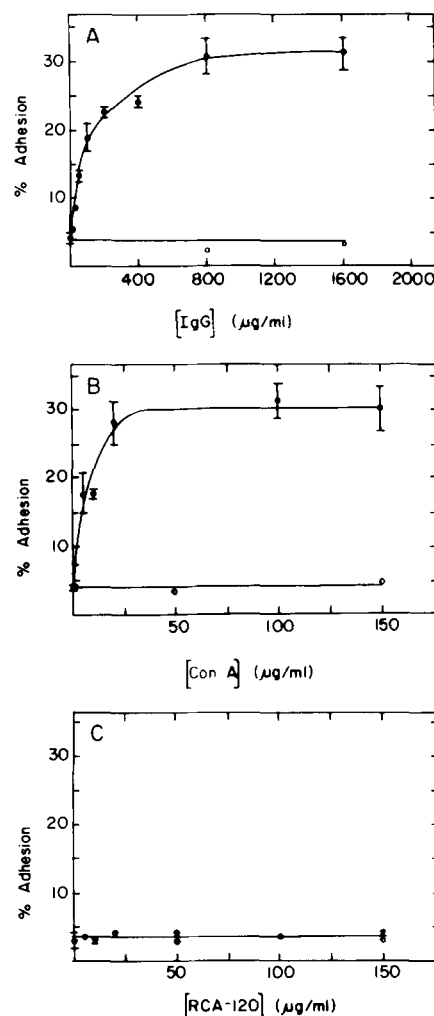


FIG. 6. Stimulation of adhesion to fixed monolayers. Fixed monolayers (●) or normal single cells (○) were preincubated for 20 min at 37 °C in Medium B containing ligand at the specified concentrations. Unbound ligand was removed by aspiration or centrifugation and the cells were washed twice with Medium B. Subsequently, 0.5 ml of Medium B was administered to the wells followed by the addition of 1.0 ml of single cell suspension. Results are represented as the percentage of cells adhering in 30 min. The range in the duplicate determinations is shown by the bars.

duced by RCA (Fig. 4). The very poor stimulation with fixed monolayers contrasted the potency of the intact anti-WC IgG. These results strongly suggest that induction of intercellular adhesion requires isotypic cross-linking of specific components.

The Requirement for Multiple Cross-links—The induction of intercellular adhesion to fixed monolayers containing bound anti-WC IgG or Con A appeared to occur with a hyperbolic dose response when plotted against the concentration of multivalent ligand used in the preincubation (Fig. 6). As multiple classes of binding sites will yield a complex curve when ligand binding *versus* ligand concentration is plotted, an improved analysis was performed. The amount of multivalent ligand bound to fixed and normal monolayers *versus* the stimulation of adhesion resulting as a consequence of ligand binding was determined. When the data were analyzed in this manner, the inducement of adhesion to unfixed monolayers by anti-WC IgG continued to exhibit a hyperbolic dose response, whereas the stimulation to fixed monolayers was more complex (Fig. 9). Clearly, a threshold quantity of anti-WC IgG must be bound to the fixed cells before an enhancement in the rate of

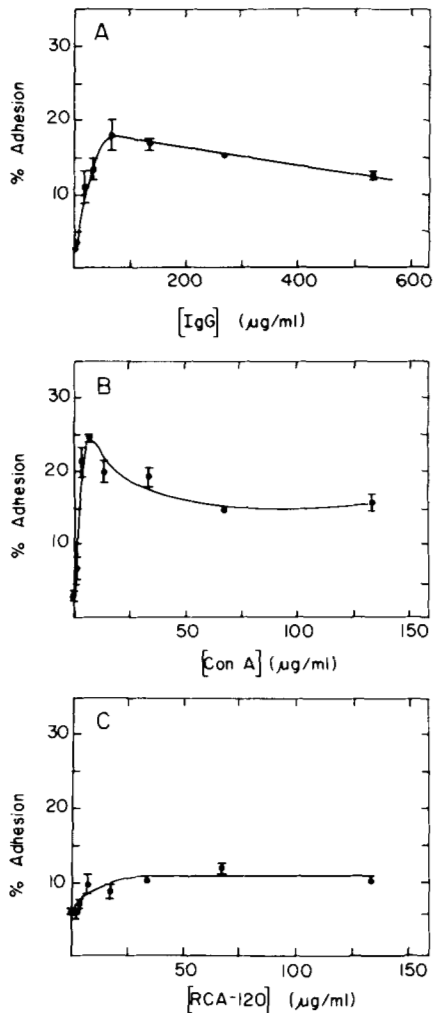


FIG. 7. Effect of introducing excess ligand on adhesion to fixed monolayers. Fixed monolayers in duplicates were preincubated for 20 min at 37 °C in 0.5 ml of Medium B containing ligand at the specified concentrations. 1.0 ml of single cell suspension was then added to each well and incubated for an additional 30 min.

