Quantitative Analysis of Intercellular Adhesive Specificity in Freshly Explanted and Cultured Cells

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ABSTRACT A new method is presented for the quantitative analysis of intercellular adhesive specificity. In this assay, two cell types are mixed, one unlabeled and the other labeled with the fluorescent dye, fluorescamine [4-phenylspiro(feran-2[3H],1'-phthalan)-3,3'-dione]. The resulting aggregates are analyzed by fluorescence microscopy to determine the number of labeled and unlabeled cells per aggregate. Random (nonspecific) aggregation was characterized by a binomial distribution, and adhesive specificity was accordingly quantified by the deviation (as determined by a chi-square test) from the calculated binomial distribution. The labeling procedure was simple and rapid, and experiments with 18 different cell types showed that it did not affect cell viability, morphology, rate and extent of adhesion, plating efficiency, and the capability of myogenic cells to undergo terminal differentiation. Most important, assays with morphologically identifiable cell pairs indicated that the fluorescent label neither induced apparent nor destroyed existing adhesive specificity.

The most pronounced adhesive specificities were observed with freshly explanted cells from adult tissues and also with mixtures of simian virus 40-transformed and nontransformed BALB/c 3T3 cells. A glucosamine-6-phosphate *N*-acetylase-deficient mutant 3T3 line (AD6), however, aggregated randomly with parental 3T3 cells. Lectin-resistant mutant Chinese hamster ovary (CHO) cells displayed marginal adhesive specificity when mixed with normal CHO cells.

Adhesive specificity, i.e., the ability of cells to recognize and adhere preferentially to certain other cells, has been implicated in a wide variety of normal and pathological biological processes such as morphogenesis, maintenance of tissue and organ integrity, hemostasis, immune responses, tissue invasion by microorganisms and tumor cells, and tumor metastasis (reviewed in references 10, 17, and 26). Elucidation of the underlying molecular mechanisms requires sensitive and accurate methods for the quantitative assay of adhesive specificity.

A number of methods have been reported for observing or measuring adhesive specificity. The original procedure, "sorting out," has been extensively studied (14, 18, 37, 40). This is a complex process in which cells in mixed aggregates relocate with respect to one another, and in which the end point is typically reached after 3–5 d. It includes not only adhesive specificity but also cell motility and can only be interpreted in qualitative terms. Collection assays focus on the earlier phases of the adhesive event and compare the initial rates of adhesion of radioactively labeled homotypic and heterotypic single cell suspensions to preformed aggregates (27), cell monolayers (46), cell-coated beads (45), or cell-coated fibers (8). Collection assays are not influenced by cell motility, and quantitative measurements are possible. However, rates of adhesion determined by collection assays may not accurately reflect intrinsic adhesion affinities of the two cell types under study. The experimental procedures used to prepare the collecting cells (e.g., monolayers or preformed aggregates) are different from those used to prepare the single cell suspensions. Single cell suspensions are often used immediately after trypsinization, whereas the collecting surface is formed 1–2 d after proteolytic treatment. During the establishment of a collecting surface, embryonic cells may not only change as they recover but may also continue to differentiate, and thus their adhesive properties may be modified further in a poorly controlled manner. Moreover, the spatial arrangement of adhesive sites on a single cell in suspension may be quite different from that on a cell that is firmly integrated into a collecting surface (e.g., preponderance of laterally oriented sites in monolayers vs. a more uniform distribution on single cells in suspension).

We have recently used another approach (20) to study adhesive specificity, in which single cells are given the choice of adhering to homotypic or heterotypic single cells or newly

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formed small aggregates. A microscopic evaluation of a sufficient number of individual aggregates formed in a mixed suspension of single cells provides a more direct means of quantifying intercellular adhesive specificity and avoids several pitfalls inherent in collection assays. In our first report (20), the two cell types were morphologically distinct and easily recognizable in aggregates. The assay, however, has not found widespread use because most cells lack the conspicuous morphological markers required for their rapid and unequivocal identification under a microscope. Our efforts, therefore, were directed at developing simple, rapid, yet gentle techniques for introducing artificial markers in cells used for aggregation experiments. Some 15 different supravital stains were tested. Most of them were found to be unsuitable because they interacted with the cells in a noncovalent fashion and thus diffused out of the labeled cells and into the previously unlabeled ones. Among those dyes that formed a covalent bond with cellular constituents, fluorescamine was found to have the most desirable properties. It stained cells very rapidly and evenly, could be applied under mild conditions, produced an intense fluorescence, did not noticeably interfere with the normal expression of intrinsic adhesive properties, and appeared to be applicable to a wide variety of different cell types. Details of the fluorescamine labeling procedure and its application in the study of intercellular adhesive specificity among freshly explanted and cultured cells (including virus-transformed and cell surface carbohydrate mutant cell lines) are discussed in the present report. Preliminary reports (31, 32) have been presented.

MATERIALS AND METHODS

Materials

Medium 199 and the alpha modification of Eagle's minimum essential medium (alpha-MEM; 36) were purchased from Flow Laboratories, Inc., (Rockville, Md.) or prepared in our laboratory according to the manufacturer's formula. Calf serum (heat-inactivated at 57°C for 30 min), fetal calf serum (FCS), trypsin-EDTA (0.5 mg of trypsin 1:250 and 0.2 mg of EDTA/ml of Hanks' balanced salt solution), and trypan blue were obtained from Grand Island Biological Co. (GIBCO; Grand Island, N. Y.); Dulbecco's medium (7) and a modified version (MDE; 20) were prepared as described. Media used for primary cultures were supplemented with penicillin (100 U/ml; GIBCO) and streptomycin sulfate (100 μg/ml; GIBCO). The pH of the culture media was adjusted to 7.3 by sodium bicarbonate/CO_2 or by the addition of HEPES (10 mM final concentration; Sigma Chemical Co., St. Louis, Mo.). Collagenase (from *Clostridium histolyticum*, type II) and trypsin inhibitor (from egg white) were purchased from Sigma Chemical Co., bovine serum albumin (BSA) from Miles Laboratories, Inc. (Elkhart, Ind.), and 4-phenylspiro(feran-2[3H],1'-phthalan)-3,3'-dione (fluorescamine) from Polysciences, Inc. (Warrington, Pa.). All other chemicals were of reagent grade or the best commercial quality available and were used without further purification.

