CALIFORNIA STATE UNIVERSITY, NORTHRIDGE

AGGLUTINABILITY OF POPULATIONS OF EMBRYONIC CELLS

A thesis submitted in partial satisfaction of the requirements for the degree of Master of Science in Biology

by

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May, 1975
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California State University, Northridge

May, 1975
ACKNOWLEDGEMENTS

I wish to thank Dr. Steven Oppenheimer for his advice and encouragement during the research and preparation of this thesis. I also wish to thank Dr. Spotts and Dr. Highkin for their help.
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ABSTRACT

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It has been demonstrated that specific changes in carbohydrate-containing cell surface lectin receptor sites occur with differentiation and maturation of sea urchin embryo cells. In this study, evidence is presented using a quantitative electronic particle counter assay to measure agglutination, which indicates that concanavalin A (ConA) mediated agglutination of dissociated 32/64 cell sea urchin embryos differs dramatically with respect to specific cell populations. The migratory cell type, the micromere, is significantly more agglutinable with ConA than the other cell types and colchicine treatment markedly increases sea urchin embryo cell agglutinability. The
results indicate that like many malignant cells which display extensive migratory behavior, specific migratory populations of embryonic cells are agglutinable with ConA. The results are discussed with respect to the possible nature of lectin receptor sites on specific populations of embryonic cells and the possible role of colchicine-sensitive structures in controlling the display patterns of these sites.
INTRODUCTION

In previous years the cell membrane was considered primarily as a static model which presumed few differences among membranes. The erythrocyte ghost had been used as the primary example. The present-day concept of the cell membrane is one of a 3-D mosaic which is capable of reflecting the diversity of cell types and is responsive to the environment. This change in attitude resulted from the discovery of the decisive roles played by the cell surface in the fundamental processes of cell growth, cell movement, and cell recognition. Much research has been done in recent years on the basis and regulation of membrane-mediated information involved in these functions.

Plant lectins, which bind to specific carbohydrate residues on the cell surface, have been used extensively in exploring the cell surface and the information that it contains. A wheat-germ preparation which agglutinated certain transformed cells but not their normal counterparts was discovered by Aub, et al (1963; 1965) and later purified by Burger and Goldberg (1967). Burger and Goldberg
characterized the activity as a plant lectin which bound to cell surface N-acetyl-D-glucosamine-like residues (Burger and Goldberg 1967). Since that time many other plant lectins have been identified which agglutinate transformed cells, but not their normal counterparts. One such lectin is Concanavalin A (ConA) which is used in this study. Con A, which is derived from the jack bean *Canavalia ensiformis*, has four binding sites which interact with \(\alpha\)-D-glucose-like and \(\alpha\)-D-mannose-like residues on the cell surface (Poretz and Goldstein 1970). Activity of Con A, like many plant lectins, is dependent on transition metal ions (Agrawal and Goldstein 1968).

Several mechanisms for the observed differences in the agglutination by lectins have been proposed. Burger (1969) proposed that the agglutination of transformed cells was due to the exposure of "masked" lectin binding sites during transformation. These binding sites were thought to be "masked" on normal cells because a brief treatment with trypsin resulted in agglutination of normal cells, but did not enhance the agglutination of transformed cells (Burger, 1969). More recently it has been found that normal, protease-treated and transformed cells have equivalent numbers of lectin binding sites per cell (Cline

It has been suggested that the difference in agglutinability of cell types might be due to a clustering in the topographical distribution of lectin binding sites on transformed cells that allows multiple cross-bridging between adjacent cells (Nicolson, 1971; Singer and Nicolson, 1972). Using ferritin labeled ConA Nicolson demonstrated that there was a clustering of ConA receptor sites in the plane of the membrane following proteolytic enzyme treatment or transformation (Nicolson, 1972).

Frye and Edidin (1970) have demonstrated that cell surface macromolecules are mobile in the plane of the membrane. Cell surface macromolecules have also been shown to be capable of having their distributions altered by the binding of multivalent ligands such as ConA (DePetris, et al., 1973; Rosenblith et al., 1973). DePetris's work indicated that the clustering of ConA receptor sites on transformed cells might be more fluid thus allowing the ConA to cluster the binding sites.

