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Evidence for another cell-adhesion molecule in *Dictyostelium discoideum*

(cellular slime mold/cell–cell adhesion/extracellular material/cell surface antigen)

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ABSTRACT **We raised a rabbit antiserum that completely blocked cell–cell adhesion of aggregating *Dictyostelium discoideum* cells in an *in vitro* assay. All adhesion-blocking activity of this antiserum was adsorbed with a *D. discoideum* fraction containing molecules having molecular weights as high as about 10^6, even after the material had been extensively digested with Pronase. The properties of this fraction indicate that the antigenic determinants in this macromolecule are saccharide residues. Antigen-rich material is found on vegetative cells but accumulates on or around differentiating *D. discoideum* cells as they aggregate. The cell surface of an aggregating cell contains about 5×10^5 antigenic sites. Antigen is also abundant in the medium of *D. discoideum* cells starved in suspension, which proved the most convenient starting material for its purification. Like several other macromolecules already discovered in *D. discoideum* by using this immunological approach, the material identified here may play a direct or indirect role in cell–cell adhesion and merits tentative consideration as a cell-adhesion molecule.**

Molecules on and between cells are believed to mediate cellular associations. Generally called cell-adhesion molecules, their identification has depended heavily on immunological techniques. Usually an antiserum is raised against a crude mixture of cellular antigens, in the hope that some of the antibodies will block cell–cell adhesion in an *in vitro* assay. This provides a basis for identifying putative cell-adhesion molecules by neutralization of adhesion-blocking activity.

This strategy was first used successfully by Gerisch and colleagues (1) in the cellular slime mold *Dictyostelium discoideum* and implicated a glycoprotein having a M_r of 80,000 in cell–cell adhesion during aggregation (1, 2). Work by others (3–5) supports this conclusion. A glycoprotein having a M_r of 95,000 apparently participates in cell–cell adhesion at a later stage of development (3, 4).

We too injected a rabbit with aggregating *D. discoideum* cells and raised an antiserum that completely blocked cell–cell adhesion. However, to our surprise, we found that the antigen that adsorbed all adhesion-blocking activity differed markedly from those already described. Here we report the identification of this material.

MATERIALS AND METHODS

Growth and Development of Cells. *D. discoideum*, strain NC-4, and *Dictyostelium purpureum*, strain 2, were grown in association with *Klebsiella pneumoniae*, harvested as vegetative cells, and differentiated in suspension or on Millipore filters, as described (6).

Medium from cells starved in suspension culture was obtained after vegetative *D. discoideum* cells, washed to remove bacteria, were suspended to 2×10^7/ml in a solution adjusted to pH 6.2 and containing (total vol, 1 liter) 1.5 g of KCl, 1.0 g of $MgCl_2$, 0.3 g of Na_2HPO_4, 1.2 g of KH_2PO_4, and 0.5 g of streptomycin sulfate. The cells were aerated by shaking at 24°C for 16 hr and, after this prolonged period of starvation, were removed by centrifugation at 1,300 × *g* for 5 min, and the remaining particulate material was removed by centrifugation at 50,000 × *g* for 1 hr.

In one experiment, we used *D. discoideum* strain AX-3, a mutant of NC-4 that grows axenically in a chemically defined medium containing no macromolecules (7). A small innoculum of spores was added to the defined medium and the cells were grown to a concentration of about 5×10^6/ml. An innoculum was diluted 1:50 in fresh defined medium and again grown to 5×10^6/ml. After 18 such passages, cells and debris were removed by centrifugation as above and medium was assayed.

Immunological Reagents. Antiserum against whole *D. discoideum* cells was raised in a rabbit by injection of cells in Freund's adjuvant as described (8), and IgG from this antiserum (anti-D.d.-IgG) was prepared with DEAE-Affi-Gel blue (Bio-Rad). Fab fragments were prepared from the IgG by the method of Porter (9).

Cell–Cell Adhesion Assays. For all such assays, *D. discoideum* NC-4 cells were differentiated on Millipore filters (6) to the stage at which loose and tight aggregates were abundant. The cells were harvested by Vortex mixing from the filters, washed, and suspended to a concentration of 2×10^8/ml in cold 17 mM Na_2HPO_4/KH_2PO_4, pH 6.2 (SP solution). Cell–cell adhesion was measured by gyrating the cells and determining the disappearance of single cells with an electronic particle counter as described (6, 8). Generally, about 50% of the cells in the assay were in aggregates at the completion of the standard assay.