Cells

Unless indicated otherwise, all cultured cells were grown on plastic tissue culture dishes in bicarbonate-buffered media at 37°C. BALB/c 3T3 fibroblasts (3T3) and simian virus 40–transformed BALB/c 3T3 cells (SV40-3T3) were grown in Dulbecco's medium containing 10% calf serum. Cell strains and culture conditions were identical with those used by Walther et al. (46). The glucosamine-6-phosphate *N*-acetylase-deficient mutant BALB/c 3T3 line (AD6; 22) and the corresponding wild-type line (3T3-A; 22), gifts from Dr. I. Pastan, were maintained in Dulbecco's medium supplemented with 10% FCS.

Chinese hamster ovary (CHO) cells resistant against the cytotoxic effects of wheat-germ agglutinin (WGA^r; line WW^{II}, 4B, 101, 4B), phytohemagglutinin (PHA^r; line WP^I, 3B, 97, 3B), and concanavalin A (Con A^r; line C321) and the corresponding wild-type lines (W5 for WGA^r and PHA^r; WTT6 for Con A^R) were gifts from Drs. P. Stanley, R. Baker, and S. Krag (3, 15, 35). All strains were cultured in alpha-MEM containing 10% FCS. The Con A–resistant line and the corresponding parental line were grown on culture dishes at 34°C. All other CHO lines were either cultured on plastic dishes or in glass roller bottles at 37°C.

Preadipocytes (also called "adipocyte precursors" or "adipoblasts") were isolated from the epididymal fat pads of young (~120 g) Sprague-Dawley rats (Microbiologial Associates, Walkersville, Md.). As a modification of the original procedures of Van et al. (44) and Rothblat and DeMartinis (28), each pair of fat pads was placed in a 15-ml polystyrene tube containing 10 ml of HEPES-buffered alpha-MEM, 2% BSA, and 0.2% collagenase. To accelerate the digestion process, each tube was mounted tangential to a rotating drum (2–3 rpm) and placed in a 37°C incubator for 90 min. The resulting stromal-vascular fraction was further purified on a Percoll (Pharmacia Fine Chemicals AB, Uppsala, Sweden) gradient as described by Björntorp et al. (1). Primary cultures of preadipocytes were initiated by placing the stromal-vascular fraction in plastic culture dishes (equivalent of one pair of fat pads per 90-mm dish) containing HEPES-buffered alpha-MEM supplemented with 15% FCS. The medium was changed daily until contaminating erythrocytes were completely removed. The medium was then replaced every second day. Confluence was typically reached after 7–9 d in culture. Primary cultures of pectoral muscle myoblasts (41) and pectoral muscle and dorsal skin fibroblasts were prepared by Dr. M. Sieber-Blum from 10-d-old White Leghorn chick embryos (Truslow Farm, Chestertown, Md.) and used 2–3 d after explantation.

Rat Leydig cells were prepared from young (250 g) Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass.), following a procedure published by Conn et al. (4). Neural retina cells (gift of Mr. K. Miller) were prepared from 10-d-old White Leghorn chick embryos (Truslow Farm) as described by Roth et al. (27). Hepatocytes (kindly provided by Drs. E. Schmell and M. Kuhlenschmidt) were isolated from young (150–175 g) male Sprague-Dawley rats (Charles River) and 3-wk-old White Leghorn chickens (Truslow Farm), respectively, by use of the collagenase perfusion technique of Seglen (30), modified as described (20).

Preparation of Single Cell Suspensions

Subconfluent monolayers were washed twice with warm Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline and then incubated for 5 min in trypsin-EDTA (5 ml/90-mm dish) at 37°C. Trypsinization was stopped by the addition of a 50% excess of trypsin inhibitor; the cells were washed once with fresh HEPES-buffered MDE, filtered through appropriately sized Nitex nylon screens (TETKO Inc., Elmsford, N. Y.), and then stored on ice until used. Cells that had been grown in suspension culture were harvested by centrifugation before they had reached the stationary phase, resuspended in fresh HEPES-buffered MDE, filtered through nylon screens, and stored on ice until used.

Cell numbers and the concentration of single cells (vs. aggregated cells) were determined in a hemacytometer. Estimates of cell viability were based on trypan blue exclusion or liberation of the cytoplasmic enzyme, lactate dehydrogenase (29). Freshly explanted cell preparations consisted typically of 80–95% viable cells and 80–95% single cells. For cultured cells, the percentages of single and viable cells were between 95 and 99%. Aggregates in the preparations contained almost exclusively two cells, with occasional aggregates containing three cells.

Labeling of Cells with Fluorescamine

To 5×10^6 cells suspended in 5 ml of cold HEPES-buffered MDE containing 5% FCS, 10 μl of a 0.5% solution of fluorescamine in dimethyl sulfoxide was added. The suspension was agitated manually immediately after the addition of the reagent to assure rapid and complete mixing. After 1 min at 0°C, the cells were harvested by low-speed centrifugation, suspended in fresh MDE, and stored in ice until used.

