DIRECT MEASUREMENT OF SPECIES-SPECIFIC COHESION IN CELLULAR SLIME MOLDS

WAYNE R. SPRINGER and SAMUEL H. BARONDES. From the Department of Psychiatry, School of Medicine, University of California at San Diego, La Jolla, California 92093

ABSTRACT

Partially differentiated cells of two species of cellular slime molds, *Dictyostelium discoideum* and *Dictyostelium purpureum,* were labeled with isothiocyanate derivatives of fluorescent dyes. The labeled cells of each species segregated promptly when mixed and placed on moist filters. We determined whether cells studied at a time when they demonstrated this capacity to segregate showed a preferential adherence to cells of the same species. When labeled dissociated cells of each species were interacted with an unlabeled immobilized layer of cells of each species under appropriate conditions, binding was, in part, species-specific.

KEY WORDS cell sorting · cell cohesion · *Dictyostelium* · slime molds · fluorescent labeling

During periods of food deprivation, the cellular slime molds undergo developmental changes, which cause them to aggregate and form fruiting bodies. When mixtures of two species of slime molds develop in this way, cells of each species eventually segregate (2, 7), a process which may be called sorting out. This is readily seen in the differentiated final products of mixed cultures of *Dictyostelium purpureum* and *Dictyostelium discoideum*. The fruiting bodies that result upon differentiation of these mixtures contain spore caps which may contain exclusively the purple spores of the former species or the white spores of the latter (2, 7). By feeding cells of one species pigmented bacteria, Raper and Thom (7) were able to identify many of these cells at earlier stages in development. They found that segregation begins during the aggregation phase.

Some cases of sorting out between mixed populations of slime molds are due to distinct diffusible chemotactic substances which act as specific aggregation signals. Thus, several species of *Dictyostelium* aggregate towards centers which emit pulses of extracellular cyclic AMP (3, 6), whereas *Polysphondylium violaceum* and *Polysphondylium pallidum* employ a peptide as the chemotactic signal for aggregation (12). These diffusible chemotactic substances could lead to segregation of a species of *Dictyostelium* from a species of *Polysphondylium*. However, sorting out of two species of *Dictyosteliaceae*, which use cyclic AMP as the chemotactic signal for aggregation, must be based on some other mechanism.

Selective cell adhesion is believed to be the other major mechanism used in sorting out of slime molds (2, 4). Yet, as Garrod has emphasized (4), there is presently no direct evidence for this. An alternative is a second chemotactic signal that is species-specific. To test for selective cellular association based on surface properties and to eliminate the effects of diffusible chemotactic signals, a direct binding assay is needed. In the present report, we employ an assay based on those previously described by Walther et al. (11), Gottlieb and Glaser (5), and Rutishauser et al. (9) which measures the selective adhesiveness of cohesive cells of *D. discoideum* and *D. purpureum*. We show that labeling of these cells with isothiocyanate derivatives of fluorescent molecules does not interfere with their segregation. We provide direct evidence that there is differential

cell adhesiveness between these two species which could mediate their sorting out.

MATERIALS AND METHODS

The species used in this study were *D. discoideum* NC-4, originally obtained from W. F. Loomis Jr., and *D. purpureum* (strain 2), originally from J. T. Bonner. Vegetative cells were grown on agar pans with *Klebsiella aerogenes* (10).

