A NOVEL ASSAY OF NEURONAL CELL ADHESION

David I. Gottlieb and Luis Glaser

Department of Biological Chemistry

Division of Biology and Biomedical Sciences

Washington University

St. Louis, Missouri 63110

Received February 19,1975

Intact cells can be made to adhere to a glass surface which is derivitized with γ-aminopropyl-triethoxysilane, and then treated with glutaraldehyde. Such cells remain fully viable and a monolayer of such cells can be formed within 1 hour after the cells are removed from an embryo. The rate of binding of radioactively labeled single cells to such a monolayer can be used as a measure of intercellular adhesion (Walther, Ohman and Roseman, Proc. Natl. Acad. Sci. USA 70, 1569-1573 (1973). The lack of toxicity of this surface is shown by the fact that it supports normal growth of C-6 rat glial cells.

Recent work in this (1,2) and other laboratories (3) has shown that embryonic brain cells have surface determinants which allow for selective intercellular adhesion. Using the inhibition of cell aggregation by plasma membranes we have defined both temporal and spatial changes in cell surface specificity. Anatomical and physiological studies show that the major brain regions are exceedingly complex with respect to cellular architecture. The optic tectum and the retina in which adhesive specificity has been most thoroughly studied consist of five and three major cellular layers respectively, the cells of which are distinguishable by numerous anatomical, physiological and biochemical criteria. In attempting to investigate the cell surface specificity of these cell types an assay capable of measuring the cell surface specificity of a small number of cells is essential.

Walther *et al*. (4) have described an assay for cell adhesion based on the adhesion of radioactive cells to a monolayer of homologous or heterologous cells. A disadvantage of this assay for studies of specificity in the devel-

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oping central nervous system is the requirement that the monolayer be established in culture for 48 hours to have adequate mechanical strength to be useful in the assay, and as we have shown (2) considerable changes in cell surface specificity can take place in a 48 hour period.

We have developed a variant of this assay in which cells can be made to adhere instantly to glass derivitized with the protein linking reagent glutaraldehyde activated γ-aminopropyl-triethoxysilane. A monolayer can be formed within 1 hour after the cells are removed from the embryo, and the cells remain fully viable.

MATERIALS AND METHODS

Standard scintillation vials were cleaned in nitric acid. 2 ml of a 10% solution of γ-aminopropyl-triethoxysilane in toluene was added to each vial, the vials were covered with a glass marble and heated on a hot plate for 1 hour under gentle reflux. The vials were allowed to cool and were thoroughly rinsed with fresh toluene and allowed to air dry (5). A 1% aqueous solution of glutaraldehyde (Sigma) was pippeted into the bottom of the vial and left for 1 hour at room temperature. The vials were thoroughly washed with distilled water and air dried (5). Single cell suspension of neuronal cells were prepared as described previously (1). The cells were suspended in Dulbecco's modified Eagle's medium (6), buffered to pH 7.3 with HEPES, and kept at 0°. 10^7 Cells in 1 ml of medium were pippeted into 2 ml of cold medium in a derivitized vial. The vials were immediately centrifuged in a Sorvall GLC-2 centrifuge with swinging bucket rotor at 162 x g for 5 min. It is important that all solutions remain cool until the centrifugation step to avoid clumping of the cells. After centrifugation the vials were placed in a 37° bath for 10 min to stabilize the monolayers. The medium was then decanted and replaced with 1 ml of calcium and magnesium-free Hanks solution containing 5 mg/ml bovine serum albumin (Sigma) (CMF-A). The monolayers were then used in adhesion assays as described in the legend to Figure 2.