Adhesion Assays

A total of 5×10^5 cells (2.5×10^5 of each type in mixing experiments) were placed in a plastic tube (Falcon #2001; Becton, Dickinson & Co., Cockeysville, Md.) in 0.5 ml of HEPES-buffered MDE (supplemented with serum as indicated) and incubated in a gyratory water bath (New Brunswick Scientific Co., Inc., Edison, N. J.) 72 cycles/min at 37°C (34°C for experiments involving Leydig cells and CHO lines C321 and WTT6). Plastic vials were preferred to glass vials because several cell types used in this study adhered to glass surfaces. Aggregation of homotypic cell suspensions was measured by monitoring the disappearance of single cells in a hemacytometer or an electronic particle counter (Coulter Electronics Inc., Hialeah, Fla.) as described by Orr et al. (21). Monolayer assays were carried out as described by Walther et al. (46) with single cell suspensions labeled with [^{3}H]leucine.

Mixed populations containing fluorescamine-labeled cells were analyzed under a Zeiss Universal microscope (Zeiss, Oberkochen, W. Germany) equipped with a vertical illuminator for epifluorescence, a 50 W DC mercury lamp, excitation barrier and reflector combinations 487702 (ultraviolet excitation) and 487703 (violet excitation), and an objective Neofluar 25/0.6 Ph-2. After a mixed cell population had been incubated for ~45 min, an aliquot of the aggregated

suspension was transferred to a hemacytometer by use of a wide-bore plastic pipette tip. About 150 aggregates containing at least 4 cells were scored systematically and the cell composition (fluorescent *vs.* nonfluorescent cells) of each aggregate was recorded. Aggregates containing fewer than four cells were not included in order to minimize the bias in favor of homotypic adhesion introduced by the low percentage of small aggregates (doublets) present in the initial cell suspensions. Extremely large aggregates, which did not permit unequivocal identification of the individual cells, were also excluded. Aggregates containing two cell types were scored as random although spatial segregation within the aggregates was frequently observed. This type of analysis has been used in earlier studies (5, 20). We wish to emphasize that the analyses are deliberately biased in favor of random or nonspecific adhesion. That is, when two cell types show homotypic or adhesive specificity, mixed aggregates generally contain spatially separated aggregates of each type. Examples of such mixed aggregates have been described (20). In the analyses, attempts are not made to correct for this segregation within the mixed aggregates, even though heterotypic adhesions within the aggregates may involve only one or two cells of each type. Instead, such aggregates are statistically treated as though they were random (entirely nonspecific).

Statistical Evaluation of Aggregation Experiments

When two cell types aggregate randomly (no adhesive preference), the frequency distribution of each cell type in a particular aggregate size class is described by a binomial curve. When the cells aggregate specifically, a nonbinomial—most often a bimodal—distribution results (Fig. 1). The deviation of the experimental distribution from the calculated binomial distribution can be quantified by a chi-square test ($\chi^2 = Ns^2/\sigma^2$; N designating the total number of aggregates counted in the particular size class, s^2 designating the variance of the experimental distribution, and σ^2 designating the variance of the calculated binomial distribution) and used as an objective measure for adhesive specificity. Because in our studies the two cell types in the mixed suspensions are aggregated at slightly different rates and to different extents, the calculation of the theoretical binomial distribution was based on the relative concentration of the cell types in aggregates rather than the usual 1:1 input ratio. A series approximation method was used to calculate the cumulative distribution (P-values) for given values of χ^2 with ν ($\nu = N - 1$) degrees of freedom (48). An example is: If the composition (A/B) of 10 four-cell aggregates formed by a mixed suspension of A- and B-cells is 4/0, 4/0, 4/0, 3/1, 2/2, 2/2, 1/3, 1/3, 1/3, and 0/4, the mean content of A-cells ($\bar{A}$) in this aggregate class is 2.2 with a variance ($s^2 = [\Sigma(A - \bar{A})^2]/[N - 1]$) of 2.18. Because the relative frequencies of A- (m) and B-cells (n) are 0.55 and 0.45, respectively, the calculated binomial distribution can be described by the equation $P(A) = 10\ (4!/[A!(4 - A)!])\ (0.55^A)\ (0.45^{1-A})$ and its variance (σ^2) is 4 mn = 0.99. Then $\chi^2 = Ns^2/\sigma^2$ becomes 10(2.18)/0.99 = 22.

Scanning Electron Microscopy

Cells were gently suspended in medium and fixed by the addition of an equal volume of 5% glutaraldehyde in medium. After the cells had been exposed to the fixative for at least 24 h, they were transferred onto polylysine-coated cover slips, washed with distilled water, dehydrated in a graded series of ethanol, and critical-point-dried from liquid CO_2. The specimens were then coated with a 150-Å film of gold/palladium (sputter-coated E 5100; Polaron Instruments Inc., Line Lexington, Pa.) and examined in a JSM-35 scanning electron microscope (JEOL USA, Electron Optic Div., Medford, Mass.) equipped with a LaB6 filament and operated at an accelerating voltage of 8 kV.

RESULTS

Labeling of Cells with Supravital Stains

The optimal conditions for labeling live cells with supravital stains were determined using primary cultures of rat preadipocytes and 3T3 fibroblasts. The conditions that gave satisfactory results with these two cell types were later found to be equally suitable for the labeling of all other cell types listed under Materials and Methods.