Observation of Sorting Out

Vegetative cells were harvested from agar pans, washed with cold distilled water to remove the bacteria, and resuspended to 2×10^7 cells/ml in 17 mM sodium-potassium phosphate buffer, pH 6.2 (SPS). The cells were aerated by shaking at 24°C for 16 h. Under these conditions of starvation, they differentiate to the point where they rapidly aggregate when plated onto a moist filter. The cells were harvested, washed once with cold water, and suspended at a concentration of 10% (vol/vol) in SPS. To 5 ml of this suspension, we added 50 μl of a solution, in dimethyl sulfoxide, containing 25 mg/ml of either tetramethyl rhodamine isothiocyanate (TRITC) (Research Organics, Cleveland, Ohio) or fluorescein isothiocyanate (FITC) (Sigma Chemical Co., St. Louis, Mo.). After shaking for 5 min, the cells were washed with cold SPS and resuspended to 2×10^8 cells/ml. Equal volumes of both species labeled with the different dyes were mixed together, and 0.5 ml was added to a black filter pad in a 60-mm petri plate. The filter pad consisted of a Millipore AABP 047 00 filter (Millipore Corp., Bedford, Mass.) on top of an absorbent cellulose pad (Millipore AP10 047 00) soaked with 1.6 ml of a solution (pH 6.2) containing 1.5 g KCl, 1.0 g $MgCl_2 \cdot 6H_2O$, 0.5 g streptomycin sulfate, 0.3 g Na_2HPO_4, and 1.2 g KH_2PO_4 in 1 l (10). The cells were allowed to develop in a moist atmosphere at 24°C. At intervals, the pads were examined and photographed with a Leitz Dialux microscope equipped with a Ploemopak 2.3 fluorescence vertical illuminator and the appropriate filters.

Adhesion Assay

Cells partially differentiated in suspension, as described above, were used after further differentiation for 1.5-2 h on Millipore filters, as described above. Some aliquots of cells were labeled with TRITC before addition to the filters, whereas others were not labeled. The cells were removed from the filter by vortexing with cold SPS (2 ml/filter). This vortexing also broke up the aggregates into single cells. 1 ml of a suspension of unlabeled cells was added to a derivatized 35-mm plastic tissue culture plate. The plate had been pretreated immediately before use with 1 ml of solution containing 10 mg/ml of concanavalin A (Sigma Chemical Co.) and 25 mg/ml of 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-*p*-toluenesulfonate (Aldrich Chemical Co., Inc., Milwaukee, Wis.) in saline pH 6.6 for 30 min at room temperature and washed three times with saline pH 6.6. Upon addition of the cells, the plates were placed in adapters and centrifuged (with centrifugal force perpendicular to the bottom of the plate) at 1600 *g* for 30 s. The cells were pelleted to the bottom of the plate which they coated completely. Excess, loosely bound cells were removed by washing twice with 1 ml of SPS containing 10 mM EDTA, and then 0.9 ml of the wash buffer containing 2 mg/ml of bovine serum albumin was added above the layer. Labeled (probe) cells, prepared as described above, were diluted to 2×10^6 cells/ml in this same solution, and 100-μl aliquots were added to the solution above the layer while the plates were being gyrated at 70 rpm on a New Brunswick G-24 gyratory shaker (1.9-cm orbit) (New Brunswick Scientific Co., Inc., Edison, N. J.). The shaker speed was reduced to 30-50 rpm, as indicated in the text. After shaking for an appropriate period (10 min in the standard assay), the plates were removed, tilted, and the supernate and unbound cells were removed by aspiration. The number of labeled cells bound to the plate was estimated by counting four fields/plate, at 63 magnification, all at a radius of 1.5 mm from the center of the plate, by epifluorescence microscopy.

RESULTS

We first examined the effects of labeling the cells with fluorescent labels. We grew cultures of *D. discoideum* and *D. purpureum* on *K. aerogenes,* harvested them at low density and allowed them to undergo some differentiation by shaking in suspension for 16 h in the absence of food. The cells become competent to aggregate under these conditions and, when placed on moist filters, aggregation is readily observed to have begun within 30-60 min. When one species of cells was labeled with TRITC, the other with fluorescein isothiocyanate (FITC), and each species was plated separately, the labeled cells behaved exactly like unlabeled controls. When a mixture of equal numbers of the labeled cells of the two species was made, segregation of cells was obvious within ~30-60 min and became progressively more prominent thereafter (Fig. 1). These results demonstrate that, 1.5 h after the labeled cells have been put down on filter pads for further differentiation, they display the properties required for species-specific sorting out. We could therefore confidently use cells at this stage to determine whether they also displayed species-specific cohesiveness.