RESULTS

Viability of cells in monolayer and stability of the monolayer. It is

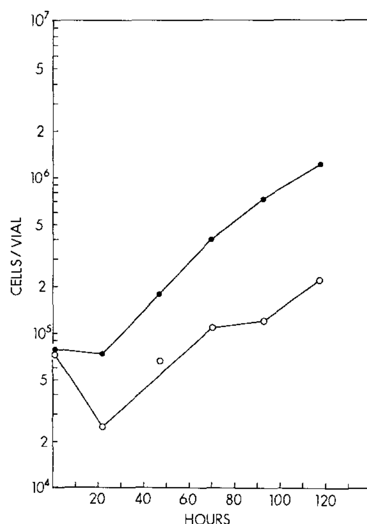

Figure 1. Growth of C-6 cells in derivitized and untreated vials.

8×10^4 Cells were inoculated into derivitized or untreated vials containing 2 ml of Higuchi's (7) medium + 10% horse serum. Vials were incubated at 37°C under seal. Cells were removed from the bottom of the vials for counting by sixty minutes digestion with 0.15% trypsin in PBS (phosphate buffered saline pH 7.3) and counted in a haeomocytameter. ●—●, cells grown in derivitized vials; O—O, cells grown in untreated vials.

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crucial that cells not be damaged during the construction of the monolayers. Three lines of evidence show that monolayers can be formed without gross injury to the cells. First, cells retained the ability to exclude trypan blue for up to three hours after being "plated" on derivitized glass. Secondly, the cells on monolayers incorporate [^{3}H]leucine into protein at the same rate as cells in suspension cultures over the first several hours after removal from the brain. Finally, we have succeeded in growing the C-6 rat glial cell line on derivitized glass and have shown that this surface is more favorable for growth than untreated glass (Fig. 1). This latter control is not strictly comparable to the formation of embryonic cell monolayers since the C-6 cells grow in the presence of serum while the monolayers are plated in its absence. It does, however, show that the treated glass is not toxic to the cells. The stability of the monolayers was estimated quantitatively by counting the numbers of cells appearing in the medium after incubation on the shaker bath. After 45 minutes incubation in CMF-A less than 0.3% of the cells were recoverable in the medium.

Evidence that added cells adhere to the monolayer. Since the ^{32}P labeled cells leak about 20% of their label into the medium, it is important to show that this material does not accumulate in the monolayer. Control experiments were performed by preincubating labeled 9 day tectum cells in CMF-A for 45 minutes, spinning out the cells and incubating the supernatant with fresh 9 day tectum monolayers. The monolayers accumulated less than 1% the number of counts when compared to monolayers incubated with an equivalent volume of cell suspension, and soluble transfer is therefore not responsible for a significant portion of the labeling of monolayers.

Unlike the monolayers studied by Walther et al (4) ours are fully confluent, with no visible gaps. Therefore, cells which stick to the bottom of the vial must be stuck to other cells. Over 85% of the bound label in one experiment was removed by gently scraping the monolayer off the vial bottom while taking pains to avoid disturbing any cells which might be clinging to the sides of the vial. Therefore, no more than 15% of the label in a typical experiment could be found on the sides of the vial. In fact this figure is probably considerably lower since fragments of the monolayer remained on the bottom of the scraped vials.

In spite of these experiments an additional control was done to show that adhering counts represent cell-cell adhesions rather than cells sticking to glass. Fig. 2 shows the effects of temperature on 9 day tectal cells sticking to homologous monolayers and to derivitized vials without a cell monolayer. The experiment shows that sticking to the monolayer is almost completely dependent on temperature, whereas binding to the derivitized vials has virtually no temperature dependence. This supports the conclusion that most of the binding in our experiments is cell-cell binding.

Patterns of specificity between embryonic brain cells. The optic tecta and telencephala of the 9 day chick embryo were chosen for study because of their large size and ease of dissection. When tectal and telencephalic single cells were tested for their ability to bind to tectal monolayers, a surprising re-

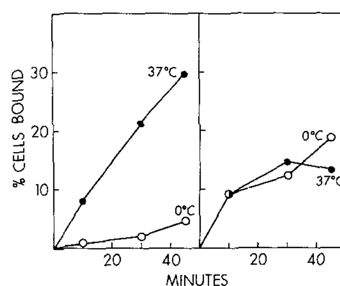

Figure 2. Temperature dependence of embryonic brain cell adhesion to monolayers and derivitized glass.