It has been found that various embryonic cell types possess similar membrane properties and exhibit similar
behavior patterns to malignant cells. Some plant lectins, particularly ConA, have been found to agglutinate embryonic and transformed cells but not normal cells without previous trypsinization (Krach, et al. 1974; Oppenheimer and Oden- crantz, 1972; Moscona, 1971; Inbar and Sachs, 1969; Kleinschuster and Moscona, 1972; Moscona, 1960). Kleinschuster and Moscona (1972) have demonstrated that chick retina cells from early embryonic stages are more agglutinable with ConA than retina cells from later embryonic stages. Krach, et al. (1974) have shown a quantitatively measured decrease in ConA mediated agglutinability of dissociated sea urchin embryo cells with increasing age of the embryo. These results indicate that during differentiation and maturation specific changes occur in the ConA receptor sites.

It has been proposed by Moscona (1971) that the presence of specific agglutinin receptor sites on the cell surface might in some way be correlated with the capacity of malignant and embryonic cells to migrate and infiltrate. The orderly shifts of individual cells and cell complexes during the course of morphogenesis may be due to differential cell surface properties (Townes and Holtfreter, 1955; Moscona, 1960; Humphreys, 1963). This would imply that
cell surface properties may vary with respect to embryonic cell type.

This work presents evidence which indicates that the cell surface properties of sea urchin *Strongylocentrotus purpuratus* embryos vary with respect to cell type as determined by ConA agglutinability. The migratory cell type, the micromere, is shown to be the most agglutinable cell type at the 32/64 cell stage of the embryo.

Singer and Nicolson (1972) suggested that the topographical organization of membrane proteins may be determined by forces extrinsic to the cell surface. It now appears that microtubules associated with the membrane inner surface may determine, in part, cell rigidity and the mobility and topographical distribution of ConA agglutinin receptor sites. Berlin and Ukena (1972) tested the effect of colchicine and vinblastine, which disrupt microtubules, on the agglutination of polymorphonuclear leucocytes by ConA. They found a decrease in ConA agglutinability. Yin, et al. (1972) found a decrease in agglutinability with ConA of transformed 3T3 cells treated with colchicine, vinblastine and colcimid. With the use of hemocyanin labeled ConA, Ukena, et al. (1974) demonstrated a change in the secondary organization of ConA receptor
sites on colchicine treated transformed 3T3 cells.

The results of our work differ from that of the above researchers. An increase in ConA agglutinability of dissociated sea urchin embryo cells treated with colchicine was demonstrated. Recently Fox (1975) has shown an increase in ConA agglutinability of transformed fibroblasts treated with colchicine. This increase in agglutinability occurred in the first 10-15 minutes, afterwards agglutinability decreased.
MATERIALS AND METHODS

Reagents and media

Ficoll, dithiothreitol (DTT, Cleland's Reagent), ethyleneglycol-bis-(\(-\beta\)-amino-ethyl ether) N N' tetracetic acid (EGTA), deoxyribonuclease (DNAase) 1X crystallized and lyophilized and colchicine were obtained from Sigma Chemical Co., St. Louis, Mo. (Hydroxymethyl) aminomethane (Tris buffer) was obtained from Mallinckroft Chemical Works, St. Louis, Mo. Calcium-magnesium-free sea water (CMF-SW) was prepared as follows; 27.0g NaCl, 1.0g Na₂SO₄, 0.8g KCL, 0.18g NaHCO₃ were dissolved in 1L of distilled water and 0.02M Tris buffer was added to adjust the pH to 8.0.

Preparation of cell suspensions

Gametes of the sea urchin Stronglocentrotus purpuratus were obtained by injection of 0.55M KCL (Oppenheimer and Odencrantz, 1972).