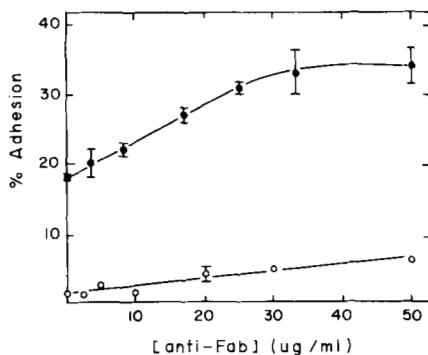


FIG. 8. Facilitated adhesion produced by heterotypic cross-linking. Monolayers and single cells were preincubated for 20 min at 37 °C in Medium B in volumes of 0.2 ml and 2.0 ml, respectively, containing 1000 μg/ml of monovalent anti-WC Fab' fragments. This concentration was demonstrated to yield the maximum stimulation of adhesion with goat anti-rabbit-Fab antibodies. Both sets of cells were washed twice in Medium B. Goat IgG, purified from antiserum raised against rabbit Fab fragments, was diluted in Medium B and 0.5 ml of the resulting solution was added to the wells, followed by the introduction of 1.0 ml of single cell suspension. Anti-rabbit-Fab concentrations shown represent the dilution at 1.5 ml. The percentage of single cells adhering, after 30 min, to normal (●) and fixed (○) monolayers was determined in duplicate determinations.

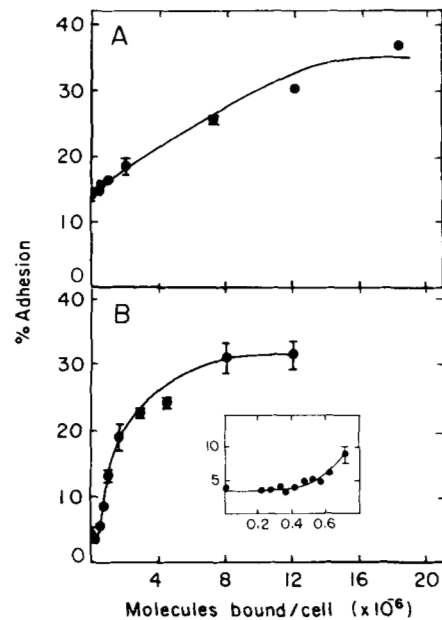


FIG. 9. Inducement of adhesion by anti-WC IgG. Monolayers were preincubated with anti-WC IgG at various concentrations in 0.2 ml of Medium B for 20 min. The monolayers were then washed with Medium B and the adhesion of labeled single cells to monolayers over 30 min was quantitated. Specific binding of IgG was measured as described under "Methods" utilizing duplicate monolayers preincubated with tritium-labeled anti-WC IgG diluted to the same concentrations as used in the corresponding adhesion assays. Results are plotted as the per cent adhesion observed using unlabeled IgG versus the quantity of IgG specifically bound to the parallel monolayers at that particular concentration of anti-WC IgG. A, inducement of adhesion to normal monolayers. B, inducement of adhesion to fixed monolayers. Inset, expanded scale showing inducement at lower concentrations of anti-WC IgG. Bars indicate the range of duplicate determinations.

adhesion occurs. Above the threshold value, an exponential increase in the stimulation was observed relative to the amount of bound IgG. At still higher values, the increase in the stimulation deviated from this behavior and more closely resembled a hyperbolic dose response. Note especially that anti-WC IgG was more potent in stimulating adhesion to fixed monolayers than to normal monolayers. This latter observation adds further support to the concept that the observed induction of adhesion involves intercellular cross-links rather than intracellular rearrangements (22).

The induction of adhesion by Con A with fixed monolayers was also examined in this respect and compared with that of anti-WC IgG. When the stimulation of adhesion to fixed monolayers by Con A was plotted against the quantity of lectin bound, a complex activity curve resulted with the same characteristics as observed with anti-WC IgG (Fig. 10).

DISCUSSION

Previous work has shown that multivalent ligands, capable of binding cell surface components, can facilitate intercellular adhesion (16). This phenomenon has been interpreted simply as a bridging of the cells, such that a multivalent ligand holding cells in close contact will promote them to adhere. The present report upholds this observation and, in addition, lends support to the hypothesis that intercellular adhesion involves an initial, specific interaction at the level of the cell membrane.

It has been demonstrated that IgG prepared against CHO cells, by introducing the proper isotypic cross-links between apposing cells, directly induces intercellular adhesion of CHO

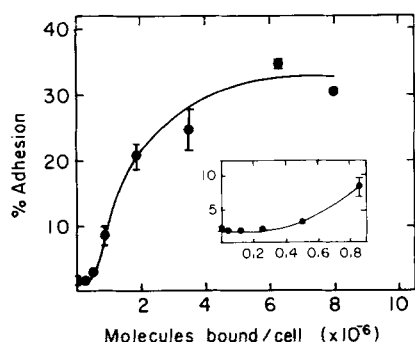


FIG. 10. Inducement of adhesion to fixed monolayers by Con A. Procedures identical with those of Fig. 9 were followed, with the exception of using Con A, rather than anti-WC IgG. Inset, expanded scale showing inducement at low concentrations of Con A.

cells, whereas heterotypic cross-links of Fab-coated cells, through anti-Fab, only results in facilitated adhesion.