Embryonic brain cells were labelled with ^{32}P by incubating triturated organs in 5 ml of Dulbeccos modified Eagle's medium (6) + 10% chicken serum with 500 µCi of carrier free $H_3{}^{32}PO_4$ for 3 hours at 37°C. The tissue was then transfered to the same medium without isotope and incubated for an additional hour. The tissue was then used to prepare single cells by standard methods of this laboratory (1,2).

For the adhesion assay, each vial was inoculated with 1 x 10^5 labelled cells (.17 cpm/cell). Vials were placed in gyratory water baths set at 0°C and 37°C and agitated at 65 rpm for appropriate times. Cell suspensions were then removed by gentle aspiration and the vials carefully washed twice with 5 ml of chilled PBS. 1 ml of 1% Triton X100 was added to the vials to dissolve cells. 3a70 Counting fluid (RPI Corporation, Elk Grove, Illinois) was added and the vials counted in a scintillation counter.

Left panel. Binding to monolayers, ●—●, 37°C,
O—O, 0°C

Right panel. Binding to derivitized glass, ●—●, 37°C
O—O, 0°C

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sult was obtained, namely that the telencephalic cells showed greater binding than the tectal cells. The converse experiment shows that this is not an expression of a universal stickiness on the part of the telencephalon cells because when the same cell suspensions are tested for their ability to stick to telencephalon monolayers, the tectal cells now show the greater affinity. This reciprocal affinity is highly reproducible. In five such experiments heterotypic binding always exceeded homotypic to both tectal and telencephalic monolayers. Part of the difference appears to be a result of the relative rates of binding as the early time points always indicate that heterotypic binding is favored over homotypic. Most experiments show that the extent of binding is greater for the heterotypic pairs although this aspect of the data

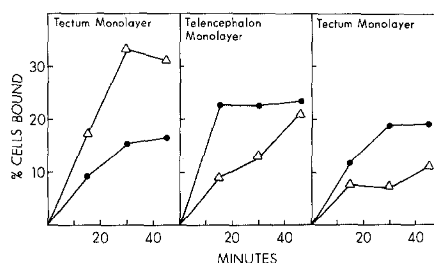

Figure 3. Specificity of cell adhesion.

9-Day tectum cells (7.7×10^4 cells, .16 cpm/cell), ●—●; 9 day telencephalon cells (7.0×10^4 cells, .15 cpm/cell), O—O; and Chinese hamster ovary cells (6×10^4 cells, .53 cpm/cell), □—□, were inoculated into vials containing appropriate monolayers. All experiments were conducted at 37°C with the platform rotating at 65 rpm. Kinetics of binding were determined as in Figure 2.

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shows more variability. Chinese hamster ovary cells (a totally heterologous cell type) essentially does not bind to tectal monolayers.

The binding of tectum and telencephalon cells to monolayers gives the surprising result that heterotypic binding is preferred to homotypic binding.

Two conclusions can be drawn from these data. 1) The specificity is a complementary type in that the heterotypic case is preferred over either homotypic pair. 2) The fact that the same result is obtained in reciprocal pairs is an indication that being placed in a monolayer does not damage the cell's specific recognition apparatus.

In this assay we measured the adhesion of 10^5 free cells to 10^7 cells in a confluent monolayer. It is probable that cells in the monolayers are altering one another's surface in complicated ways. For example, homotypic binding sites may be masked by "self" interactions in the monolayers. The effect of cell-cell interactions in the monolayers on cell surface specificity is under investigation.

The derivitized glass products provide useful new methods for the study of cell adhesion and a potentially useful new surface for cell growth studies.

This work has been supported by Grants NIH GM 18405 and NSF BMS75-22638 and NIH F22-NS 166.

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