In the experiments in which we studied the effects of anti-D.d.-IgG on cell–cell adhesion, we used conditions established previously (8) as follows. Twenty microliters of the cell suspension was diluted with 200 μl of cold SP solution containing anti-D.d.-IgG at various concentrations and the mixture was incubated on ice for 30 min. Then, the mixture was vigorously mixed on a Vortex for 15 sec to dissociate agglutinated cells, and a 50-μl aliquot was removed from the mixture and added to 550 μl of SP solution containing 10 mM EDTA, bovine serum albumin at 2 mg/ml, and Fab fragments of goat anti-rabbit IgG at 0.5 mg/ml (Cappel Laboratories) to inhibit agglutination by the

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Abbreviation: Anti-D.d.-IgG, IgG from an antiserum raised against whole *D. discoideum* cells.

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divalent IgG (8) while gyrating the medium at room temperature in a vial at 200 rpm on a New Brunswick G-24 gyratory shaker (19-mm orbit, New Brunswick). This mixture was gyrated for 10 min, 10 ml of ice-cold 0.15 M NaCl was added, and the diluted cell suspension was analyzed in an electronic particle counter (Coulter) adjusted to count 95% of the single cells. Cell–cell adhesion was measured as the loss of single cells during gyration as described (8).

The assay was modified to study the effects of Fab prepared from anti-D.d.-IgG on cell–cell adhesion. A 20-μl aliquot of cell suspension was incubated with 200 μl of SP solution containing Fab at various concentrations and, as with IgG, a 50-μl aliquot was added to 550 μl of SP solution containing 10 mM EDTA, bovine serum albumin at 2 mg/ml, and Fab at the given concentration. It was necessary to add this additional Fab to the assay mixture to maintain high enough levels for an effect.

The ability of various materials to block antibody inhibition in the above assays was determined by previous incubation of IgG or Fab with the material for 30 min at 4°C prior to addition of the cells. For controls, preimmune IgG, nonimmune IgG, or Fab were used.

Antibody Binding to Cells. Anti-D.d.-IgG, iodinated (10) with ^{125}I, was allowed to react on ice for 30 min with aggregated *D. discoideum* cells at 2×10^7/ml in SP solution containing normal rabbit IgG at 5 mg/ml. An aliquot of 100 μl of the mixture was then layered onto 400 μl of 10% Ficoll (M_r, 400,000; Sigma), and centrifuged for 2 min in a Beckman microcentrifuge. The liquid above the cell pellet was removed by careful aspiration, and the portion of the tube containing the cell pellet was cut off with a wire cutter and assayed in a gamma scintillation counter. The results were corrected for the small amount of radioactivity that was found in the bottom of the tube when no cells were present.

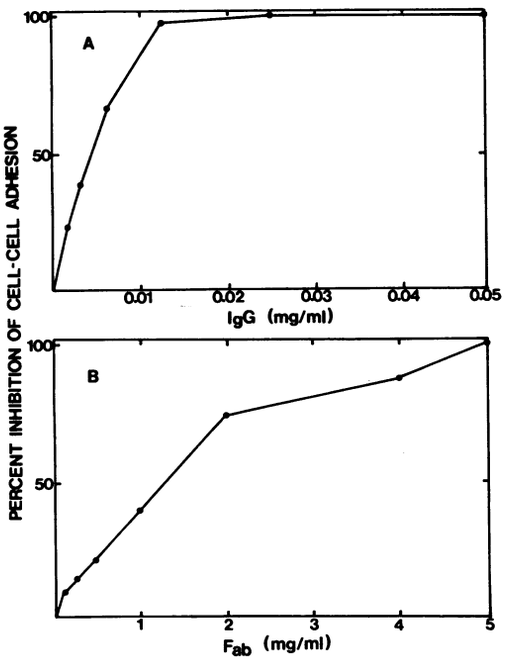

FIG. 1. Inhibition of adhesion by anti-*D. discoideum* antibodies. Cell–cell adhesion was assayed with or without anti-D.d.-IgG (*A*) or Fab (*B*) prepared from this IgG. The concentrations of antibodies shown are those in the final cell–cell adhesion reaction mixture. IgG was first incubated at a concentration 12-fold higher than shown and then diluted into the assay buffer. Fab was incubated and assayed at the same concentration.

