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Cell-Surface Interactions: Differential Inhibition by Proflavine of Embryonic Cell Aggregation and Production of Specific Cell-Aggregating Factor

(cell ligands/cell recognition/differentiation/acridines)

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ABSTRACT **Cell recognition and morphogenetic aggregation of embryonic cells into tissues are mediated by specific macromolecules in the cell surface (cell-aggregating factors). A factor specific for embryonic neural retina cells was demonstrated; its synthesis is required for histotypic reaggregation of retina cell suspensions. We show that proflavine (3,6-diaminoacridine) preferentially and reversibly suppresses production of the cell-aggregating factor and thereby inhibits normal cell reaggregation. If such proflavine-treated retina cells are exogenously supplied with the retina-specific factor, they reaggregate. The selectivity of these effects supports the postulated significance of specific cell-surface components in cell association into tissues; the results indicate that proflavine may be a useful molecular probe for studying formation of specific cell-surface components and their role in various cell interactions.**

Cell recognition and morphogenetic interactions of embryonic cells are mediated by specific macromolecular components and configurations located in the cell membrane and between cells. It has been suggested that these components function as tissue-specific cell-ligands and phenotypic determinants (recognition sites) of cell surfaces (1–3). Disruption of these cell-ligands by proteolytic treatment makes it possible to disperse the cells. In order to reaggregate into a tissue, the dispersed cells must reform on their surfaces the specific cell-ligand components in appropriate configurations; for this, macromolecular synthesis is necessary (3, 4).

The concept of specific cell-ligands has been supported by demonstrations that embryonic cells produce macromolecular factors with tissue-specific cell-ligand activity (cell-aggregating factors); these factors are released in cell cultures into the culture medium, from which they have been recovered (3, 5). Such tissue-specific factors, when added to fresh suspensions of homologous cells, strikingly enhanced cell reaggregation (3, 5). Thus, cell-aggregating factor obtained from embryonic neural retina cells enhances the reaggregation only of neural retina cells (3); the factor from cerebrum cells preferentially enhances the reaggregation of cerebrum cells, but not of cells from other brain regions, or from other tissues of the same embryo (5). The selective effects of these factors on cell aggregation closely match the adehesion specificities and recognition properties of embryonic cells, as seen in cell-aggregation experiments *in vitro;* they also correspond to the presence on embryonic cell surfaces of immunologically detectable tissue-specific determinants (1, 2, 6).

Earlier studies showed that gene-mediated protein synthesis is necessary for histotypic cell reaggregation. Complete inhibition of protein synthesis by puromycin or cycloheximide, or of RNA synthesis by actinomycin D, blocks the reaggregation of trypsin-dissociated embryonic cells by preventing production of the cell-aggregating factor (3, 4). A search for more specific chemical probes for analyzing macromolecular synthesis in cell aggregation led us to proflavine (3,6-diaminoacridine), a molecule which intercalates into DNA and interferes with the actions of DNA and RNA polymerases (7). Proflavine differentially inhibits a limited range of gene expressions in embryonic neural retina, including the induction of glutamine synthetase (EC 6.3.1.2) (8). We describe here the preferential and reversible inhibition by proflavine of the reaggregation of embryonic retina cells and the production of retina-specific cell-aggregating factor.

MATERIALS AND METHODS

Suspensions of Neural Retina Cells (Fig. 1*A*) were prepared by trypsinization of retina tissue from 10-day chick embryos as described (9). Reaggregation of the cells was studied in rotation cultures in flasks with cell suspensions in serum-free medium on a gyratory shaker by standard procedures (8). 3-ml Aliquots of retina-cell suspension (2×10^7 cells) were dispensed into 25-ml Erlenmeyer flasks in medium [Eagle's basal medium with 1% glutamine, 25 μg/ml of DNase (4), and 1% penicillin–streptomycin]. The flasks were placed on a gyratory shaker (70 rpm) for 24 hr at 38° (9). After 24 hr of rotation, control cultures contained cell aggregates of characteristic and consistent size distribution (Fig. 1*B*) with practically no single cells left; within the aggregates, the cells had reconstructed neuroretinal tissue (9, 10). Under these assay conditions, the size (volume) of cell aggregates provides a baseline measure for assessing inhibition of cell aggregation by drugs, and its enhancement by the cell-aggregating factor.