Layers of cells of either species could be immobilized on tissue culture plates treated with concanavalin A. In all cases, a fairly uniform layer of

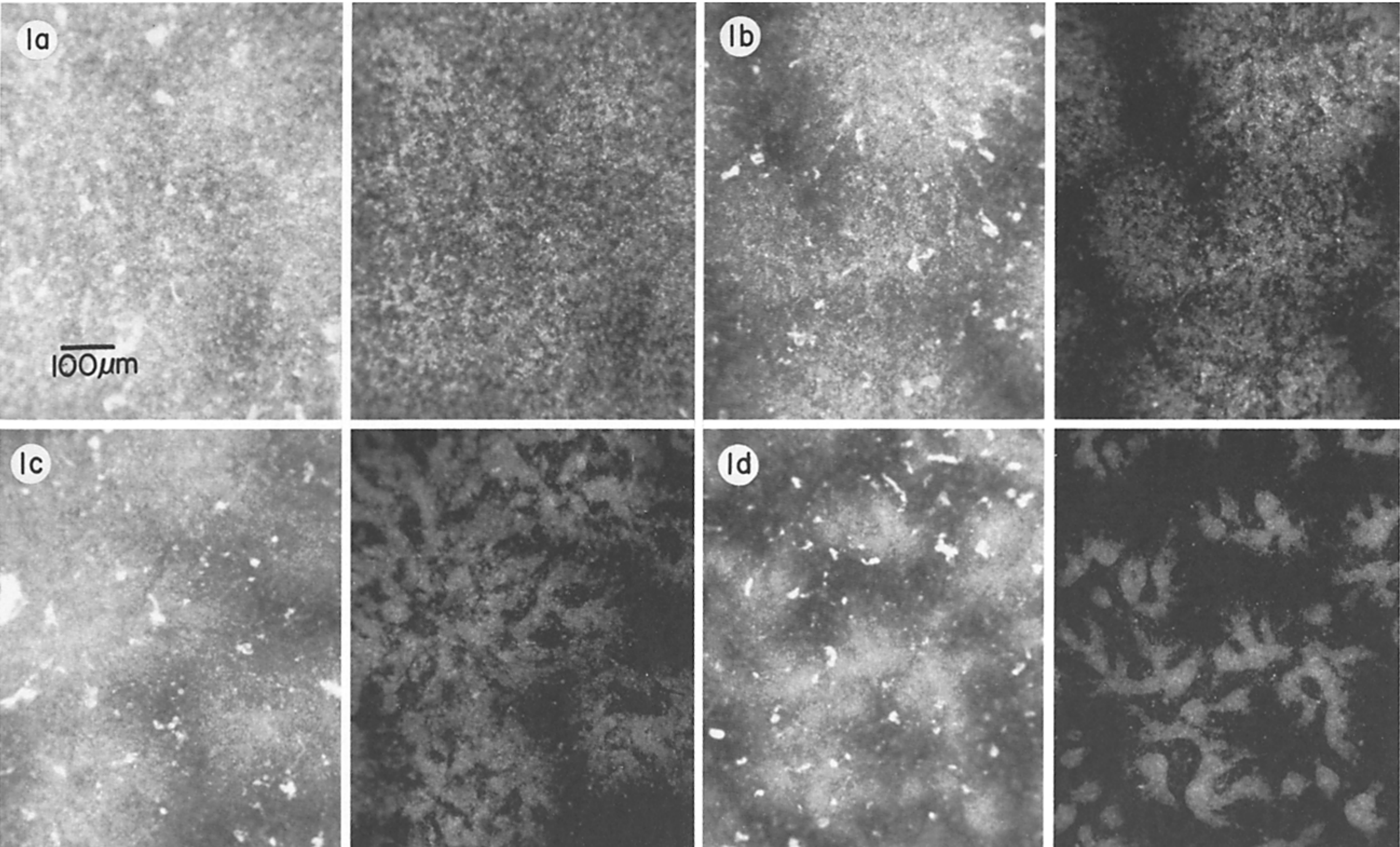

FIGURE 1 Sorting out between two species of cellular slime molds. Cells of the two species, *D. discoideum* and *D. purpureum*, were labeled, mixed, and put on filter pads as described in Materials and Methods. Photographs were taken at the indicated times (in minutes) after adding the cells to the pads. The left portion of each photograph is a field taken with filters to expose total fluorescence (TRITC and FITC) whereas the right is the same field's TRITC fluorescence. *D. purpureum* cells were labeled with TRITC and *D. discoideum* with FITC. Mixed cultures were photographed at (*a*) 30 min, (*b*) 60 min, (*c*) 90 min, and (*d*) 120 min after plating.

these cells could be achieved with very few gaps (Fig. 2*a*). Probe cells of one or the other species labeled with TRITC were interacted with the layer, and the number of probe cells remaining associated with the layer could be counted, by direct observation, with a fluorescence microscope (Fig. 2*b*). Probe cells bound to layer cells and were not concentrated over the rare cell-free gaps. The number of labeled probe cells bound to the layer cells approximated the steady-state condition at 10 min under these experimental conditions with all cell combinations in these experiments (for example, see Fig. 3). The number of cells bound was directly related to the