A FINE STRUCTURAL ANALYSIS OF SURFACE
INTERACTIONS OF AGGLUTINATED SARCOMA.180 CELLS

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by

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ABSTRACT

AN ULTRASTRUCTURAL ANALYSIS OF THE FINE STRUCTURE
AND SURFACE INTERACTIONS OF AGGLUTINATED SARCOMA 180 CELLS

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Sarcoma 180, a mouse ascites tumor, was examined ultrastructurally using transmission electron microscopy. Its general fine structure, and the cell surface interactions occurring as a result of agglutination were examined. Three types of agglutinins were used, Concanavalin A (Con A), an ascites "factor", and manganese ion.

Ultrastructurally, Sarcoma 180 had the features of many tumors: A large heterochromatic, multilobate nucleus with numerous nucleoli, reduced amounts of endoplasmic reticulum, and numerous microvilli on the cell surface. The mitochondria were somewhat pleomorphic. Large lipid droplets were seen in addition to several other large, darkly stained bodies in the cytoplasm. Cell surface interactions between untreated cells were not commonly seen.

Three surface interactions were seen after agglutination: 1) Microvillous associations characterized by the interaction of villi
with adjacent cell surfaces; 2) intermediate associations in which adjacent cell membranes were approximately parallel, crosslinked by patches of lightly stained material, and separated by greater than 100Å; and 3) close association in which the cells were separated by less than 100Å, commonly 20Å, and resembled gap junctions.

All three types of associations were seen after Con A agglutination. The intermediate association may represent actual crosslinking by Con A aggregates. The close associations, it was argued, resulted from interactions between cell surface components. Microvillous associations were most commonly seen, though their significance was not clear.

The ascites "factor" at high concentration induced pinocytosis and vacuolization. Agglutination resulted from large aggregates of flocculent material crosslinking the cells. At lower concentration, intermediate associations were seen, small aggregates of "factor" possibly crosslinked the cells together.

Manganese ion produced a very rapid agglutination mediated totally by villous interactions. Points of interaction were very tight, with no obvious gap between membranes. It was thought that ionic bridging, or denaturation of surface components was involved.
Introduction

In recent years several lines of investigation have shown that the surfaces of transformed and malignant cells are different from those of "normal" cells. These differences include biochemical alterations, changes in cell-cell adhesion and of cell surface charge, and agglutinability.

Gasic and Gasic (1963, 1962) provide the first convincing evidence of a carbohydrate-rich layer external to the plasma membrane of mammalian cells. This cell coat was conceptualized for all cells as the 'glycocalyx' by Bennett (1963). The work of several investigators seems to confirm that the cell coat of neoplastic cells differs from that found on normal cells. Both increases and decreases of various constituents in the glycocalyx have been reported (Buck, et al., 1970; Brady, et al., 1969; Martinez-Palomo, et al., 1969; Meezan, et al., 1969; Mora, et al., 1969; Wu, et al., 1969; Morgan, 1968; Ohta, et al., 1968; Kraemer, 1966; Purdom, et al., 1958).

Coman (1944) demonstrated a general decrease in adhesiveness between epithelial tumor cells in comparison with normal epithelium. This reduction in adhesion has been postulated to account for the change in "social" behavior demonstrated by transformed cells. Transformed cells show a loss of contact inhibition of growth and mobility (Dulbecco, 1970; Abercrombie and Ambrose, 1962; Vogt and Dulbecco,
1962). This change in adhesion is not a simple change reflected in an overall decrease in adhesion to other cells and substrates, but specifically cell-cell adhesion. Indeed, it can be shown that some transformed cells stick to other cell types and substrates quite well; for example glass, plastic, or millipore filters (Ambrose, 1967). This change in adhesion may be the cause of metastatic behavior in tumors.

It has been suggested that the change in cell surface components leads to a greater number of negative charges and therefore greater repulsion between tumor cells. This is coupled with decreased adhesion (Abercrombie and Ambrose, 1962). Purdom, Ambrose, and Klein (1958) have shown that the potential at the cell surface of different sublines of MCIM mouse sarcoma increased as they progressed from the solid tumor to the ascites form.

While these studies are suggestive in the understanding of cell adhesion, intensive research has failed to reveal the nature of the mechanism(s) involved in adhesion and their relationship to tumor behavior and growth.

One of the most exciting areas in membrane research in recent years has involved the use of plant lectins or agglutinins to probe the structure of the cell surface. Lectins are proteins or glycoproteins with highly specific, differential saccharide-binding capabilities. Examples are Concanavalin A (Con A) which binds α-D-mannopyranosyl, α-D-glucopyranosyl and sterically related terminal sugar residues (Agrawal and Goldstein, 1967), and wheat germ agglutinin
(WGA) which binds N-acetyl-D-glucosamine and similar groups (Burger and Goldberg, 1967). The work of Burger and Goldberg, showing increased agglutinability by transformed cells as compared with normal cells using WGA, provided a stimulus for the use of lectins in the investigation of tumor cell surfaces. In general, it has been shown that transformed cells agglutinate with much lower concentrations of lectin than needed for the corresponding normal cell line (Sela, et al., 1971; Inbar and Sachs, 1969; Burger and Goldberg, 1967).

It was originally thought that the increased agglutinability demonstrated by transformed cells with Con A was due to increased numbers of, or the unmasking of "cryptic", Con-A-binding sites. A large amount of evidence now suggests these interpretations are not correct and, in fact, nearly the same amount of Con A will bind to normal cells as will bind transformed cells of a particular cell line (Arndt-Jovin and Berg, 1971; Cline and Livingston, 1971; Ozanne and Sambrook, 1971; Sela, et al., 1971).