Proflavine (free base; Nutritional Biochem.) was added to the culture medium in solution at the same time as the cells, except when otherwise noted. Proflavine concentrations were monitored spectrophotometrically; all solutions and cultures with proflavine were protected from light.

Retina Specific Cell-Aggregating Factor was prepared essentially as in earlier work (3, 5) from supernatant medium collected from primary monolayer cultures of 10-day embryonic retina cells. The monolayer cultures were established in Falcon tissue-culture flasks ($4–5 \times 10^7$ cells per ml) in medium with 5% fetal-bovine serum; after 24 hr the cultures were thoroughly washed and maintained thereafter in the serum-free medium, either with or without 12 μM proflavine. Medium was collected at 24-hr intervals and dialyzed as the first step in purification of the cell-aggregating factor (3, McClay, D. R. & Moscona, A. A., in preparation). The factor and/or proflavine were added to rotation cultures of cell suspensions, as explained in the text.

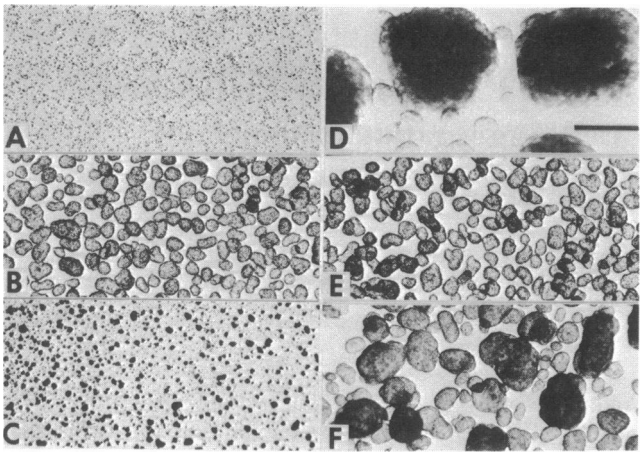

FIG. 1. (*A*) A freshly prepared suspension of neural retina cells (trypsin-dissociated retina tissue from a 10-day chick embryo). (*B*) 24-hr Cell aggregates obtained in rotation cultures of cell suspension. (*C*) 24-hr Rotation culture of cells in the presence of 12 μM proflavine. (*D*) 24-hr Aggregates obtained in the presence of the retina-specific cell-aggregating factor. (*E*) 24-hr Cell aggregates obtained in the presence of factor preparation collected from proflavine-treated cultures. (*F*) Effect of addition of active, specific retina cell-aggregating factor on retina cell aggregation in 12 μM proflavine (24-hr cultures). Length of bar: 0.5 mm.

Cell Reaction with Lectins (11) was examined with concanavalin A (10–100 μg/ml) and wheat-germ agglutinin (50–250 μg/ml). Lectins were added to suspensions of trypsinized retina cells in serum-free medium, and cell agglutination was examined in rotation cultures (12). The effects on proflavine-treated and control cells were compared.

RESULTS

Inhibition of Cell Aggregation by Proflavine.. Addition of 12 μM proflavine to freshly prepared retina-cell suspensions inhibited cell reaggregation in rotation cultures. After 24 hr, cultures with proflavine contained only small cell clumps and single cells, while control cultures contained the characteristic multicellular aggregates (Fig. 1*A–C*). The effectiveness of proflavine was dependent on its concentration (Fig. 2); kinetic studies showed that 12 μM proflavine completely halted cell aggregation in 3–4 hr. It is significant to considerations of the site of the proflavine action that the nonspecific clumping of the cells (3, 4, 9) which occurs in the first 1–2 hr was not noticeably inhibited; unlike the subsequent phases of reaggregation, this initial cell clumping does not require protein synthesis or optimal temperatures (3, 4) but depends on temporary conditions of the cell surface, and these are not altered by proflavine.

Preferential Nature and Reversibility of the Proflavine Effect. That the inhibition of cell aggregation by proflavine was not due to general alteration of the cell surface was shown by the following results. Proflavine did not abolish the typical agglutination of embryonic retina cells by concanavalin A and by wheat-germ agglutinin (12); thus, the receptor sites for these lectins on the cell surface are not inactivated or hindered by proflavine. This finding is of further interest because it indicates that these receptor sites are not directly involved in histotypic reaggregation of retina cells.