RESULTS

Anti-D.d.-IgG (Fig. 1*A*) or Fab prepared from it (Fig. 1*B*) inhibited cell–cell adhesion. About 500 times as much Fab was needed for complete inhibition. Therefore, it would not have been practical to use Fab for extensive studies. Fortunately, our assay circumvented the agglutination effects of IgG (8), permitting its use in all further experiments, except where indicated. Given the results of Fig. 1, we routinely incubated 4×10^6 cells with 40 μg of anti-D.d.-IgG in 0.22 ml and then diluted 50-μl aliquots in 11 vol of assay buffer. The final concentration in the assay, 33 μg/ml, inhibited 90–100% of cell–cell adhesion.

To purify the antigens that bind the adhesion-blocking IgG, we initially assayed $NaDodSO_4$ extracts of aggregating cells or cells starved for 16 hr in suspension. The two extracts contained comparable amounts of antigen. However, the most convenient source was the medium from *D. discoideum* cells that had been starved in suspension for 16 hr. This contained about half the inhibitory activity of detergent extracts of the whole cells (Table 1) but only a few percent of the protein. It could completely neutralize the adhesion-inhibitory activity of either the immune IgG or Fab.

The antigenic material in the medium is derived from *D. discoideum* rather than from bacteria since it was not found in bacterial growth medium (Table 1). Furthermore, cultivation of an axenic strain of *D. discoideum* in growth medium formulated without macromolecules (7) generated antigen in the medium (Table 1). The antigenic material was not found in medium of *D. purpureum* cells (Table 1), indicating that it is species specific.

Release of antigenic material is not unique to suspension culture, since cells starved and differentiated on a filter also released antigen (Fig. 2). Some was also detectable in association with vegetative cells (Fig. 2), in contrast with the M_r 80,000 glycoprotein implicated in cell adhesion (1, 2). Indeed, vegetative cells could totally adsorb the inhibitory antibody and $NaDodSO_4$ extracts of vegetative cells contained as much antigenic material as similar extracts of aggregating cells.

The antigenic material from cells allowed to differentiate on

Table 1. Relative amounts of antigenic material in media and cells

Source	Cells, no./ml	Relative amount per ml
D. discoideum medium after starvation in suspension	10^7	100
D. purpureum medium after starvation in suspension	10^7	<1
D. discoideum axenic defined growth medium	5×10^6	120
K. pneumoniae growth medium	10^9	<1
$NaDodSO_4$ extract of starved *D. discoideum* cells	10^7	180

D. discoideum and *D. purpureum* cells were starved in suspension and *D. discoideum* were also grown axenically in a chemically defined growth medium containing no macromolecules (7). *Klebsiella pneumoniae* growth medium was obtained by growth of the bacteria to 10^9 cells per ml in nutrient broth and removal of the bacteria by centrifugation. $NaDodSO_4$ extracts of *D. discoideum* cells starved in suspension were obtained by centrifuging the cells after 16 hr of starvation and then suspending them at 10^8/ml in 0.1% $NaDodSO_4$. This mixture was dialyzed extensively against SP solution, diluted 1:10 with SP solution, and centrifuged to remove insoluble debris. Serial 1:2 dilutions of each antigen were incubated with anti-D.d.-IgG and the minimal concentration that adsorbed at least 90% of the inhibitory activity was determined. Relative amounts of antigen are in arbitrary units with *D. discoideum* starvation medium taken as 100.