In an alkaline environment, fluorescamine reacts with primary amino groups with a half-time of <1 s yielding highly fluorescent products (excitation maximum, 390 nm; emission maximum, 475 nm), while excess reagent is concomitantly hydrolyzed to a nonfluorescent product with a half-time of a few seconds (42). Initial attempts to label cells in 0.2 M borate buffer, pH 9, while they were still attached to the culture dish

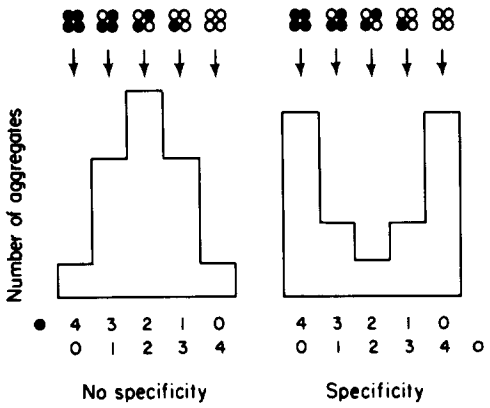

FIGURE 1 Principle of statistical evaluation of aggregates explained for the four-cell aggregate size class: nonspecific (random) aggregation of cell types A (●) and B (○) results in a binomial frequency distribution (left panel), specific aggregation in a nonbinomial (in this case bimodal) distribution (right panel). The greater the deviation of the bimodal from the binomial distribution, the greater the degree of specificity.

(13) and then use them for adhesion experiments did not yield the desired results: although the cells were brightly fluorescent, the fluorescence intensity varied considerably, the labeled cells developed large surface protrusions, they no longer aggregated, and their viability dropped by 50% within the first 15–20 min after labeling. Suspending the cells in borate buffer before labeling made them more uniformly fluorescent but the other problems were not significantly alleviated. We chose, therefore, to use very low concentrations of fluorescamine that were well below the saturating concentration and to keep the cells suspended in a complete culture medium (MDE + 5% FCS) that was buffered at pH 7.3. Cells stained under these mild conditions (Fig. 2) were less fluorescent than those labeled in borate buffer, pH 9, but the label was still readily recognizable even on strongly autofluorescent cells (such as rat hepatocytes) and under simultaneous phase-contrast illumination. Based on a visual examination under the fluorescence microscope, each cell appeared to contain about the same amount of fluorochrome. This was confirmed quantitatively by a flow-cytofluorometric analysis of fluorescamine-labeled 3T3 and SV40-3T3 cells in a fluorescence-activated cell sorter (FACS II; Becton, Dickinson & Co., Mountain View, Calif.). A two-parameter analysis (light scatter/fluorescence) indicated a close correlation between fluorescence and cell size. Clustering, patching, or capping of fluorescamine-derivatized surface components was not observed. Cells that had been derivatized in borate buffer were labeled only at their surface. In cells that had been derivatized at physiological pH, however, fluorescent label was found at the surface, in the cytoplasm, and on the nuclear membrane. The fluorescence observed in these different cellular compartments was probably attributable to direct derivatization with reactive fluorescamine rather than to uptake or adsorption of fluorescamine-labeled components of the medium (e.g., amino acids). Exposure of cells to fluorescamine-treated medium did not produce a detectable level of fluorescence on or within cells. A Folch (9) extraction of fluorescamine-labeled rat hepatocytes with chloroform-methanol (2:1 as described by Radin [25]) indicated that hydrophilic and hydrophobic cell constituents had been derivatized to a similar extent. Experiments with various amounts of reagent showed that the standard fluorescamine concentration derivatized ~3% of all accessible amino groups (rat hepatocytes). An exchange

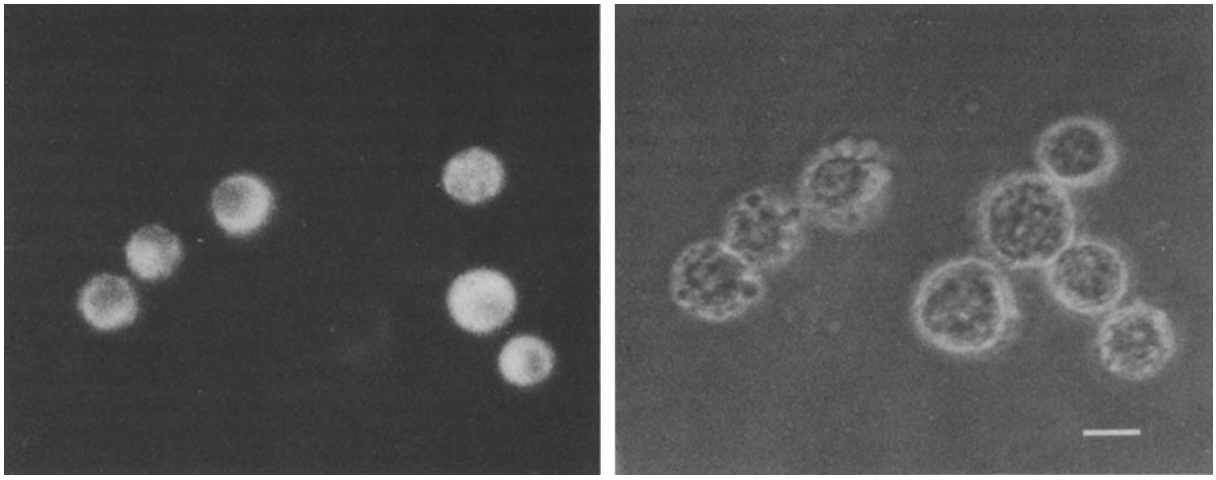

FIGURE 2 Typical aggregates formed by mixing fluorescamine-labeled SV40-3T3 and unlabeled 3T3 cells. The three-cell aggregate contains only fluorescamine-labeled SV40-3T3 cells, whereas the five-cell aggregate contains both cell types. Left panel, fluorescence microscopy; right panel, phase-contrast microscopy. Bar, 0.01 mm.

of fluorescent label between labeled and unlabeled cells or leakage into the medium was not detected during the course of a standard aggregation assay.

