Failure To Detect Immunocytochemically Reactive Endogenous Lectin on the Cell Surface of *Dictyostelium discoideum*

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ABSTRACT The endogenous lectins of *Dictyostelium discoideum*, called discoidins I and II, have been implicated in cell cohesion during the associative phase of this organism. In an effort to repeat and extend the studies of these putative cell-surface proteins, we attempted a variety of immunocytochemical techniques. Antibodies to a mixture of the purified discoidins were raised in rabbit. Both living and fixed cells were examined by indirect immunoferritin labeling using whole antiserum and by direct immunolabeling using purified specific IgG adsorbed to colloidal gold. Cells, at the appropriate stage, of strains A3, NC-4, and WS-582 were tested. In no instance were cell surface antigens detected despite meticulous efforts to duplicate the published techniques and to extend and refine them. Specific localization was found only in the cytosol and on the cytoplasmic face of certain endomembrane vesicles, and much less so on outer nuclear and mitochondrial membranes, in inadvertently disrupted cells. In no case was specific label found on either side of the plasma membrane or on food vacuoles. Exogenously supplied discoidins, bound to cells, were successfully localized by our technique. We conclude that the discoidins are not present on the cell surface, or are there in undetectable quantities, during the associative phase. We suggest that previous demonstrations of these proteins at the cell surface were artifacts resulting from the way in which the cells were handled, which caused the binding of externalized discoidins, possibly those released from lysed cells. We believe that the current notion that the discoidins play a direct role in cell cohesion by virtue of their carbohydrate-binding capacity should be reexamined. We suggest that the true role of the discoidins is solely intracellular.

The elucidation of the molecular basis for cell-cell recognition is fundamental to understanding the mechanisms that underlie the complex cellular interactions occurring in the morphogenesis and development of multicellular organisms. An increasing amount of evidence suggests that cells have on their surfaces the means to specifically recognize and bind to other cells (2, 19). Specific cellular interactions are thought to occur through the association of complementary cell surface proteins and glycoproteins.

Because of their unique life cycle, the cellular slime molds have been used by many workers for studies of intercellular recognition and adhesion. The most extensively studied organism has been *Dictyostelium discoideum*. In this species, the concentration of two lectins, discoidins I and II, initially discovered in soluble extracts containing hemagglutination activity (35), increases dramatically upon differentiation of the organism from a unicellular to a multicellular state. During this time the cells become mutually cohesive. Evidence suggesting the presence of the discoidins on the surface of cohesive amoebae has been presented (1, 4, 10, 13, 28, 36, 39, 41). These data suggest the involvement of the discoidins in the cell cohesion process. Lectins have also been discovered in all other species of cellular slime molds so far examined (36). The accumulated data on the discoidins and other slime mold lectins have led to the proposal of the "lectin hypothesis" of cell-cell cohesion. The theory states that species-specific cellular cohesion is accomplished through these lectins by virtue of their carbohydrate-binding ability (2, 7).

Recently, the presence of hemagglutination activity during development of the sexual stage in *D. discoideum* has been reported (16). This activity has been shown to be due to the presence of the discoidins (18). In view of the evidence sug-

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gesting the involvement of the discoidins in cell cohesion during the asexual life cycle, we wished to determine whether and when the discoidins were present on the surface of cells undergoing sexual development. To set up a positive control, we conducted experiments to repeat and extend the previously reported immunocytochemical localization of cell surface discoidin on asexually grown cohesive cells (13).

In the present study, we present evidence that suggests that the discoidins are not present on the surface of cohesive cells during asexual development, nor are they present on the surface of cells undergoing sexual development. In addition, we present evidence suggesting that the location of the discoidins is solely intracellular.

MATERIALS AND METHODS

Culture Conditions: Vegetative cells were grown on nutrient agar (3) in aluminum pans by inoculating 5×10^6 spores of strains NC-4 or WS-582 (compatible mating type to N-4) with the food bacterium, *Escherichia coli*, strain B/r, and incubating them at 23°C for 36 h. The cells were then harvested in cold deionized water, washed three times by centrifugation at 175 *g* for 10 min, and resuspended in 17 mM Na_2HPO_4-KH_2PO_4, pH 6.2, containing 50 μg/ml streptomycin sulfate (4, 33) at a concentration of $1–3 \times 10^7$ cells/ml. Aggregation-competent cells were produced by suspension differentiation of vegetative cells in the above buffer for 12 h on a rotary shaker at 23°C. In some instances, cells were grown on 0.5% Bacto-lactose, 0.5% Bacto-peptone, 1.5% Bacto-agar (5LP) in petri plates until the time when the cells had just begun to form streaming aggregates. The axenic strain, A3, was grown in the HL-5 medium of Loomis (26) to $6–7 \times 10^6$ cells/ml.

For mating, cultures were grown in liquid media by the method of Chagla et al. (12). Sexual cultures were also produced on solid medium by the method of Erdos et al. (17). In some instances, 18–20-h sexual cells were pelleted from liquid culture medium, resuspended in 1 mM Tris-HCl, pH 6.5, containing 1 mM $CaCl_2 \cdot 2H_2O$ (31), and allowed to proceed through the sexual cycle on glass coverslips in a moist chamber within a light-tight box.