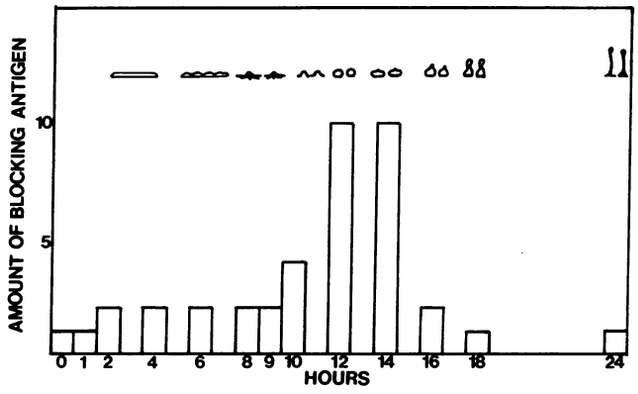

FIG. 2. Accumulation of the blocking antigen on or around differentiating *D. discoideum* cells. Vegetative *D. discoideum* cells were washed five times by centrifugation at 1,300 × *g* for 5 min to remove bacteria and allowed to differentiate on Millipore filters (6). At the indicated times, two filters were Vortex mixed for 15 sec in 10 ml of SP solution. The resulting suspension was centrifuged at 1,300 × *g* for 5 min and then at 50,000 × *g* for 1 hr to remove cells and particulate material. The supernatants were concentrated and assayed for antibody blocking activity as described in Table 2. Blocking activity in samples from cultures allowed to differentiate for 12 hr is arbitrarily set at 10, and other results are expressed relative to this. The drawings indicate the approximate morphological stage of the cells at the designated times.

filters (not shown) and from suspension medium behaved as a large molecule or a molecular aggregate having a M_r of up to 2×10^6, based on gel filtration on Sepharose 4B (Fig. 3). After

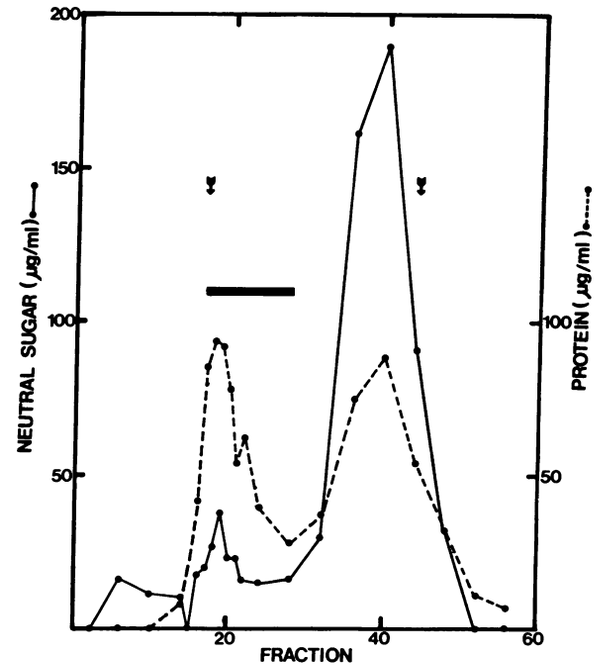

FIG. 3. Fractionation on Sepharose 4B. Five milliliters of concentrated medium from *D. discoideum* cells starved in suspension was layered onto a 130-ml Sepharose 4B column and eluted with SP solution. Three-milliliter fractions were collected and assayed for neutral sugar (11), protein (12), and inhibition of anti-D.d.-IgG in the cell–cell adhesion assay. The heavy bar indicates the fractions that totally inhibited the antibody effect at the concentrations at which they eluted from the column and that were pooled for the next step in purification. These fractions contained more than 80% of the antigenic activity recovered, with the remainder in adjacent fractions. Arrows indicate blue dextran (M_r, 2×10^6) and phenol red (total volume) peaks.