concentration added over the range used in this assay. In the experiment shown in Fig. 3, ~15% of the applied probe cells had bound to the layers within 10 min. This 15% is not a subpopulation of cells that are the only ones capable of binding, but represents the dynamic equilibrium between binding and dissociation under these conditions. This was shown by studying the binding aspects of the residual probe cells aspirated after interaction for 10 min with a layer of cells in a standard assay. When added to a fresh layer, 15% of the remaining probe cells bound to the layer.

Using this assay, we were able to demonstrate species-specific adhesion, provided we used appropriate rates of gyration. Results with gyration during the assay at 40 and 50 rpm are summarized in Table I. At 50 rpm, *D. discoideum* (*D.d.*) probe cells bound about six times better to the *D.d.* layer than to the *D. purpureum* (*D.p.*) layer. Conversely, *D.p.* probe cells bound about twice as well to the *D.p.* layer as to the *D.d.* layer. When the assay was conducted at 40 rpm, the selective association of *D.d.* probe cells with the

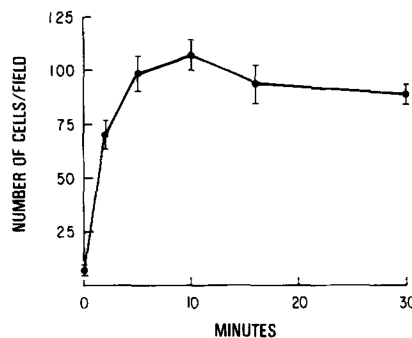

FIGURE 3 Kinetics of binding of *D. discoideum* probe cells to a layer of *D. discoideum*. Cells were prepared and assayed as described in Materials and Methods. The time of shaking at 50 rpm was varied from 0 to 30 min. The figure shows the average number of cells (± SEM) bound to the monolayer per field (three plates per time point) after each of the times tested.