Cell Extract Preparation: Crude cell extracts were prepared by a method modified from that of Frazier et al. (20). Suspension-differentiated NC-4 or WS-582 cells and axenically grown A3 cells were pelleted by centrifugation and resuspended in PBS, pH 6.4 (36) at a concentration of 2×10^8 cells/ml. Cell-free extracts were prepared by liquid nitrogen freeze-thaw in the presence of 2×10^{-4} M phenylmethylsulfonyl fluoride, and by subsequent centrifugation at 100,000 *g* for 75 min. Twofold serial dilutions of the resultant supernatants were assayed for hemagglutination activity in V-microtiter plates (Dynatech Laboratories, Alexandria, VA) using glutaraldehyde-fixed sheep red blood cells (35).

Affinity Purification: Discoidins I and II were co-purified from cell-free extracts of strains A3 and NC-4 on acid-treated Sepharose 6B (9). Protein concentrations were determined by the method of Lowry et al. (27) using BSA as a standard. The purity of the preparations was verified by PAGE.

Preparation of Discoidin(s)-Sepharose: Sepharose 4B-CL was activated with cyanogen bromide in acetonitrile by the method of March et al. (29). A mixture of the discoidins (7 mg), in 0.2 M $NaHCO_3$ containing 0.3 M D-galactose, was coupled to the activated Sephrose for 24 h at 4°C. After this coupling, the gel was stirred gently for 2 h at room temperature with 0.5 M 2-aminoethanol, pH 8.0 (38). The gel was then washed with 0.2 M glycine-HCl, pH 2.3, and finally with PBS, pH 7.4. The resultant discoidin(s)-Sepharose contained ~1 mg discoidin(s)/ml Sepharose.

Antibody Preparation and Characterization: Rabbit antibodies against the discoidins were prepared by the following protocol: 1 mg of the discoidins (~90% discoidin I, 10% discoidin II) in complete Freund's adjuvant was injected subcutaneously twice, 3 wk apart. 3 wk after the second injection, 1 mg in incomplete Freund's adjuvant was injected intravenously. Bleeding was performed 2 wk later, and subsequently 1 mg in incomplete Freund's adjuvant was injected subcutaneously. Bleeding was performed again 2 wk later.

The antiserum was tested by immunodiffusion (32) in 1% agarose, 0.15 M Tris buffered normal saline, pH 7.2, against purified discoidins from strains A3 and NC-4 using agarose immunodiffusion tablets (Bio-Rad Laboratories, Richmond, CA) prepared with 0.3 M D-galactose. Immunodiffusion against A3, NC-4, and WS-582 cell-free extracts was also performed. The agarose gels were dried, stained in Crocein scarlet (15) for 30 min, and then destained in 0.3% acetic acid. The dried gels were contact printed to give a negative image on the photographs.

Affinity Purification and Characterization of Antidiscoidin(s) Antibody: Ammonium sulfate precipitation (22) of the antiserum was performed to prepare a gamma globulin fraction (IgG), which was then exhaustively dialyzed against PBS, pH 7.4. The dialyzed fraction was incubated for 24 h at 4°C with the discoidin-Sepharose, and any unbound material was subsequently removed by extensive washing with PBS. The bound antibody was then eluted with 0.2 M glycine-HCl, pH 2.3, neutralized, and dialyzed against PBS for 36 h. The purified antibody was tested by immunodiffusion as above. Ammonium sulfate precipitation of the preimmune serum was also performed.

Preparation of the Gold Marker: Colloidal gold was prepared according to the method of Frens (21) as modified by Horisberger and Rosset (24). The particles produced were ~15–20 nm diam. The optimum amount of protein needed to stabilize the colloid was determined by the method of Roth et al. (37). Antidiscoidin IgG and preimmune IgG were adsorbed to the colloidal gold.

Electron Microscopy Studies: Electron microscopy of aggregating or aggregation-competent NC-4, WS-582, and A3 cells was performed. Studies were performed on suspension-differentiated cells and cells allowed to differentiate in situ on agar cultures. In the latter case small blocks of agar, carrying cells at the appropriate stage, were inverted on small polyethylene squares. The agar was carefully lifted away, leaving the developing cells in much the same orientation that they had on the culture plate. The cells were then carried in this manner throughout the experiment. Both unfixed cells and cells rapidly fixed in glutaraldehyde were treated with antibody. The fixation method of Chang and co-workers (13, 14) was used. In their studies they determined that only the briefest exposure to the fixative could be used. Cells were fixed in 1% glutaraldehyde in 20 mM Na_2HPO_4-KH_2PO_4, pH 7.2 (Na-K), for 15–30 s followed immediately by three washes of 5 min each in 0.02 M glycine in Na-K to halt further fixation. Cells, whether fixed or not, were treated with 0.2% BSA three times for 5 min each (13).