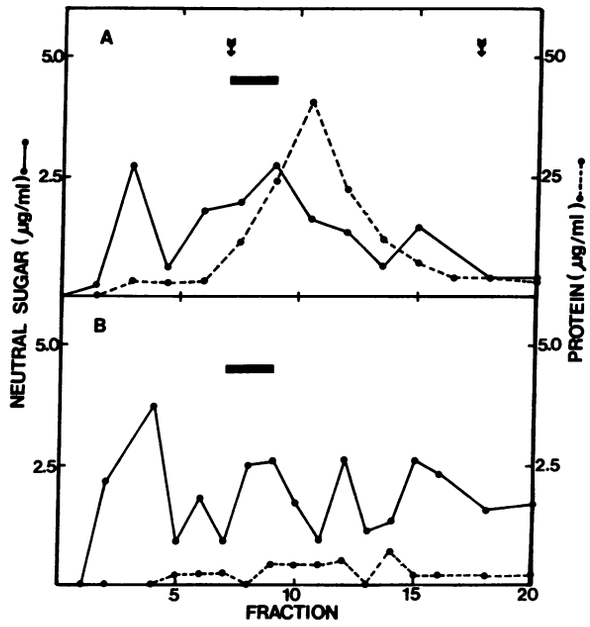

FIG. 4. Gel filtration of the blocking antigen before (*A*) and after (*B*) digestion with Pronase. The blocking antigen was purified through the antibody affinity column step. An aliquot (*A*) was dissolved in 6 M guanidine hydrochloride and applied to a Sephacryl S-300 column (Pharmacia) equilibrated with 6 M guanidine hydrochloride. Another aliquot (*B*) was digested with Pronase as described in Table 2 and then applied to the same column in 6 M guanidine hydrochloride. Fractions were dialyzed extensively against SP solution. Neutral sugar, protein, and inhibition of anti-D.d.-IgG were determined as described in Fig. 3. The heavy bar indicates the fractions that totally inhibited the effect of anti-D.d.-IgG at the concentrations obtained from the column. These fractions contained more than 80% of the antigenic activity recovered, with the remainder in adjacent fractions. Arrows indicate blue dextran (M_r, 2×10^6) and phenol red (total volume) peaks.

further purification by binding to and elution from an antibody column, the antigen retained a M_r of up to 2×10^6 in the presence of 6 M guanidine hydrochloride (Fig. 4). Pronase digestion of the antigen prior to the gel filtration reduced the amount of protein associated with the antigen-rich fractions to barely detectable levels without reducing its neutral sugar content or its apparent molecular weight (Fig. 4*B*).

A scheme for purifying the antigen is summarized in Table 2. The antigen is resistant to Pronase digestion (Tables 2 and 3) and to boiling (Table 3). It has no detectable nucleic acid, based on spectrophotometric analysis. Since none of the recovered antigen was found in the organic phase on chloroform/methanol extraction (13) and its apparent molecular weight is much larger than would be expected of lipid micelles, it does not appear to be a lipid or a simple glycolipid. Based on its size, the presence of neutral sugar, the virtual absence of protein after Pronase digestion, and its inactivation by periodate oxidation (Table 3), we conclude that the antigen is a saccharide-rich macromolecule such as a large polysaccharide. However, the native molecule might also be a proteoglycan with a significant protein component and could contain lipid.

The availability of partially purified antigen permitted us to evaluate the number of antibody binding sites on the surface of aggregating cells. We estimated this by comparing binding to cells of ^{125}I-labeled anti-D.d.-IgG previously incubated with or without a large excess of antigen (Fig. 5). Most of the IgG still bound to the cells (Fig. 5) after previous incubation with the antigen. However, the antigen blocked the binding of about 5×10^5 molecules of IgG per cell (Fig. 5), indicating that there

Table 2. Purification of antigenic material

Material	Activity, total units	Neutral sugar,* mg	Protein,† mg	Specific activity, units/mg of sugar	% recovery
Starvation suspension medium	400	1.2	1.8	330	100
Sepharose 4B fractions	200	0.61	0.44	330	50
Antibody column eluate	120	0.13	0.07	920	30
Pronase digest	240	0.09	<0.005	2,700	60
Sephacryl S-300 fractions	200	0.04	<0.005	5,000	50