Proflavine did not abolish adhesion of retina-cell suspensions to tissue-culture dishes and did not prevent the establishment of cell monolayers in Falcon flasks; this demonstrates an important difference in the nature of cell adhesion to plastic, and of histotypic attachment of cells to each other. Retina cells from 10-day chick embryos are mostly post-mitotic and their formation of monolayer cultures involves little cell proliferation (13).

Inhibition of cell aggregation by proflavine was not due to general or irreversible toxicity. In the continued presence of 12 μM proflavine, monolayer cultures of retina cells remained alive for more than 1 week and showed no gross changes in cell morphology or in number of cells staining with nigrosin dye. For measurement of inhibition of macromolecular synthesis by proflavine, retina cells were labeled with [^{14}C]-aminoacids, [^{3}H]GlcN, and [^{3}H]U; they were cultured in the presence or absence of 12 μM proflavine for 2 or 24 hr and labeled for 60 min before harvesting. The results showed that proflavine inhibited the incorporation of [^{3}H]U by less than 10% and the incorporation of [^{14}C]aminoacids and [^{3}H]-GlcN into proteins and glycoproteins, respectively, by 20%. The total uptake of these precursors was not affected by proflavine, indicating no major changes in their transport into the cells. Similarly, when retinas were labeled for 24 hr in the continuous presence of 12 μM proflavine, accumulation of labeled RNA was reduced by less than 10%, and that of labeled protein or glycoprotein by less than 30% (Fig. 2). Even after 72 hr of continuous exposure to 12 μM proflavine, accumulation of [^{3}H]U-labeled material was not reduced by more than 10%.

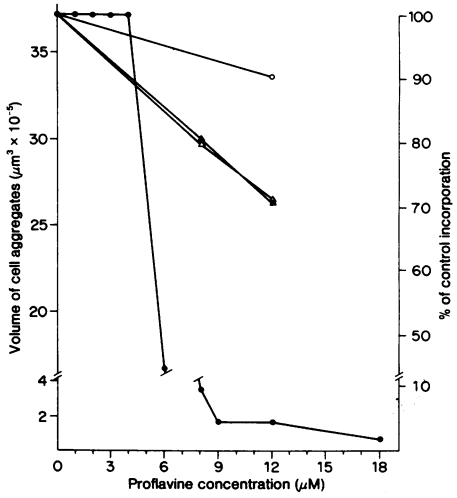

FIG. 2. Dose-dependent inhibition by proflavine of retina-cell aggregation in rotation cultures, and of macromolecular synthesis in monolayer cultures of retina cells. Proflavine was added to the culture medium at the concentrations indicated. The cultures were supplied for 24 hr with [^{3}H]U and [^{14}C]aminoacid mixture, or [^{3}H]GlcN (1.0 μCi/ml, New England Nuclear Corp.). Isotope incorporation was measured in material precipitated with cold trichloroacetic acid from sonicates of the cells (8). (●) Average volume of cell aggregates in 24-hr cultures (to estimate this, all aggregates in the culture flask were photographed, their sizes were measured, and average volumes calculated); (○) 24-hr incorporation of [^{3}H]U expressed as percentage dpm/mg of protein of untreated controls; (▲) 24-hr incorporation of [^{14}G]aminoacids expressed as above; (△) 24-hr incorporation of [^{3}H]GlcN expressed as above.

Inhibition by proflavine of macromolecular synthesis and cell aggregation was not due to a generalized suppression of protein synthesis; this was shown by the following experiment. Suspensions of neural retina cells were treated for 24 hr with 0.15 μg/ml of cycloheximide, which inhibited 30% of total protein synthesis (i.e., equivalent to the effect of 12 μM proflavine on protein synthesis in these cells). This treatment did not significantly prevent retina-cell aggregation; only at higher concentrations of cycloheximide, which blocked more than 90% of protein synthesis, was cell aggregation suppressed (4).

Although acridines may affect mitochondrial functions, the inhibition by proflavine of cell aggregation cannot be explained as being due to its possible action at the level of mitochondrial protein synthesis; chloramphenicol, a preferential inhibitor of mitochondrial protein synthesis (14), did not block cell aggregation at concentrations of 10–400 μg/ml.