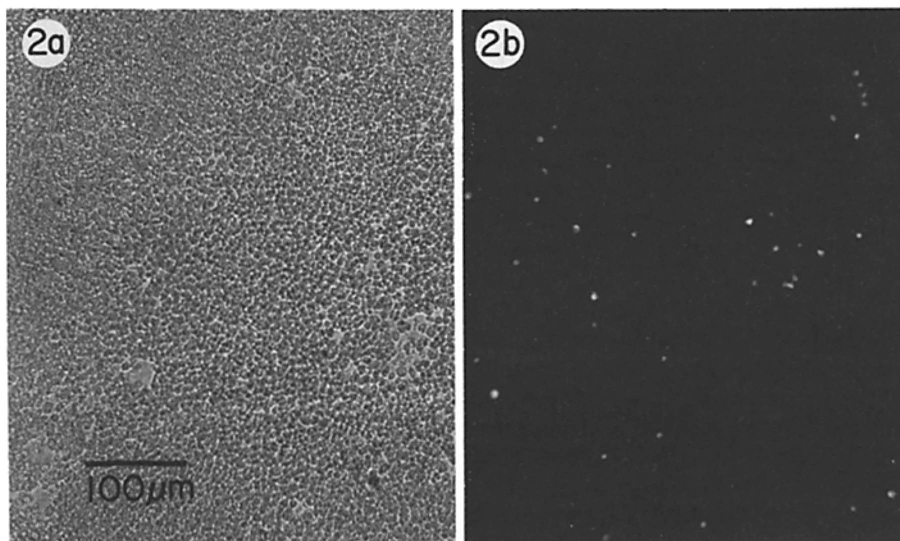

FIGURE 2 Typical layers and probe cell distributions in the adhesion assay. The adhesion assay was done with *D. discoideum* layer and probe cells. The layer cells were photographed with phase-contrast illumination and the probe cells with epifluorescence. (*a*) Layer of *D. discoideum* after 10 min at 50 rpm. (*b*) Labeled *D. discoideum* probe cells bound to the above layer. Note that some of the probe cells appear elongated due to the fact that the probe cells could migrate over the layer of cells.

TABLE I
*Interspecific Slime Mold Adhesion**

Layer	Probe			
	50 rpm		40 rpm	
	D.d.	D.p.	D.d.	D.p.
	number of cells bound per field			
D.d.	115 ± 10	53 ± 4	128 ± 7	61 ± 13
D.p.	18 ± 3	109 ± 7	92 ± 5	147 ± 6

* Cells of *D. discoideum* (*D.d.*) and *D. purpureum* (*D.p.*) were assayed at 40 and 50 rpm as described in Materials and Methods. The results are given as the mean number of probe cells binding per 63 × microscopic field (± SEM) for 2×10^5 cells added to each of the designated layers. Three independent experiments were done for each probe-layer pair at each rpm. The number of cells binding to the homotypic layer is significantly higher ($P < 0.001$) in all cases. Significance was determined using Student's *t* test. An estimate of total number of added probe cells visible per field was obtained by counting multiple fields after settling and without aspiration. This estimate was 638 ± 47 when 2×10^5 cells were added, as in these experiments. If 638 is taken as an indication of 100% of the probe cells added, then binding of 115 cells, as in one case above, represents binding of 18% of the probe cells.

D.d. layer as compared with the *D.p.* layer was still apparent but less dramatic. This was consistently observed in three experiments. When the assay was conducted at 30 rpm, little, if any, species-specific association could be observed (data not shown). Binding in this assay is therefore a combination of species-specific and nonspecific components. The latter are apparently less avid inasmuch as they are more sensitive to shear forces applied by gyration. With appropriate shear, the species-specificity can be demonstrated. In the limiting cases, the assay is either ineffective or nonselective. At very low shear, cell binding tends to be higher and nonselective, whereas at very high shear, there was little if any cell binding (data not shown).

DISCUSSION

These results demonstrate that, under labeling conditions that do not interfere with sorting out, we can demonstrate species-specific cell adhesion between two species of *Dictyostelium* in an assay in which chemotactic signaling cannot play a role. Any putative species-specific chemotactic signaling that would call cells together would be eliminated by the gyration. Their ability to remain associated while being subjected to shear is undoubtedly a property of their cell surfaces. The surfaces therefore show the capacity to make species-specific discriminations, which could lead to the segregation of cells that is observed when the cells differentiate in mixed culture. However, binding is not completely specific. Association across species could be due to truly nonspecific factors and/or to the considerable cross-reactivity which might exist among putative cell surface adhesion molecules of the two species.

This assay can now be applied to study species-specific cohesiveness in slime molds in more detail and in other species. It can be used to determine the conditions and factors necessary for selective cohesiveness. It might test the possible role in species-specific association of the developmentally regulated and species-specific cell surface carbohydrate binding proteins identified and purified from a number of species of slime molds (1, 8)[1] including *D. discoideum* and *D. purpureum*.

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