A linear dose response of facilitated adhesion, with respect to anti-Fab concentration, is observed with normal monolayers. In contrast, intact anti-WC IgG exhibits a hyperbolic stimulation. Anti-WC IgG retains its ability to stimulate adhesion when fixed monolayers and normal single cells, or the converse, are employed and only the fixed cell is preincubated with the ligand. No stimulation of adhesion is observed if the unfixed cells are preincubated with ligand. Heterotypic cross-linking of Fab'-coated cells by goat anti-Fab yields only a slight stimulation with a linear dose response when fixed monolayers are utilized. It is likely that two different mechanisms of stimulating intercellular adhesion are observed.

Heterotypic cross-linking of the crucial induction antigens with a nonspecific assortment of antigens on the apposing cells is insufficient to induce adhesion in the manner observed with anti-WC IgG. The implication of these results is that isotypic cross-links of certain specific antigens promotes an intercellular acceptor-receptor interaction, and that one antigenic entity, or complex of cell surface components, comprises the cellular acceptor and receptor constituents.²

The use of various lectins have proven very useful in the elucidation of the stimulatory mechanism of anti-WC IgG. Con A exhibits a hyperbolic dose response, while RCA demonstrates a linear dose response. These findings can be compared with the experiments of fixed monolayers where excess, unbound lectin was removed prior to the assay. Con A continued to be a very potent stimulator, whereas RCA could not promote the adhesion of live single cells to the fixed monolayers.

All of these observations are consistent with a model of isotypic cross-linking of one or several specific antigens to trigger intercellular adhesion. Con A is known to bind many cell surface glycoproteins of CHO cells, yet these represent only a fraction of the total cell surface composition (24). Therefore, cross-linking produced by Con A would be expected to include isotypic and heterotypic forms. The work with antibodies implies that Con A may also promote the formation of the essential isotypic cross-links to induce adhesion.

The marked impotency of RCA to stimulate intercellular adhesion of CHO cells, in comparison with Con A, despite their similarities in binding and intercellular bridging abilities, argues against the suggestion that Con A simply anchors cells together to enhance intercellular adhesion as a secondary event. The stimulation by RCA closely resembles that when heterotypic cross-links are favored, as was done with anti-Fab antibodies. Since RCA will bind to a different subset of cell

surface carbohydrates than Con A, the implication is that RCA does not bind to essential antigen(s) required for induction of adhesion, as do anti-WC IgG and Con A. Rather, RCA-stimulated adhesion is described by the conventional model of facilitated adhesion by multivalent ligands.

The inability to block fully the induction of adhesion to fixed monolayers in the presence of high ligand concentrations is partially due to incomplete saturation of the single cells with ligand. Firstly, note that in Fig. 7 the single cells were not preincubated with the multivalent ligand, and also that over 50 min is required before a cell-ligand binding equilibrium is attained (Fig. 5). Secondly, when neither the monolayers nor the single cells are fixed, an optimal concentration of ligand to cause induction is not observed (Figs. 2 and 4), suggesting recycling of the induction antigens on the cell surface. These possibilities can account for the unusual concentration dependence seen when normal single cells are induced to adhere to fixed monolayers in the presence of excess ligand.

The triggering of adhesion of single cells to fixed monolayer cells by anti-WC IgG and Con A clearly demonstrates that a threshold quantity of ligand must first be bound to the receiving cell. Just beyond this extent of binding an exponential rise in the adhesive rate is observed relative to the amount of bound ligand. These findings demonstrate that induction of adhesion by these multivalent ligands requires the formation of multiple intercellular cross-links. Whether a minimum number or a specific geometrical array has to be achieved cannot be determined. A similar observation was made with respect to the adhesive response of hepatocytes towards synthetic carbohydrate substrates (25). A cooperative stimulation is not seen with unfixed monolayers, most likely since the mobility of cell surface components may readily allow for intracellular cross-linking of the induction sites by the antibodies, and also for internalization and recycling of the pertinent receptors.

Little is known about the molecular mechanism of intercellular adhesion. The data presented here may reflect on the second step in the model of intercellular adhesion (7), such that anti-WC IgG intercellularly cross-links specific intrinsic membrane glycoproteins and thereby promotes acceptor-receptor interactions which release the message that induces the complex, energy-requiring step.² The 4-min lag period (Fig. 3) could then represent the time after which the cells are induced until rigid intercellular bonds are formed. Since the lag period is not shortened by anti-WC IgG, it seems less likely that the energy-requiring step is affected by the antibody cross-linking specific cell surface receptors.

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