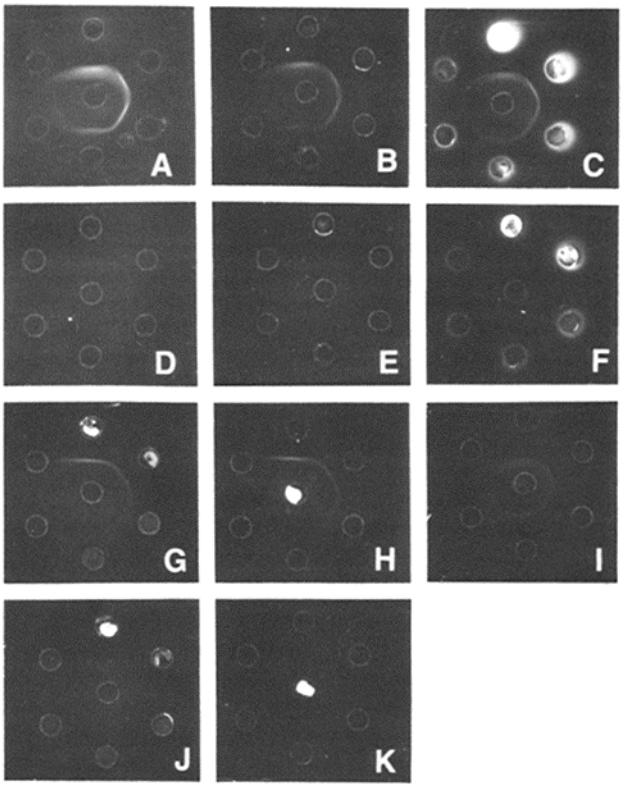


FIGURE 1 Immunodiffusion analyses. Center wells contain the serum or antibody tested. Outer wells contain serial twofold dilutions of antigen. (*A*) Purified A3 antigen tested against antiserum and (*D*) preimmune serum. (*B*) Purified NC-4 antigen tested against antiserum and (*E*) preimmune serum. (*C*) Crude cell-free extract of A3 tested against antiserum and (*F*) preimmune serum. (*G*) Crude cell-free extract of NC-4 tested against antiserum and (*J*) preimmune serum. (*H*) Crude cell-free extract of WS-582 tested against antiserum and (*K*) preimmune serum. (*I*) Purified A3 antigen tested against purified antidiscoidin antibody.

For indirect immunoferritin labeling, the cells were incubated with a 1:400 dilution of the antiserum in BSA Na-K for 1 h, washed several times in BSA NA-K over 1 h, treated with ferritin-conjugated goat anti-rabbit IgG (Miles Laboratories, Elkhart, IN) for 1 h, and washed again. Controls consisted of preimmune serum or buffer in place of the antiserum. For direct labeling, purified specific antibody adsorbed to colloidal gold was used as the primary label. Cells were treated and washed as described above. Controls consisted of unlabeled colloidal gold or preimmune IgG-gold in place of the antidiscoidin-gold.

Following antibody labeling, the cells were fixed in 2.5% glutaraldehyde in Na-K for 15 min, postfixed in 1% OsO_4 in Na-K for 30 min, dehydrated, and embedded in Spurr's resin (42). Thin sections were viewed either unstained or stained with uranyl acetate or uranyl acetate followed by Reynolds' lead citrate (34).

To determine whether the rapid fixation used was denaturing the antigen such that the antibody would not bind, exogenously supplied lectin was bound to glutaraldehyde-fixed cells after the method of Reitherman et al. (33). Both purified discoidins and cell-free extract of a high hemagglutination titre were used as the source of the exogenous antigen. The fixed cells were incubated for 2 h with either the purified discoidin at ~300 μg/ml, or the cell-free extract and then extensively washed. Next, they were either treated directly with antidiscoidin-gold or subjected to the rapid glutaraldehyde fixation (as above) before being treated with the labeled antibody. After being washed, they were processed as described above for electron microscopy. BSA Na-K was substituted for the exogenously supplied discoidins as a control.

RESULTS

Immunological Characterizations

Antiserum to the discoidins was characterized by immunodiffusion against purified A3 and NC-4 discoidins and crude soluble extracts (Fig. 1, *A–C* and *G*). Two very closely spaced precipitin lines could sometimes be detected when the initial protein concentration was sufficiently high (Fig. 1 *C*). This result is interpreted as an indication that the antiserum contains antibodies against both discoidins I and II. No attempt was made to separate the antibodies against discoidin I from those recognizing discoidin II. Recently, it has been reported by Berger and Armant (10) that cross-reactivity exists