Fifty milliliters of medium from cells starved for 16 hr in suspension was concentrated to 4 ml by ultrafiltration and applied to a 130-ml column of Sepharose 4B. The active fractions were isolated as in Fig. 3 and applied to a column containing 15 mg of anti-D.d.-IgG coupled to 10 ml of cyanogen bromide-activated Sepharose 4B (Pharmacia). The column was washed extensively with SP solution and bound antigen was eluted with 3 ml of 6 M guanidine hydrochloride followed by SP solution. The active material was dialyzed extensively against SP solution and then concentrated to 2 ml and digested for 3 days at 37°C with Pronase (5 μg/ml; added at 0, 24, and 48 hr) in 50 mM Tris·HCl, pH 7.8/1.5 mM $CaCl_2$. After digestion, EDTA was added to a concentration of 2 mM and the mixture was boiled for 20 min and centrifuged. The supernatant was adjusted to 6 M guanidine hydrochloride and applied to a 120-ml Sephacryl S-300 column (Pharmacia) equilibrated with 6 M guanidine hydrochloride. Active fractions were collected as described in Fig. 5, dialyzed extensively against SP solution, and concentrated. The antibody-blocking activity of the fractions was determined by incubating 100 μl of serial 1:2 dilutions of antigen in SP solution with 40 μg of anti-D.d.-IgG in 100 μl of SP solution for 30 min at 4°C and then assaying the inhibition of cell–cell adhesion. One unit of activity is the reciprocal of the highest dilution that blocked at least 90% of the antibody. For example, if the highest dilution of a sample that blocked 90% of the antibody was 1:50, this solution contained 50 units in 100 μl or 500 units/ml. The total number of units was determined by multiplying by the volume (number of ml) of the sample.

* Determined with the anthrone reagent (11).

† Determined by the method of Bradford (12).

are approximately this number of relevant antigenic sites on the cell surface.

The experiment summarized in Fig. 5 also indicates that the anti-D.d.-IgG contains many antibodies that bind to surface antigens without influencing cell–cell adhesion. At all of the ^{125}I-labeled IgG concentrations used, 3% to 4% of the total bound to the cells, indicating that we had not approached saturation of all the cell surface antigens. Yet reaction of the anti-D.d.-IgG with our antigen neutralized the adhesion-blocking activity of the entire antibody mixture. It is this specificity that defines our antigen as a cell-adhesion molecule, in contrast with other cell surface antigens that bind other antibodies in anti-D.d.-IgG.

Because the antigen was purified on the basis of neutralization of anti-D.d.-IgG, we considered the possibility that the antigen might not have been identified with the more conventional approach (1), which uses Fab. This was excluded because antigen purified through the antibody affinity column step completely neutralized the effect of Fab made from anti-D.d.-IgG.

Although the antigenic material that we identified is clearly different from the M_r 80,000 glycoprotein previously implicated in cell–cell adhesion of aggregating *D. discoideum* cells (1, 2), we sought to determine a possible relationship by trying to adsorb anti-D.d.-IgG with high concentrations of the purified glycoprotein (5). When we incubated 40 μg of anti-D.d.-IgG with 40 μg of this purified glycoprotein (a gift of Ben Murray, University of California at San Diego, La Jolla, CA) we found no detectable neutralization of our antibody in the cell–cell adhesion assay, indicating that the cell-adhesion molecule recognized by anti-D.d.-IgG is distinct. In contrast, antigenic material purified as described in Table 2, which contained 0.2 μg of neutral sugar and less than 0.02 μg of protein, completely blocked the inhibitory effect of 40 μg of anti-D.d.-IgG.

Table 3. Effects of various treatments on partially purified antigen

Treatment	Relative activity
None	100
100°C, 30 min	100
Pronase digestion	100
Periodate oxidation	<2

Antigen purified through the antibody column step was treated as described, and the amount of activity remaining was determined by the cell–cell adhesion assay. Activity is expressed relative to that of untreated material, which is arbitrarily taken as 100. Pronase digestion was carried out as described in Table 2. Periodate oxidation was done with 45 mM sodium metaperiodate in SP solution for 2 days in the dark at room temperature. The reaction was stopped with 2% ethylene glycol and the mixture was dialyzed against SP solution before assay. The oxidized sample was compared with a control in which ethylene glycol was added before sodium metaperiodate.

DISCUSSION

The immunological approach to identification of cell-adhesion molecules has been successful because few of the antibodies raised against cell surface antigens block cell–cell adhesion. In the case of *D. discoideum*, a glycoprotein antigen having a M_r of 80,000 has generally been taken to be critical for cell adhesion of aggregating cells (1–5); one with a M_r of 95,000 apparently plays a role later (4, 5) and there is also some evidence for participation of a glycoprotein with a M_r of 150,000 (14) in cell–cell adhesion. As observed previously and confirmed here, binding of antibodies to many other cell surface antigens does not apparently block cell–cell adhesion. In the case of chicken neural retina in which this approach has also been applied, a glycoprotein with a M_r of about 150,000 has been implicated in cell–cell adhesion (15). As in the present case, its initial purification used conditioned medium from cultured cells (16). Sugar-rich macromolecules eluted from cell surfaces or found in culture media have also been implicated in cell–cell adhesion by using other approaches (see e.g., refs. 17–20; reviewed in ref. 21).