In order to obtain single cells from urchins at the 32/64 cell stage, it was necessary to prevent formation of the fertilization membrane as previously reported (Krach
et al., 1974; Vacquier, et al. 1972). Each 10 ml of lightly packed eggs was resuspended in 50ml of millipore-filtered 0.02M Tris-buffered sea water at pH 8 (MFSW). This suspension was added to 50ml of 0.02M DTT in MFSW and gently stirred for 4 minutes and diluted with MFSW. The eggs were allowed to settle out of this solution and washed with 1L of MFSW. The DTT treated eggs were then collected in 100ml of MFSW and 0.1ml of undiluted sperm was added to the egg suspension. The suspension was slowly stirred for 45 sec and diluted with 2L of MFSW to prevent clumping of the zygotes. The zygotes were distributed into beakers and allowed to develop at 17 C.

When most embryos reached the 32 cell stage (with some at 64; 32/64 cell stage) they were collected and dissociated by a modification of the method of Guidice (1962), as previously reported (Krach, et al. 1974). The embryos were washed 3X in CMF-SW (pH 8). 2 ml of 0.01M EGTA in CMF-SW (pH 8) was then added to each 3ml of packed embryos. This suspension was incubated for 10 minutes at 17 C and then gently pipetted (10X) with a Pasteur pipette to complete dissociation. The suspension was diluted with 10ml of CMF-SW, centrifuged and resuspended in CMF-SW.
Cell separation

The dissociated embryos were separated into two groups of cell types: the micromeres and a combined mixture of mesomeres and macromeres. The separation was accomplished by using a slightly modified version of a method developed by Hynes and Gross (1970). The cell suspension (8-10ml) was layered over a 5-15% Ficoll gradient. The beaker was placed in a 17°C incubator and allowed to stand for 3 to 4 hours, until the bands were well separated.

Cells from each band were carefully removed with a Pasteur pipette. The separated cell suspensions were diluted with an equal volume of CMF-SW and centrifuged. The collected cells were washed once in CMF-SW and resuspended in CMF-SW plus 10 g/ml DNAase. Photomicrographs of the bands confirmed that the light band consisted of 90% micromeres with 10% contaminating larger cells. The heavier cell band consisted of 80% mesomeres and macromeres with 20% contaminating micromeres.

Agglutination assay

A reliable quantitative method for measuring cell agglutination has been developed and described previously (Oppenheimer and Odencrantz, 1972). This assay measures the disappearance of single cells into aggregates in a
rotating suspension with an electronic particle counter.

In this study a model 112 LT Celloscope (Particle Data, Inc. Elmhurst, Ill.) was employed to measure agglutination. The settings, for single cells, which excluded most clumps and debris, were as follows: current 1/16, gain 12 with a window of 100-700. In all cases the decrease in cell numbers as a function of time was due to clumping of viable cells and not cell lysis. Microscopic examination, dye exclusion tests, and use of the electronic particle counter debris window were used to verify viability and agglutination as previously reported (Krach, et al., 1974; Oppenheimer, et al. 1969).

Cells were suspended in CMF-SW with or without varying concentrations of ConA. Some experiments included preincubations with colchicine. DNAase (10 g/ml) was added to all cell suspensions to prevent non-specific cell agglutination. This enzyme had no effect on ConA mediated agglutinability as previously described (Oppenheimer and Odencrantz, 1972). Aliquots of cell suspensions (0.2ml) were placed in 1 dram screw cap vials on a gyratory shaker with a 4 5/8 inch diameter of gyration and rotated at 68 rpm at 17 C. At various times the vials were placed on ice, diluted with 10ml of CMF-SW and counted with the
electronic particle counter. The average variation of replicate vials was 4% with the maximum variation occasionally running as high as 9%. Experimental results were reproducible under the standard conditions of the assay procedure.
RESULTS

Reliability of data

Hapten inhibition studies were performed using α-methyl-D-glucoside and N-acetyl-D-glucosamine. Table 1 shows that even at very low concentrations (10^{-2} M) α-methyl-D-glucoside decreases ConA mediated agglutination while N-acetyl-D-glucosamine does not decrease agglutination. Photomicrographs of the separated cell suspensions were taken and analyzed to determine if the cell separation procedures produced an adequate quality of separation. Band I, the micromeres, was approximately 90% pure while band II, the mesomeres and macromeres, was approximately 80% pure. In repeated experiments the absolute percentages of agglutination varied but in all cases the relative degrees of agglutination for the various experimental and control samples remained the same. For example, in 3 separate experiments band I cells agglutinated 75, 76, and 58% after 10 minutes with 1 mg/ml ConA while band II cells agglutinated 40, 20 and 20% under the same conditions, respectively.
Agglutination of separated cell suspensions