Except for the fluorescent marker, there were no obvious morphological differences between labeled and unlabeled cells when they were examined at the light microscope level. Samples of labeled and unlabeled SV40-3T3 cells were also compared in the scanning electron microscope and found to be indistinguishable. The cell size distribution as determined by a Coulter counter analysis also did not change in response to the fluorescamine labeling, indicating that labeled cells were still capable of maintaining the correct intracellular concentration of osmotically active substances. The viability of labeled and unlabeled aliquots of the same cell preparation was determined (by trypan blue exclusion and lactate dehydrogenase liberation) immediately after the labeling and up to 6 h later, and no differences were found. Further, the reaction with fluorescamine did not affect the ability of cells to attach to polystyrene substrates. No significant difference ($P > 0.1$) was found in the plating efficiency of labeled and unlabeled SV40-3T3 cells. Labeled chick embryo myoblasts continued to differentiate and fuse into multinucleated myotubes on schedule.

The rate and extent of intercellular adhesion were the same for labeled and unlabeled rat hepatocytes using the Coulter assay (21) and for 3T3, SV40/3T3, and embryonic chicken neural retina cells (data not shown). When labeled and unlabeled cells of the same type were mixed, the two populations aggregated randomly (Fig. 3), indicating that the fluorescent marker did not induce apparent adhesive specificity. Conversely, the labeling procedure did not eliminate an existing adhesive preference. This was shown by using mixtures of rat hepatocytes with rat preadipocytes, rat Leydig cells, or chicken hepatocytes (20). Mixed aggregates formed by these cell pairs could be analyzed equally well in the presence or absence of the fluorescamine marker because the cells were readily identifiable by size or intrinsic autofluorescence. Each mixture of cells aggregated specifically with the same degree of intercellular adhesive specificity in the presence or absence of the fluorescamine label.

Several other supravital stains were tested, but were found to have less desirable properties than fluorescamine: for example, the labeling intensity obtained by incubating cells with

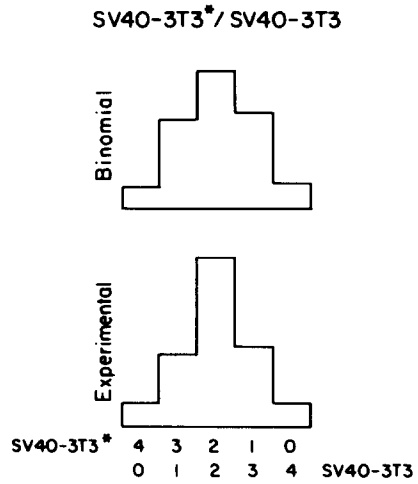

FIGURE 3 Aggregation of a mixture of fluorescamine-labeled (*) and unlabeled SV40-3T3 cells in MDE supplemented with 5% FCS. The same results were obtained in serum-free MDE. The frequency distribution that was determined experimentally did not differ significantly from the calculated binomial distribution, indicating that labeled and unlabeled cells did not discriminate against each other and aggregated randomly. The four-cell aggregate size class (shown) was representative of all aggregates analyzed: 46 aggregates were scored and included 91 SV40-3T3* and 93 SV40-3T3 cells; χ^2, 43.94; $P > 0.5$ (not significant). Note that in this and subsequent figures the asterisk indicates fluorescamine-labeled cells.

the disodium salt of 4-acetamido-4-isothiocyano stilbene 2,2'-disulfonic acid was too weak to be of much practical use. Fluorescein isothiocyanate and tetramethylrhodamine isothiocyanate had a higher resistance to fading and offered the possibility of double-labeling experiments (34, 39). However, they reacted much more slowly than fluorescamine, requiring incubation times of 30–60 min as compared with only 1 min required for derivatization with fluorescamine. Furthermore, after the labeled cells were washed twice with fresh medium, fluorescent material continued to be released into the medium during the subsequent aggregation experiment. Because the released material was absorbed by previously unlabeled cells, distinction between the two cell populations became increasingly difficult. Release of fluorescent material was also a prob-

lem when cells were stained with thiolyte-monoquat (Calbiochem-Behring Corp., American Hoechst Corp., La Jolla, Calif.). In addition, intensely fluorescent cells were only obtained when they were labeled at 37°C. To prevent the cells from aggregating at this elevated temperature, we placed them in capped tubes that were tangentially mounted on a rotating drum (3 rpm).

All dyes that did not form covalent bonds with cell constituents (fluorescein diacetate, fluorescein dibutyrate, acridine orange, ethidium bromide, Hoechst H-33258 (16), 3,3′-ditetradecanoyl indocarbocyanine iodide (diI-C_{14}-(3)) (33), tetracycline, neutral red, and Nile blue) were gradually released into the medium and absorbed by previously unlabeled cells. In addition, labeling with neutral red and Nile blue interfered with the ability of the cells to aggregate.

Quantitative Evaluation of Aggregates

After incubating cell suspensions for 45 min at 37°C as described under Methods, we found that 50–80% of the original single cells in the suspension had aggregated into small planar aggregates. Most aggregates consisted of fewer than 20 cells and were rarely more than one cell layer thick, which greatly facilitated visual analysis. Fading of the fluorescamine label under prolonged ultraviolet irradiation was noticeable but was too slow to interfere with the identification of cells.