In some types of cells, addition of exogenous glutamine can enhance cell reaggregation (15). In the present case, additions of 68–685 mM glutamine to retina cells in 12 μM proflavine did not overcome the inhibition of cell aggregation. This result does not exclude the possibility that proflavine interferes with utilization of glutamine in a biosynthetic pathway for products essential for normal cell reaggregation.

Proflavine Inhibits Production of Cell-Aggregating Factor. The following experiments showed that proflavine selectively prevents production of the specific retina cell-aggregating factor and suggest that this effect accounts for the inhibition of retina-cell aggregation. Proflavine (12 μM) was added to monolayer cultures of retina cells (in serum-free medium) 24 hr before collection of the cell-aggregating factor; control cultures received no proflavine. After removal of proflavine by dialysis, the preparations were assayed on fresh retina cell suspensions for the presence of cell-aggregation enhancing activity. In contrast to the untreated monolayer cultures which yielded, as usual, retina cell-aggregating factor (Fig. 1*D*), no factor activity was obtained from proflavine-treated cultures (Fig. 1*E*). Thus, proflavine either directly inactivated the factor, or prevented its production by the cells.

The second of the above alternatives is supported by experiments that showed that cells would aggregate in the presence of proflavine if supplied with exogenous cell-aggregating factor. Retina cell-aggregating factor was added to rotation cultures of retina-cell suspensions in the continuous presence of 12 μM proflavine. Without the added factor, cell aggregation was inhibited (as in Fig. 1*C*); addition of the factor caused the cells to form aggregates of sizes that depended on the amount of factor supplied (Fig. 1*F*). This "repair" of the proflavine-caused defect in the retina cell surface was obtained only with the retina-specific cell-aggregating factor, and not with cerebrum cell factor (5), which is entirely consistent with the known tissue specificity of these cell-aggregating factors.

From the above experiments it can be provisionally concluded that: (*1*) Since the cell-aggregating factor is not inactivated by direct exposure to proflavine, absence of factor activity in preparations from proflavine-treated cells is most likely due to interference with factor production; accordingly, inhibition of cell aggregation by proflavine results from lack of active factor. (*2*) Proflavine does not hinder reaction of the cell surface with the specific cell-aggregating constituents in the factor preparation (cell-ligands); this is evident from the

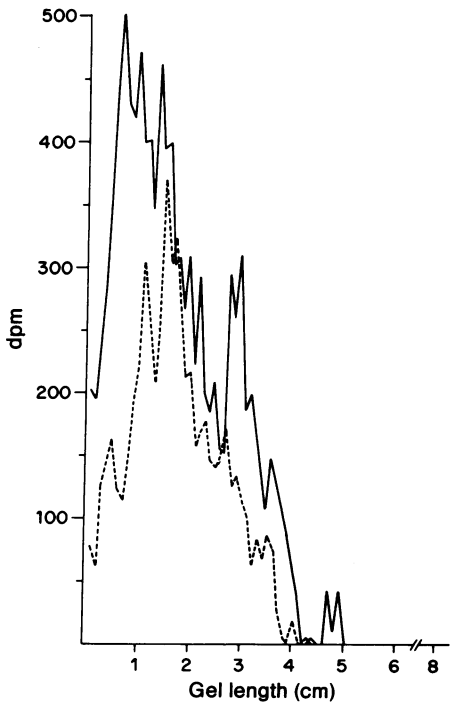

FIG. 3. Distribution of radioactivity on 10% Na dodecyl sulfate–polyacrylamide gels (8 cm) after electrophoresis of partially purified retina cell-aggregating factor, after Sephadex and subsequent DEAE–cellulose column fractionation (McClay, D. R. & Moscona, A. A., in preparation). Factor was collected from monolayer cultures of retina cells that were supplied for 24 hr with [^{3}H]GlcN (1.0 μCi/ml, New England Nuclear Corp.). Gels were loaded with 30 μg of protein. *Solid line,* factor preparation from control cultures; *dashed line,* factor collected from cultures treated with 12 μM proflavine. Proflavine caused a reduction in the amount of [^{3}H]GlcN-labeled protein banding in the region of 50,000 daltons (1.0 cm) and in a low-molecular-weight region. Molecular weights were estimated with markers, according to Weber and Osborn (16). The direction of migration was from *left* to *right.* Consecutive 1.25-mm slices were solubilized in NCS (Amersham Searle), and radioactivity was measured in a liquid scintillation counter. Dpm were determined after quench correction by ^{133}Ba standardization and subtraction of background radioactivity.