Fig. 1 shows that after correcting for background aggregation, when the ConA concentration was 1 mg/ml, after 45 min, 79% of the micromeres agglutinated. Under the same conditions only 14% of cells of the mesomere-macromere band were agglutinable. This latter result could be due to contaminating micromeres. In a 50% mixture of both groups of the separated cells, 42% of the cells agglutinated. As experiments were conducted in CMF-SW, this necessitated the addition of exogenous divalent cations to promote ConA mediated agglutination. 0.05 ml of a solution containing 0.001M CaCl$_2$ and 20 mg/ml of ConA was added to 1 ml of the cell suspension. This resulted in a final ConA concentration of 1 mg/ml. CaCl$_2$ was added to the control suspensions.

Fig. 2 describes the effect of colchicine on the agglutination of different populations of sea urchin embryo cells. By 5 min 75% of the micromeres preincubated with colchicine (0.001M were agglutinated with ConA and 55% of these cells which were not preincubated with colchicine were agglutinated with ConA. In both cases the figures were corrected for background aggregation. This represents a 20% increase in agglutination of micromeres produced by
treatment with colchicine. Since this assay only measures the disappearance of single cells into aggregates, it is not capable of measuring the size of the aggregates. It was observed by microscopic examination that most of the micromeres treated with colchicine were clumped into 2 or 3 large aggregates. The micromeres not treated with colchicine were clumped into many small groups of cells. Thus, the 20% increase in agglutination of micromeres treated with colchicine may be an underestimate of the increased agglutinability. 43% of the mesomere-macromere groups of cells agglutinated by 5 min when the cells were preincubated for 30 min with colchicine before addition of ConA. Only 29% of this same group of cells were agglutinated with ConA without colchicine treatment. These figures were corrected for background aggregation. Therefore, colchicine increased agglutination of the mesomere-macromere group of cells by 14%. This increase in agglutination could represent increased agglutination of contaminating micromeres or an actual increase in agglutination of the mesomeres and macromeres. In any case, colchicine clearly increases ConA mediated agglutinability of sea urchin embryo cells.
TABLE I

Effects of Hapten Sugars on ConA Agglutination of Sea Urchin Embryo Cells.

<table>
<thead>
<tr>
<th>Sample Number</th>
<th>% agglutination after 10 min. before subtraction of background aggregation</th>
<th>% agglutination after 10 min. after subtraction of background aggregation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13</td>
<td>--</td>
</tr>
<tr>
<td>2</td>
<td>33</td>
<td>20</td>
</tr>
<tr>
<td>3</td>
<td>46</td>
<td>33</td>
</tr>
<tr>
<td>4</td>
<td>20</td>
<td>7</td>
</tr>
</tbody>
</table>

Cells were prepared and assayed as described in the Materials and Methods. (1) mixture of all cell types without ConA; (2) mixture of all cell types with ConA (1mg/ml). (3) mixture of all cell types with ConA (1mg/ml) and $10^{-2}$M N-acetyl-D-glucosamine; (4) mixture of all cell types with ConA (1mg/ml) and $10^{-2}$M Methyl-D-glucoside.
DISCUSSION


Embryonic and transformed cells exhibit extensive migratory movements and malignancy may therefore represent a regression to the embryonic state with respect to certain cell surface characteristics. For example, ConA, which binds to receptor sites containing D-mannose-like or D-glucose-like residues (Goldstein and So, 1965) is known to agglutinate both embryonic and neoplastic cells (Krach et al.; Oppenheimer and Odencrantz, 1972; Inbar, et al. 1971; Inbar and Sachs, 1973; Inbar and Sachs, 1967; Moscona, 1960; Sharon and Lis, 1972; Inbar and Sachs, 1969;
It has been suggested that this agglutinability may be due to reappearance of embryonal antigen display patterns on the surface of malignant cells (Kleinschuster and Moscona, 1972; Stonehill and Bendich, 1970).