Four-cell aggregates, on which the routine statistical analysis was based, represented the largest aggregate size class, comprising ~30% of all aggregates formed. A semiquantitative analysis of the larger aggregates agreed with the results obtained from four-cell aggregates. When live (unfixed) cells were examined, ~130–150 individual aggregates could be scored before the cells began either to attach to the surface of the hemacytometer and spread out (which makes a distinction between labeled and unlabeled cells more difficult) or to form surface protrusions and eventually lyse. Thus ~40 four-cell aggregates were available for statistical analysis. For the detection and statistical corroboration of moderate to pronounced adhesive preferences, a sample size of 40 was adequate. Where only marginal adhesive preferences were suspected (see, for instance, experiments with CHO cells), a larger sample was clearly desirable. This requirement was routinely met by combining the results of two or more independent experiments. Although it was not permissible to increase the sample size by simply pooling the raw data, one could subject the data of each experiment to a separate chi-square test and then take advantage of the additive properties of the chi-square distribution ($\chi^2 = \chi_1^2 + \chi_2^2 + \ldots + \chi_\kappa^2$, with $\nu = \nu_1 + \nu_2 + \ldots + \nu_\kappa$). This approach was preferred over the two following procedures because the sample size could be increased ad libitum and the cells were not subject to any additional (potentially harmful) manipulations. Sample size could be slightly increased by repeated sampling from aggregated cell suspensions that had been stored on ice. (Low temperatures almost completely inhibit the formation of new aggregates without dissociating existing aggregates.) Some aggregated cell suspensions were also fixed immediately after completion of the incubation by the addition of an equal volume of 5% glutaraldehyde in MDE. Visual examination of the cell suspension under the microscope before and after addition of the fixative, as well as Coulter counter assays, confirmed that this fixation procedure preserved aggregates as aggregates and single cells as single cells. The only exception so far has been with mature rat adipocytes, which formed large aggregates after addition of the glutaraldehyde. When glutaraldehyde-fixed cells were stored in the dark at 5°C, they retained their fluorescamine fluorescence for several days. The rate of appearance and final intensity of glutaraldehyde-induced fluorescence varied from preparation to preparation. An unequivocal distinction between fluorescamine-labeled and (glutaraldehyde-induced) autofluorescent cells was usually possible for several hours, occasionally for up to 1 or 2 d. When the difference between labeled and autofluorescent cells became too blurred, the initial contrast could be partially restored by extracting the fixed cell suspension with methanol at −10°C for 15 min (47).

Applications of the Fluorescamine Labeling Technique

ADHESIVE BEHAVIOR OF PARENTAL AND VIRUS-TRANSFORMED CELLS: Fig. 4 shows that after a 45-min incubation at 37°C, mixed populations of freshly trypsinized 3T3 and SV40-3T3 fibroblasts displayed marked adhesive specificity ($P < 10^{-9}$, $\nu = 197$). Cells derived from confluent instead of subconfluent cultures showed the same adhesive preferences and the fluorescent marker could be applied equally well to either of the two cell lines. It is unlikely that the few small aggregates (1–5% doublets) present in the initial cell preparations significantly affected the outcome of this experiment (by simulating adhesive specificity), because mixtures of labeled and unlabeled cells of the same type aggregated randomly ($P > 0.3$, $\nu = 85$) (Fig. 3). We have previously reported (46) that 3T3 and SV40-3T3 fibroblasts showed no adhesive specificity when tested by the collecting monolayer assay. These experiments were repeated with the cell lines used for the fluorescamine assay, and the monolayer assay again indicated no adhesive specificity. The apparent discrepancy between the two methods is discussed below.

ADHESIVE PROPERTIES OF MUTANT CELL LINES WITH ALTERED CELL SURFACE CARBOHYDRATES: AD6 is a mutant cell line derived from 3T3 fibroblasts defective in the enzyme that *N*-acetylates glucosamine-6-phosphate, a key step

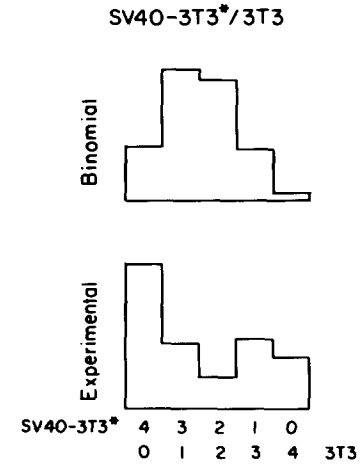

FIGURE 4 Adhesive behavior of a mixture of labeled SV40-3T3 cells and unlabeled 3T3 cells. The frequency distribution found experimentally differed significantly from the calculated binomial one, indicating that the two cell types showed a strong preference for homotypic adhesion in MDE (not shown) or MDE supplemented with 5% FCS (shown). The four-cell aggregate size class (shown) was representative of all aggregates analyzed: 56 aggregates were scored, and included 139 SV40-3T3* and 85 3T3 cells; χ^2 138.24; $P < 10^{-8}$ (highly significant).

in the metabolic pathway leading to biosynthesis of the complex carbohydrates (19, 22–24). The mutant phenotype is characterized by altered morphology, reduced adhesion to the substrate, a change in the pattern of iodinatable plasma membrane proteins, and a reduced content of most sugars (22, 23). Despite all of these differences, AD6 cells aggregated randomly ($P > 0.2$, $\nu = 194$) with cells from the parental 3T3-A line (Fig. 5). The rate and extent of adhesion as determined by a Coulter counter assay were also very similar for the wild-type and mutant cell lines.

All lectin-resistant mutant CHO cells (Fig. 6) showed a small but significant preference for homotypic adhesion when mixed with the corresponding wild-type lines. The respective P-values were $<10^{-4}$, $\nu = 280$ for WGA^r, $<10^{-4}$, $\nu = 150$ for PHA^r, and $<10^{-5}$, $\nu = 114$ for Con A^r. P-values for control experiments (labeled vs. unlabeled cells of the same type) were 0.2, $\nu = 90$ or higher.