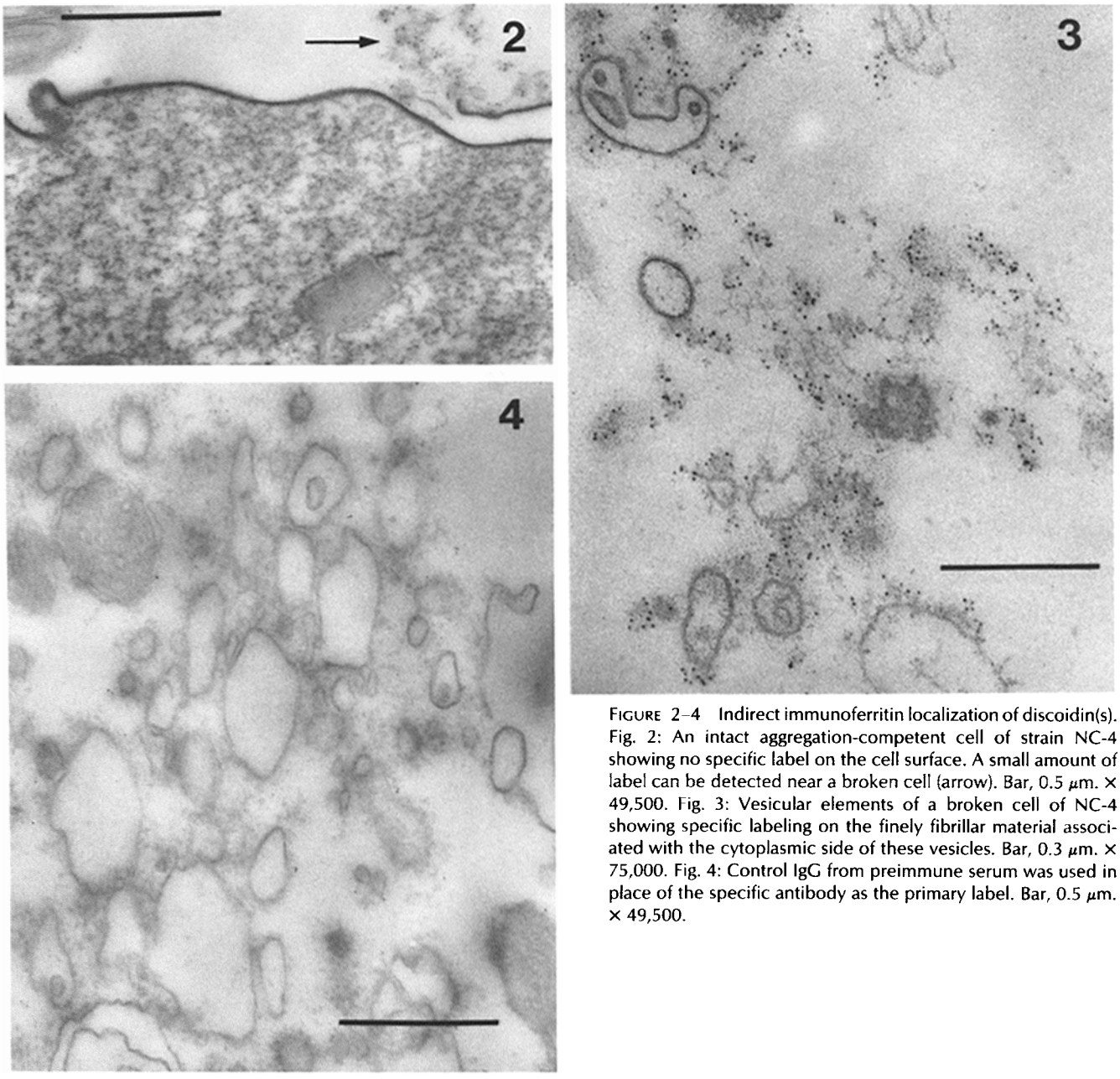


FIGURE 2–4 Indirect immunoferritin localization of discoidin(s). Fig. 2: An intact aggregation-competent cell of strain NC-4 showing no specific label on the cell surface. A small amount of label can be detected near a broken cell (arrow). Bar, 0.5 μm. × 49,500. Fig. 3: Vesicular elements of a broken cell of NC-4 showing specific labeling on the finely fibrillar material associated with the cytoplasmic side of these vesicles. Bar, 0.3 μm. × 75,000. Fig. 4: Control IgG from preimmune serum was used in place of the specific antibody as the primary label. Bar, 0.5 μm. × 49,500.

between antisera produced separately against the discoidins. Fig. 1*H* depicts the results of immunodiffusion analyses against WS-582 (compatible mating type of NC-4) crude cell extract. Both strains show reactivity with antiserum produced against the discoidins. In all of the immunodiffusion analyses which were performed, we saw no reaction with preimmune serum (Fig. 1, *D–F* and *J–K*). Immunodiffusion analysis of affinity-purified antibody against the discoidins showed that reactivity was still retained after purification (Fig. 1*I*).