If the material we identified in the present study is really important in cell–cell adhesion, why was it not detected previously? One possibility is that the rabbit we immunized was peculiarly responsive to this antigen, a situation observed previously (22). What is certainly relevant is that, in contrast with previous studies (1), we did not adsorb our antiserum with vegetative cells because they contain similar material. Whereas the nature of the antigenic molecule in vegetative cells could be different from that in the aggregating cells, adsorption with

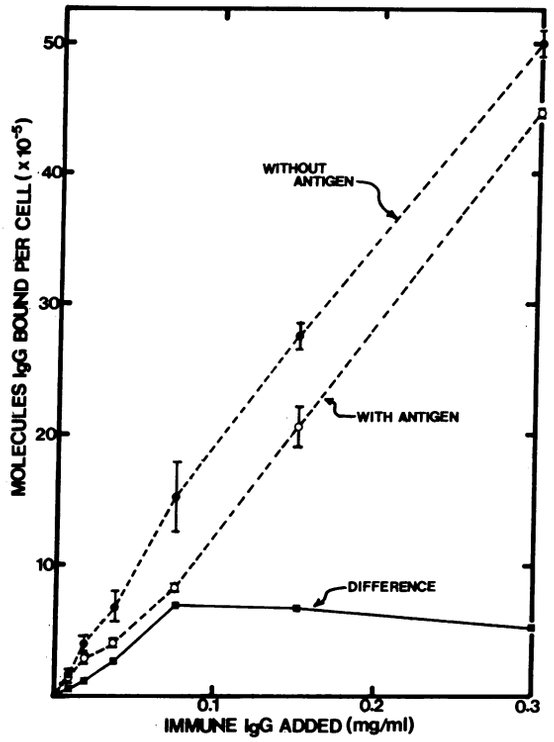

FIG. 5. Binding of ^{125}I-labeled anti-D.d.-IgG to *D. discoideum* cells in the presence and absence of partially purified blocking antigen. Binding of ^{125}I-labeled anti-D.d.-IgG in the presence of nonimmune IgG at 5 mg/ml was determined in the presence (○) or absence (●) of antigen purified through the antibody affinity column step. The range of concentrations of ^{125}I-labeled IgG and the cell concentration (2×10^7/ml) used were similar to those used in the incubation step of Fig. 1*A*. Antigen containing 15 μg of sugar and the indicated concentrations of IgG were incubated at 4°C in 225 μl for 30 min before addition to the cells. This antigen concentration is 10 times that needed to neutralize anti-D.d.-IgG at 0.3 mg/ml. Results represent mean ± range of duplicate determinations. ■, Difference in binding after incubation with or without partially purified blocking antigen.

vegetative cells would have removed our antibody.

The fact that our antigen is apparently present in vegetative cells does not preclude its role in cell–cell adhesion, because some other factor may limit the acquisition of adhesiveness with development, as considered in detail previously (23). For example, the material we have identified may participate in cell–cell adhesion only after its modification, a change in its cellular position, a general change in cellular properties, or the synthesis of a specific ligand with which it interacts. On the other hand, the evidence presented here does not prove that this material plays a specific role in cell–cell adhesion (23).

Because of evidence that discoidin I, a developmentally regulated lectin from *D. discoideum* also plays a role in cell–cell adhesion (24–26), we assessed its interaction with material purified as described in Table 2. This material markedly inhibited the binding of discoidin I to a neoglycoprotein rich in lactosyl residues (unpublished experiments). However the relevant antigen is probably not completely purified by our procedure, so it is not clear whether the same molecules that bind the lectin are also responsible for adsorbing our antibody. This will be resolved after further purification of the active materials. It is notable that the culture medium of another cellular slime mold, *Polysphondylium pallidum*, contains a highly glycosylated macromolecule that interacts with its endogenous lectin (27). That lectin too has been implicated in cell–cell adhesion (26, 28).

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