Chick embryo retina cells have been shown to be more agglutinable with ConA at early embryonic stages than at later stages (Kleinschuster and Moscona, 1972). A similar correlation between agglutinability and embryonic age has been shown to exist in the sea urchin system (Krach et al., 1974). The specific changes which occur on the ConA receptor sites during development may be manifested in one of several ways. Less receptor sites could be incorporated into the cell membrane as it is synthesized; the receptor sites may be masked during development such that they are less accessible for interaction with ConA (Burger and Goldberg, 1969; Burger, 1969). Other possibilities involve the arrangement of ConA receptor sites. During differentiation the receptor sites might move from a clustered distribution to a more random distribution which could reduce lectin mediated agglutination without affecting the ability of cells to bind ConA (Nicolson, 1971; Nicolson, 1972). The mobility of ConA receptor sites
might be reduced during maturation which could prevent
lectin-induced rearrangements of the ConA receptor sites

Until now it was not known if the age dependent ConA
mediated agglutinability of dissociated sea urchin cells
was due to agglutinability of all the cells, or just
certain cell populations. Our results clearly indicate
that one specific cell type, the micromere, is responsible
for most of the agglutination of dissociated sea urchin
cells at the 32/64 cell stage.

Moscona has suggested that ConA receptor sites may
function in cell contact, cell organization and cell recog-
nition during development (Moscona, 1971). Of the cell
types in the 32/64 cell stage of the sea urchin embryo,
the micromeres exhibit behavior most similar to malignant
cells. These presumptive mesenchymal cells exhibit a
great deal of movement and migration during gastrulation.

Recently microtubule and microfilament components
attached to the inner membrane surface have been implicated
in controlling the mobility and topographical distribution
of agglutinin receptor sites (Berlin and Ukena, 1972; Yin
et al., 1972; Ukena, et al., 1974; Knapp and Oppenheimer,
In Preparation; Edelman, et al. 1973). Colchicine and
vinblastine (which disrupt microtubules) have been shown to decrease ConA mediated agglutinability of polymorph-nuclear leucocytes (Berlin and Ukena, 1972). These drugs have also been shown to decrease ConA mediated agglutinability of transformed 3T3 fibroblasts. Ukena, et al (1974) demonstrated that colchicine treatment of transformed 3T3 fibroblasts caused a change in the secondary distribution of ConA receptor sites when labeled with ConA hemocyanin before fixation. (Several investigators (Berlin and Ukena, 1972; Yin, et al. 1972; Ukena, et al. 1974; Edelman, et al. 1973) have proposed that the mobility of the receptor sites is regulated by a colchicine-sensitive assembly near the plasma membrane. Our results show an increase in ConA mediated agglutinability of dissociated sea urchin cells treated with colchicine. These results support the contention that microtubules play a role in determining the display of ConA receptor sites.

In summary, our experiments indicate that a specific population of sea urchin embryo cells, the micromeres, which display extensive migratory activity are most agglutinable with ConA, while other populations of sea urchin embryo cells do not exhibit this increased agglutinability. We also show that treatment of sea urchin embryo cells with
colchicine increases agglutination, suggesting that colchicine-sensitive structures are involved in controlling the display of cell surface receptor sites. As micromeres differentiate with increasing embryonic age migratory ability and agglutinability decrease (Krach, et al. 1974). This latter characteristic, or perhaps, both phenomena may result from: decrease in numbers of surface ConA receptor sites for interaction with lectin, randomization of lectin binding sites or reduced receptor site mobility (Inbar and Sach, 1973; Nicolson, 1973; Burger and Goldberg, 1969; Burger, 1969; Nicolson, 1971; Nicolson, 1972). The results with colchicine suggest that structures sensitive to the drug may anchor ConA receptor sites in a pattern which inhibits the mobility of these sites and cell agglutination. Disruption of these structures with colchicine could free lectin binding sites, allowing increased mobility and cell agglutination.
REFERENCES


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