Immunoelectron Microscopic Studies

Numerous attempts were made to repeat the cell surface localization of the discoidins on asexually developing wild-type NC-4 cells as reported by Chang et al. (13). Immunocytochemical localization experiments were also performed on unfixed cells of NC-4, WS-582, and A3, and on NC-4 and WS-582 cells briefly fixed with glutaraldehyde, using either direct or indirect labeling methods. In all cases, the results were essentially the same regardless of the technique employed, that is, no significant cell surface labeling was detected. Only a few examples of the various permutations attempted are presented. Using the indirect immunoferritin technique, we detected occasionally a small amount of cell surface label on cells that were adjacent to inadvertently lysed cells (Fig. 2), but the cell surface was essentially free of any label. The escaping cytosol did label positively. Significant specific label was observed on the cytoplasmic side of certain vesicular elements released from the small number of inadvertently broken cells (Fig. 3) found in our preparations. In these cases most of the cytosol had been lost on lysis and hence heavier labeling was not observed. Fig. 4 is a control in which preimmune serum was used as the primary label. With a direct label (antidiscoidin-gold), a similar pattern emerges. The escaping cytosol labels heavily (Fig. 5) but intact cells show no label on their surface (Fig. 6). Again, certain membrane vesicles label preferentially (Figs. 7–9), including vesicles containing protein paracrystals (Fig. 9), which indicates that the vesicles are right side out. Less label was found associated with outer mitochondrial and outer nuclear membranes (not shown). No label was found associated with the cytoplasmic side of food vacuole membranes (Fig. 7 and 10) or with either side of the plasma membrane (Fig. 10) under these circumstances. As with the indirect ferritin method, some cell surface label could be found on intact cell when a broken cell was nearby (not shown).

When discoidins from an exogenous source (either purified discoidins or cell free extract of cohesive cells) were applied to the cells, surface label could be detected. The results were essentially identical regardless of whether or not the bound discoidin had been subjected to the rapid glutaraldehyde fixation prior to the application of the labeled antibody (compare Figs. 10–14). The spotty distribution of label is much like that presented by Chang et al. (13), who claimed to be localizing endogenous cell surface discoidin.

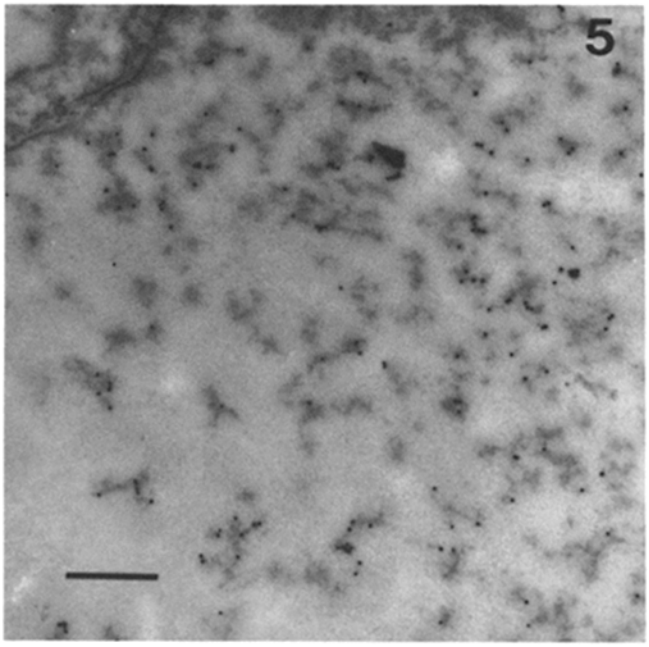
DISCUSSION

Repeated attempts to localize the discoidins on the cell surface of asexually developing NC-4, WS-582, or A3 cells or sexually grown cells were unsuccessful, although intracellular discoidins could be detected in inadvertently lysed cells. This result shows that the antiserum produced in this study can recognize the discoidins in situ. If the discoidins are involved in cell-cell cohesion, then these active sites should be exposed on the cell surface. Alternatively, the possibility exists that the low amounts of cell surface discoidins (10, 40) were somehow lost during the fixation process or subsequent treatments. However, every effort was made to treat the cells as gently as possible to minimize any loss of antigen that might occur. Additionally, the fact that we could successfully localize exogenously supplied lectin supports our contention that our technique is not the cause of our failure to localize any endogenous surface lectin. Further, it also shows that our antibody will recognize the discoidin that has bound to surface receptors.

The results of this study suggest that there is no cell surface discoidin on wild type NC-4, WS-582, or A3 cells or on cells undergoing sexual development. The negative findings with NC-4 aggregation-competent cells are contrary to those of Chang et al. (13). This contradiction has yet to be resolved despite meticulous efforts to precisely duplicate their technique as described in the literature. Bartles et al. (9) have reported that a small amount of discoidin I is "externalized" into the extracellular medium during suspension differentiation. There has been, however, no proposed mechanism for this "externalization." It is possible that the earlier evidence for cell surface discoidin may have been the result of the binding of this externalized discoidin to the membrane due to some undescribed handling of the material. Furthermore, it is also possible that some discoidin could have been released from lysed cells and subsequently been bound to the cell surface. Our observation that label could occasionally be found on cell surfaces when a lysed cell was close by and that application of exogenous lectin to intact cells could produce results similar to those presented previously (13) lends credence to this idea.