"repair" experiment which showed that addition of the specific factor to proflavine-treated cells caused them to reaggregate by supplying the lacking cell-ligands. Accordingly, a causal relationship exists between inhibition by proflavine of factor production and of cell reaggregation; this conclusion is consistent with the view that the cell-ligands in the factor represent specific components of the cell surface which are normally used for histotypic cell aggregation. Dispersed cells in monolayer cultures produce and release these components from the cell surface into the medium, and this process is inhibited by proflavine.

Further evidence that proflavine prevents the production of cell-aggregating factor was obtained by Na dodecyl sulfate–polyacrylamide gel electrophoresis (16) of radioactively labeled factor derived from retina cells cultured in medium with [^{3}H]GlcN in the presence and absence of proflavine. Other studies have shown that the characteristic activity of the retina cell-aggregating factor is associated with a carbohydrate–protein complex banding in the 50,000-dalton region. In the present experiments, analysis of the distribu-

tion of radioactivity on the gels and staining with Coomassie blue and by the periodic acid-Schiff reaction demonstrated that proflavine inhibited preferentially the production of [^{3}H]GlcN-labeled proteins banding in the region of 50,000 daltons, and in a low-molecular-weight region (Fig. 3). The nature of the low-molecular-weight material has not been determined. The amount of labeled protein associated with the factor activity is estimated to be less than 1.0% of the total amount of newly synthesized protein.

Effect of Other Acridines on Cell Aggregation. Other acridines were tested to examine the specificity of the proflavine molecule (3,6-diaminoacridine) in the inhibition of retina-cell aggregation. The effect was not obtainable with molecules lacking the amino groups (acridine), or with long side chains (quinacrine); the presence of only one amino group (9-aminoacridine) or of methylated amino groups (acridine orange) considerably reduced inhibitory effectiveness. Thus, the effects of proflavine on cell aggregation are not common to acridines in general; a detailed survey of other acridines and other cell types is required to clarify the exact molecular basis of proflavine specificity in this context. It is of interest that a similar specificity exists in the inhibition by proflavine of glutamine synthetase induction in the retina (8).

DISCUSSION

Our results are consistent with the postulated role of tissue-specific cell-ligands in morphogenetic cell aggregation. They confirm earlier findings that cell-aggregating factors obtainable from live embryonic cells contain the characteristic activity assigned to those cell-surface components. Proflavine inhibits, differentially, production by cells of specific macromolecules (protein–carbohydrate complexes) associated with the specific cell-aggregating activity of the factor; it therefore suppresses the histotypic reaggregation of embryonic cells. The results reported refer to embryonic neural retina cells; however, work in progress indicates that the conclusions may apply, in general, also to other types of embryonic cells. Projecting from these findings, proflavine suggests itself as a potentially useful molecular probe for studies on cell-surface components in cell contact and differentiation in normal growth (17), and possibly also in cell-membrane changes associated with neoplasia (18).

The detailed site of proflavine action and the metabolic mechanisms by which it inhibits cell aggregation and production of cell aggregating factor remain to be elucidated. Since embryonic cell aggregation does not require ongoing DNA synthesis, inhibition by proflavine of DNA replication is unlikely to be causally implicated in the suppression of cell aggregation. The fact that RNA and protein syntheses are reduced suggests that one or both may be the primary level of proflavine action. Preliminary tests point to transcriptional or pretranslational processes as the relevant site.

Another situation in which proflavine has a strikingly differential inhibitory effect is the hormonal induction of glutamine synthetase in the embryonic neural retina (8). It is not known whether these two effects of proflavine are in some way related, even though glutamine has been implicated in cell aggregation (15). Since, as we have shown, addition of excess glutamine to retina cells in proflavine does not overcome the suppression of cell aggregation, such a relationship, if proven, would be circuitous. Conceivably, formation of cell ligands in retina cells may require some secondary product of the glutamine synthetase system, or a glutamine derivative that is not formed in the presence of proflavine. Alternatively, the two processes may share no cause and effect relationship, but their control mechanisms might be preferentially sensitive to proflavine.

It would be of obvious interest to examine the effects of proflavine and related molecules in other systems that involve cell-surface reactions, specific changes in cell-surface properties, or the formation of differentiation products.

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