Single cell suspensions of WGA^r, PHA^r, and wild-type CHO cells were also directly harvested from roller-bottle suspension cultures. These nontrypsinized cells displayed the same degree of adhesive specificity as did their freshly trypsinized counterparts. The lack of a more pronounced adhesive specificity among freshly trypsinized CHO cells could not be attributed, therefore, to the trypsinization procedure used to prepare single cell suspensions from monolayer cultures (38, 39).

TISSUE-SPECIFIC AGGREGATION OF FRESHLY EXPLANTED POSTEMBRYONIC CELLS: Freshly explanted cells from anatomically different sites of adult rats displayed a marked preference for homotypic adhesion. Figs. 7 and 8 give representative examples of the adhesive behavior of mixed populations of Leydig cells and hepatocytes ($P < 10^{-6}$, $\nu = 27$) and preadipocytes and hepatocytes ($P < 10^{-9}$, $\nu = 119$), respectively. The same degree of specificity was observed when one experiment (rat preadipocytes mixed with rat hepatocytes) was repeated with cells derived from isohistogenic Wistar Lewis rats, therefore suggesting that there must be at least one cell surface tissue-specific component involved in cell-cell adhesion.

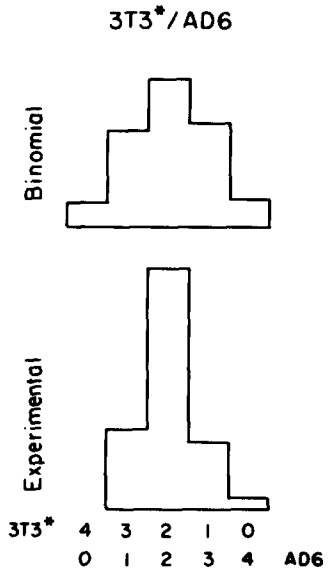

FIGURE 5 Adhesive behavior of a mixture of fluorescamine-labeled 3T3 and unlabeled AD6 mutant cells in MDE supplemented with 5% FCS. In this particular experiment the variance of the experimental distribution happened to be smaller than the variance of the calculated binomial distribution. The difference between the two distributions was, however, only marginal and an analysis of a larger sample composed of several independent experiments (see text) clearly argued against a preference for heterotypic interactions. These two cell types thus aggregated randomly despite their marked cell surface differences. The four-cell aggregate size class (shown) was representative of all aggregates analyzed: 35 aggregates were scored, and included 69 3T3* and 71 AD6 cells; χ^2, 17.48; $P \cong 0.99$ (not significant).

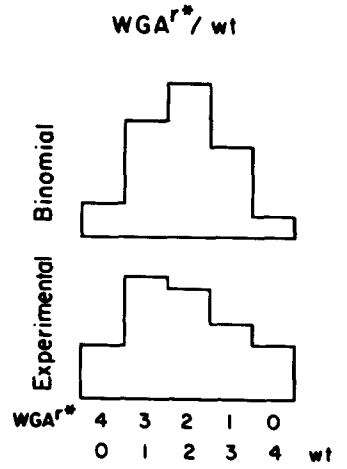

FIGURE 6 Adhesive behavior of a mixture of monolayer-derived WGA-resistant CHO cells *WGA*^{r}* and monolayer-derived wild-type CHO cells (*wt*) in MDE supplemented with 5% FCS. A very small degree of adhesive specificity was consistently found. Nontrypsinized cells derived from roller cultures and mixtures of Con A– and PHA-resistant mutant cells and wild-type cells behaved very similarly (not shown). The four-cell aggregate size class (shown) was representative of all aggregates analyzed: 61 aggregates were scored, and contained 129 WGA^r* and 115 wild-type cells; χ^2, 93.93; $P < 0.005$ (moderately significant).

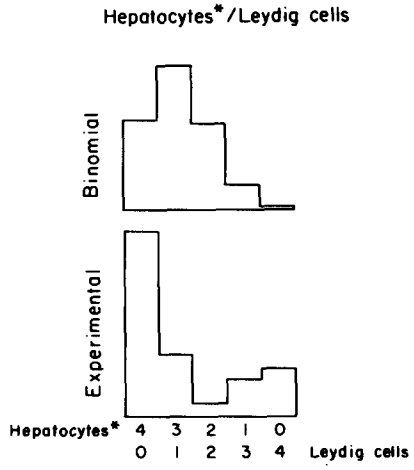

FIGURE 7 Adhesive behavior of a mixture of freshly explanted rat hepatocytes and rat Leydig cells. A very pronounced preference for homotypic adhesion was displayed in Ca^{2+}-free MDE (shown), random adhesive behavior was observed in complete MDE (not shown) or MDE containing Ca^{2+} but no Mg^{2+} (not shown). Leydig cells aggregated poorly when compared with hepatocytes. The four-cell aggregate size class (shown) was representative of all aggregates analyzed: 28 aggregates were scored containing 80 hepatocytes and 32 Leydig cells; χ^2, 80.69; $P < 10^{-6}$ (highly significant).