The question of whether lysed cells could be the source of the cell surface discoidin has never been addressed adequately by those who have reported its presence except for in the work of Armant and Berger (1), who estimated that their procedure for dissociating cells released ~0.5% of the soluble discoidin. They make no further estimates of release during subsequent steps. Considering that the most recent estimate of the amount of total cellular discoidin that can be found on the cell surface is ~0.02% (9), very little cell lysis need occur to release

FIGURES 5–9 Direct immunolabeling of discoidin(s) using colloidal gold bound to specific antibody or control IgG. Fig. 5: Specific label of the cytosol of a ruptured cell of WS-582. Bar, 0.5 μm. × 24,000. Fig. 6: An intact, aggregation-competent cell of A3 showing no label associated with the cell surface. Stained with uranyl acetate only. Bar, 0.5 μm. × 50,000. Fig. 7: Specific localization of discoidin on the cytoplasmic side of vesicles from a broken cell of WS-582. Note no label associated with the food vacuole (*FV*). Bar, 0.5 μm. × 42,900. Fig. 8: Similar material as Fig. 7 except that control IgG-gold was used as the label. Bar, 0.5 μm. × 50,000. Fig. 9: Specific label associated with a vehicle containing a protein crystal (arrow) indicating that it is the cytoplasmic side of these vesicles that is exposed to the label. Bar, 0.5 μm. × 42,900.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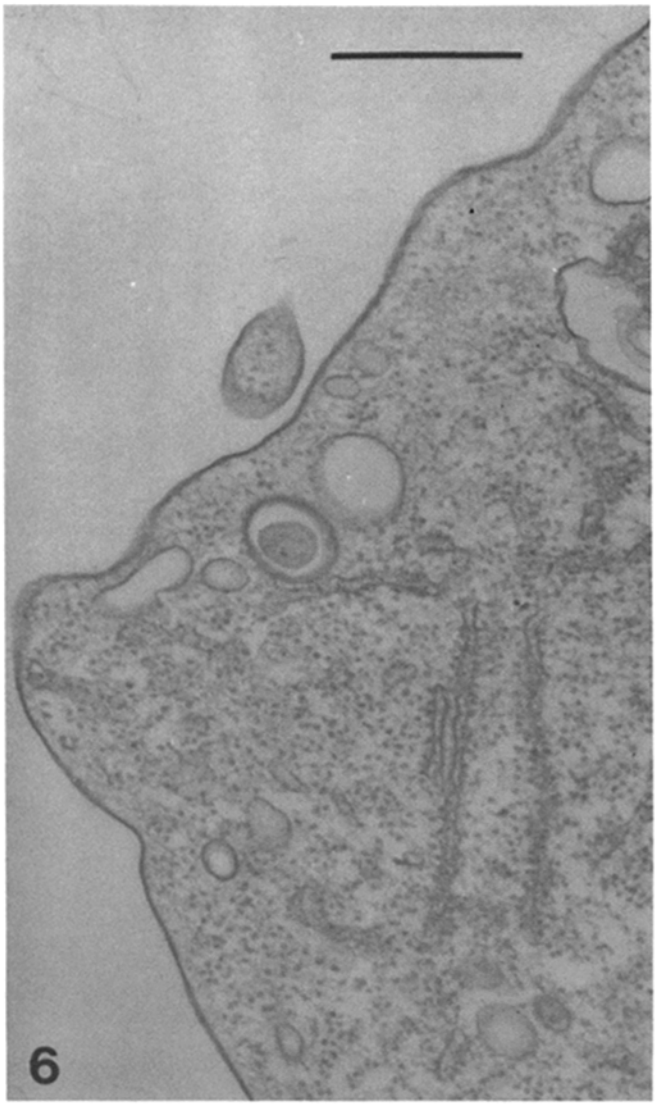
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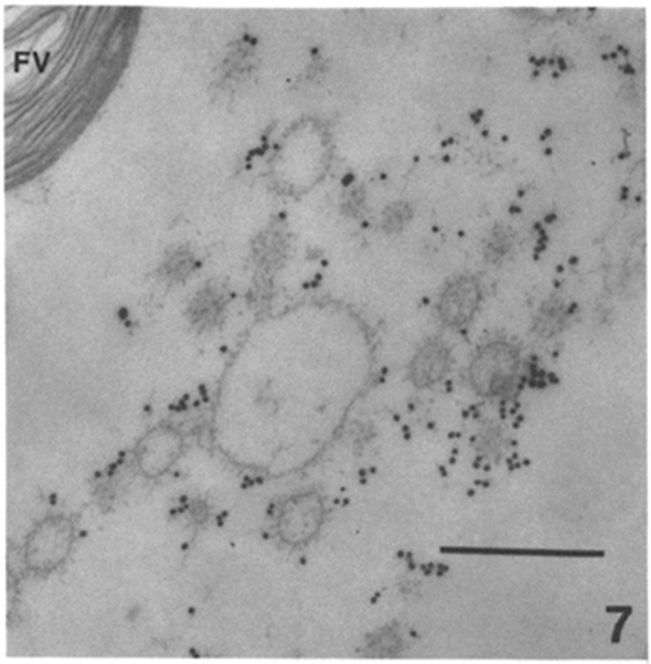
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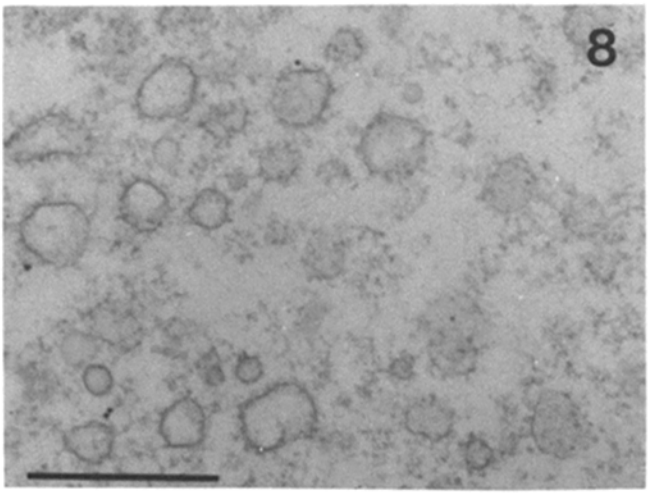
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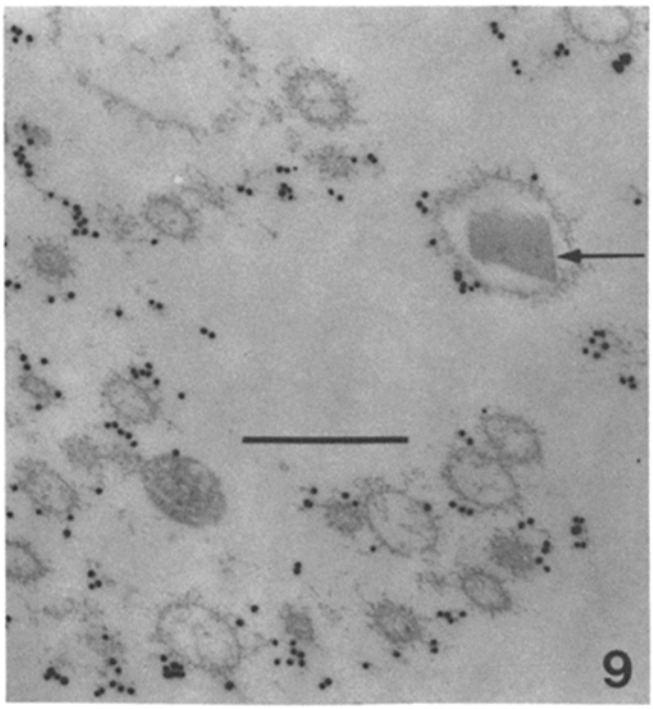
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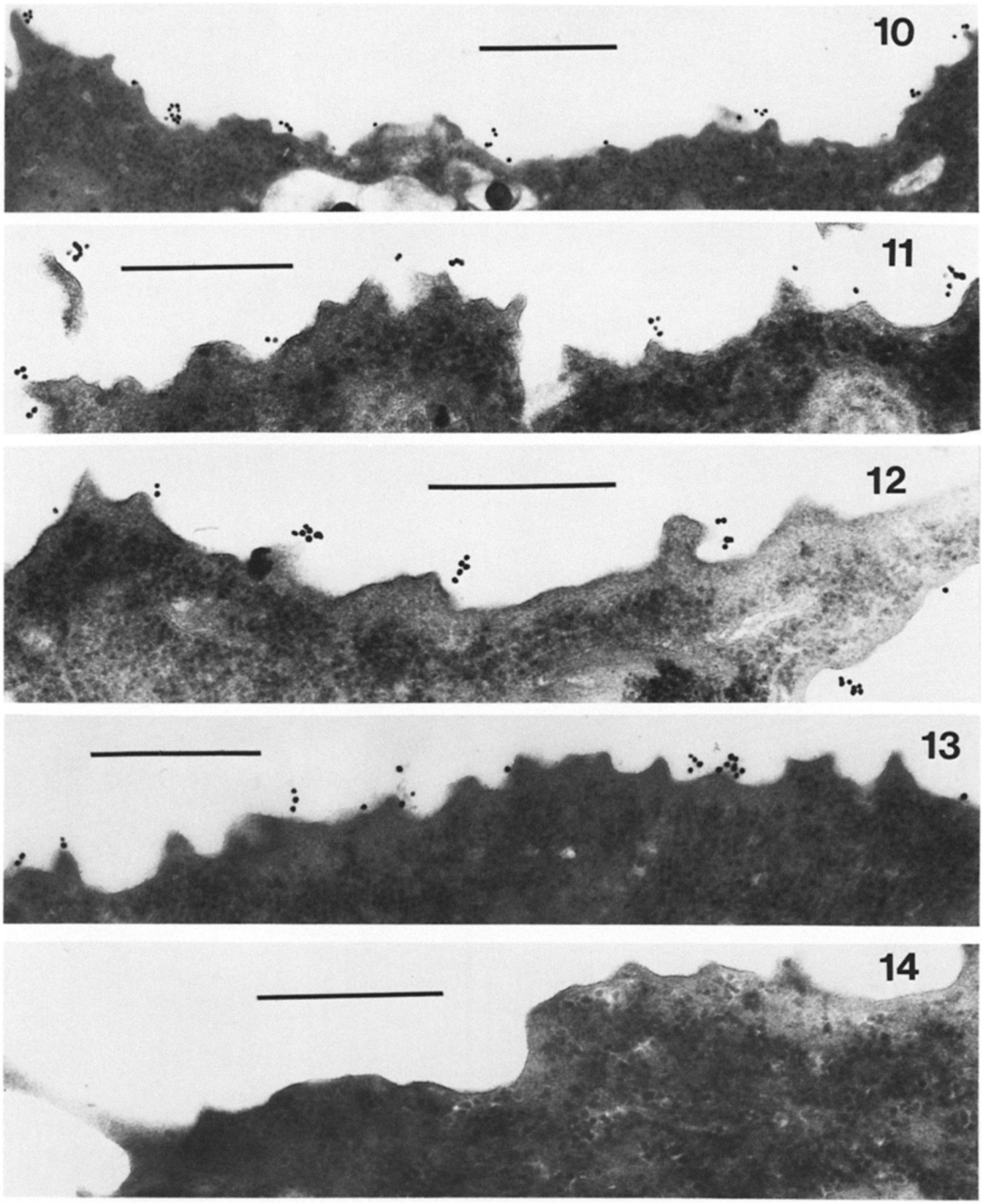

FIGURES 10–14 Cell surface localization of exogenously supplied discoidins bound to cells previously fixed with glutaraldehyde. Fig. 10: Cell treated with purified discoidins and then subjected to the rapid glutaraldehyde fixation before immunolabeling (as used in the localization of the endogenous lectin). × 46,000. Fig. 11: Same as in Fig. 10 but not fixed following application of discoidins. × 60,000. Fig. 12: Cell treated with cell-free extract of cohesive cells followed by rapid glutaraldehyde fixation before immunolabeling. × 66,000. Fig. 13 same as Fig. 12 but not fixed after treatment with cell-free extract. × 59,800. Fig. 14: Control cell not supplied with exogenous discoidin before immunolabeling. Bars, 0.5 μm.

enough discoidin to occupy all of the receptors on the surfaces of all the cells. How one is to monitor or determine such low levels of cell breakage during handling is a difficult problem and, at this time, we can provide no further insight as to how one might approach this problem. A similar difficulty has been encountered in the study of some vertebrate lectins (11, 25), where evidence for surface lectin has been found but the majority of the lectin is cytoplasmic. These authors were unable to rule out the possibility that the surface lectin they detected immunocytochemically had actually come from lysed cells or cell-fragments. In examining the work of others, Kaufman and Lawless (25) found reason to doubt cell surface immunolocalization of lectins. (See their discussion for details of their reservations.)

We have also observed, over the years, that amebae, under stress, will cast off small bits of their protoplast by plasmotomy. This is also characteristic of amebae in liquid culture. These membrane-bounded packets of cytosol would contain the lectins. One would expect that these structures would not be very stable and would soon lyse, thus releasing the lectins into the surrounding medium where they could bind to intact cells. This could also be the source of spurious cell surface localization.

Evidence against the involvement of the discoidins in cell-cell cohesion has been reported and has been extensively considered by Bartles and colleagues (5, 6, 9). These data deal with the effect of competitive inhibitors and antagonists of the discoidins on cell cohesion. Springer and Barondes (40) reported that the binding of antidiscoidin IgG plus goat anti-rabbit Fab fragments, or antidiscoidin Fab fragments alone, to differentiated *D. discoideum* cells, had little inhibitory effect on cell-cell adhesion. Similarly, Gerisch et al. (23) have reported that Fab against discoidins I and II did not significantly inhibit cell adhesion. However, this Fab preparation did inhibit the lectin-mediated agglutination of erythrocytes, which indicated that the Fab fragments were interfering with the carbohydrate-binding ability of the lectins. In addition, the lectins did not detectably adsorb adhesion-blocking Fab. Electrostatic interaction of discoidin I with biological membranes has been reported by Bartles and co-workers (7, 9). However, inhibitors of this interaction, such as heparin, have no effect on the EDTA-resistant cohesion of *D. discoideum* cells (8, 9). These investigators have also failed to detect immunoreactive discoidin I on the surface of aggregation-competent NC-4 or A3 cells (6, 9). In addition, *D. discoideum* cells can apparently display maximal cohesiveness while exhibiting miniscule amounts of discoidin I on their surfaces or in the surrounding medium (9). Attempts have also been made to bind a number of labeled asialoglycoproteins to cohesive cells (G. W. Erdos, unpublished observation). Although some of these should bind to the discoidins, none was ever successfully bound to the surface of these cells.

The negative findings for cell surface localization of the discoidins, together with their localization within aggregation-competent and aggregating *D. discoideum* cells and the large quantities of lectin that can be isolated from soluble extracts of these cells, suggest that the discoidins are more likely involved with intracellular functions than with one directly involving them in cell-cell cohesion. Alternatively, the discoidins may play a regulatory role in the development of *D. discoideum*, as suggested by Marin et al. (30).

In light of the findings presented in this study, and those of other workers previously cited, we suggest that the cell cohesion theory proposing the direct involvement of the discoidins in cell-cell cohesion be reassessed and that the possible involvement of the discoidins in intracellular functions be further investigated.

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