DISCUSSION

In this communication we present evidence that a microscopic analysis of individual aggregates formed by mixed cell suspen-

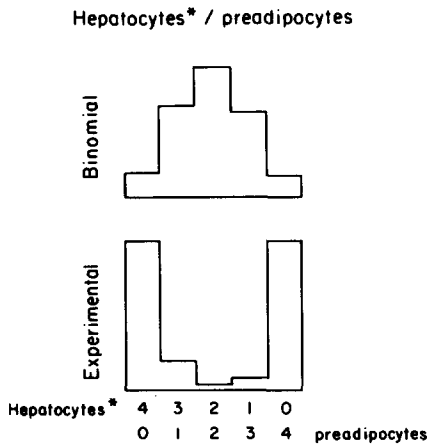

FIGURE 8 Adhesive behavior of a mixture of freshly explanted rat hepatocytes and rat preadipocytes. The same pronounced preference for homotypic adhesion was observed in MDE (not shown), MDE supplemented with 10% FCS (shown), and MDE supplemented with 20% rat serum (not shown), after a gyratory incubation (shown) or a stationary incubation (not shown). The four-cell aggregate size class (shown) was representative of all aggregates analyzed: 58 aggregates were scored and included 119 hepatocytes and 113 preadipocytes; χ^2, 210.47; $P < 10^{-9}$ (highly significant).

sions can be used to assess intercellular adhesive specificity. The method avoids most of the problems inherent in collection assays and, unlike sorting out experiments, allows quantitation of the results. We also show that a wide variety of different cell types can be labeled with the fluorescent dye, fluorescamine, at pH 7.3 in complete tissue culture medium, without affecting either their adhesive properties or several other vital cell functions such as exclusion of dyes, adhesion to substrates, proliferation, and fusion of myoblasts into myotubes (32). The microscopic assay of intercellular adhesive specificity is thus applicable to a variety of cells and is no longer limited to the analysis of those with unique morphological features. The use of fluorescamine offers several advantages over previously used cell-labeling procedures (2, 12, 34, 39): (*a*) The reagent is readily available and simple to use. (*b*) The reaction takes place under mild conditions and is almost instantaneous. (*c*) The label is easily visualized, even on highly refractive round cells, a situation in which most conventional histochemical stains cannot be detected. (*d*) The labeling procedure does not affect the adhesive specificity of the cells. (*e*) The cells are subject to fewer and shorter manipulations before and after the aggregation experiment. Thus, the method is particularly well suited for studying fragile or short-lived cells or cells suspected of undergoing rapid changes in adhesive behavior.

In the present studies, single cells in mixed suspensions were exposed to either homotypic or heterotypic cell types. It seems likely that the composition of the resulting aggregates reflected the adhesive preferences of the participating cells. Although the fewest assumptions would be required in an experimental model limited to the analysis of two single cells aggregating into a doublet, the analysis of this size class is also most prone to be biased by small (two-cell) aggregates already present in the initial cell suspensions. We therefore based the statistical analysis on slightly larger (four-cell) aggregates. Measurement of the deviation between the empirically determined frequency distribution and the calculated binomial distribution by a chi-square test was an essential part of the assay: it provided an objective criterion according to which a distribution could be classified as significantly different (adhesive specificity) or not different (no adhesive specificity) from a random distribution. It also allowed quantitation of the degree of adhesive specificity, provided the *P*-values that were compared with each other were derived from samples of equal or at least comparable size. The only serious limitation of the assay is the considerable amount of time required for the visual examination of a sufficient number of individual aggregates. However, preliminary experiments with a fluorescence-activated cell sorter suggest that an automated analysis should be feasible with very small aggregates. Some of the results obtained with the fluorescamine method are discussed below.

Variable results have been reported using 3T3 cells and cell lines derived from these cells. The monolayer (46) and aggregate (6) collecting methods indicated no adhesive specificity, whereas Gershman et al. (11) reported a moderate degree of "sorting-out" between 3T3 and SV40-3T3 cells. The present method clearly indicates adhesive specificity in mixtures of 3T3 and SV40-3T3 cells. We do not know why the cells show dissimilar results with different assays, although several explanations are possible. For example, cell-cell adhesion is a complex and multistep (43) phenomenon; different assays may be measuring different stages in the process, some of which may be specific and some nonspecific. Another possibility is that although adhesive sites are present they are not equally available in all assays. In the monolayer assay, for instance, adhesive sites may be clustered laterally rather than being on the dorsal surface and thus might not be available for the binding of single cells.

We have previously suggested (26) that cell surface complex carbohydrates may be involved in cell-cell recognition and adhesive specificity, and it was of interest to test cells deficient in such components. To our knowledge no cell line is available in which cell surface carbohydrates are completely depleted. A 3T3 cell line derivative, AD6 (22), which adheres poorly to tissue culture plastic, was found to adhere randomly when mixed with 3T3 cells. Although these results at first suggest that cell surface carbohydrates are not involved in intercellular adhesion of this cell type, the mutant is "leaky" (23, 24) so that significant quantities of complex carbohydrates are, in fact, synthesized by the cells.

In previous studies with parental CHO cells and lectin-resistant mutants,[1] the cell monolayer assay gave variable results. Apparently very small changes in growth or assay conditions had significant effects on the monolayer assay. Similar results were obtained with the present method; there was a slight preference for homotypic adhesion, but the degree of specificity was marginal. The mutant cell lines do contain cell surface carbohydrates (3, 35), but greatly reduced (to negligible) levels of such molecules that bind the respective lectins used for selection. Thus, it seems likely that the latter, at least, are not involved in specific cell adhesion.

Finally, the results reported here confirm and extend our earlier report (20) showing that cells derived from adult tissues exhibit adhesive specificity, an important result because it validates the use of such tissues in attempts to isolate the cell surface molecules involved in adhesive specificity.

In summary, we have presented a method for labeling cells with fluorescamine without apparently affecting their adhesive behavior, viability, and several other functions. This technique permits quantitative assessment of adhesive specificity with a

[1] B. T. Walther and S. Roseman. Unpublished observations.

relatively small number of cells, and should be applicable to a wide variety of cell types.

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