The relative agglutinability of a cell has now been correlated with the topological distribution of Con A bound to the cell surface. In normal cells the Con A binding sites appear to be randomly dispersed while these sites are clustered on the transformed cell (Nicolson, 1971). The concentration of binding sites in these clusters appears to promote crosslinking between cells by the tetravalent Con A resulting in cell agglutination. Apparently, Con A binding sites have an increased freedom of movement in transformed cells allowing these sites to cluster under the influence of Con A (Nicolson, 1973;

Agglutination can be induced by other mechanisms as well. For example, there have been some studies in which poorly defined "factors" from the ascites fluid of tumor bearing mice have been used to bring about agglutination. In the case of a mouse teratoma, this factor was shown to be possibly a glycoprotein and to possess some degree of specificity for the cell line from which it was isolated (Oppenheimer and Humphreys, 1971). That these "factors" have any importance in cell-cell adhesion is unknown.

Specific metal ions such as manganese and lanthanum have been shown to increase cellular adhesion to substrates and to cause cell agglutination. But again, the mechanism of their action or its biological significance has yet to be demonstrated (Rabinovitch and De Stefano, 1973; Levinson, et al., 1972; Oppenheimer, personal communication).

There are several structural specializations which can occur normally between cells and hence are implicated in cell adhesion. These associations are called junctional complexes. They involve intercellular, plasma membrane, and cytoplasmic specializations. These structures are found universally throughout the animal kingdom. Hence, they appear to have an important role in cell-cell associations, though what this role is has not been completely elucidated (McNutt and Weinstein, 1973).

Junctional complexes are classified into four major types: 1) the zonula adherens, 2) the gap junction or nexus, 3) the desmosome, and
4) the tight junction or zonula occludens (see McNutt and Weinstein, 1973 for a complete review). There is some evidence to suggest these structures are involved in adhesion between cells. The use of hypertonic media (Brightman and Reese, 1969), or the incubation of cardiac muscle in Ca\(^{++}\)-free media (Muir, 1967), reveals tight junctions, gap junctions and/or desmosomes which remain after these treatments, keeping the cells attached. The gap junction has been implicated as the pathway for electrical coupling and the passage way of small molecules between cells. It has been suggested that some of the "social" behavior of cells may be mediated by communication through this type of junctional complex. (Bennett, 1973; Lowenstein, 1973).

In general, a relationship appears to exist between the number of junctional complexes and cell normalcy, tumor cells having fewer junctions or none at all (McNutt and Weinstein, 1973; Martinez-Palomo, 1971, 1970; McNutt, et al., 1971; Beneditti and Emmelot, 1968; Emmelot and Beneditti, 1967).

The fact that solid tumor cells have reduced numbers of junctions suggests a possible change in the plasma membrane which could in turn be responsible for differences in the adhesion and "social" behavior of tumor cells. One could depict the tumor cell surface as being modified in such a way that the components which mediate the formation of a particular type of junction are missing, in which case one would not expect junctional formation under any set of conditions unless these components were regenerated at the cell surface.
On the other hand, these components could be present within the cell but are not able to interact because of any of several reasons, such as greater repulsive forces between the cells, or cryptic burying of the components involved. Obviously, if the proper environmental conditions are met, the time required for formation of junctions would be shorter in the latter case since the components are present and do not need to be regenerated.

"In vitro" agglutination appeared to be a useful approach to probe the phenomenon of junctional complex formation in tumor cells; agglutination acting to bring cells in close contact in the presence of an exogenous component. In this study, the problem of membrane interactions in a mouse ascites tumor line, Sarcoma 180 (S-180), was examined over short and extended periods in culture using thin section and transmission electron microscopy (TEM) after agglutination by Con A, an ascites "factor", and manganese ion.
Methods and Materials

Maintenance and preparation of S-180.

The ascites form of S-180 was originally obtained by Dr. Steven Oppenheimer from Dr. Melvin Cohen of the Salk Institute and from Mr. Samuel M. Porley of the National Cancer Institute. It was maintained by intraperitoneal transfer every 4-6 days in Swiss-Webster, young, male mice. Tumor bearing mice, inoculated 4-6 days prior, were killed by cervical dislocation. The abdominal skin was opened and the ascites fluid was aspirated with a syringe bearing a 19 gauge needle or by cutting open the peritoneum and pouring the ascites fluid directly into a plastic petri dish. The cells were harvested and washed twice by low speed centrifugation in Hanks' Balanced Salt Solution (BSS) before use at an appropriate dilution.

Viability Test.

Viability was determined using the Nigrosin dye-exclusion test. The test was performed by mixing a sample of cells with an equal volume of a 1% solution of Nigrosin in Hanks' BSS. The cells were examined after five minutes under a light microscope using low power. Stained cells were considered dead. The percentage of dead cells was determined by counting at least 100 cells.

Electron Microscopy.

Fixation of cells was carried out in either a 1:1 dilution of Karnovsky's fixative (Karnovsky, 1965) with 0.1 M sodium cacodylate.
buffer pH 7.4 (Shea, 1971), (referred to as "modified Karnovsky's"); or with 2.5% glutaraldehyde in 0.06 M sodium cacodylate buffer at pH 7.2-7.4. Fixation was at room temperature for 2-4 hours and at times continued overnight at 4°C. The cells were washed three times in

0.1M cacodylate buffer pH 7.4 then postfixed in 2% osmium tetroxide in 0.1 M cacodylate buffer pH 7.4 for two hours. The cells were then washed twice in cacodylate buffer and dehydrated in a graded series of acetone. The cells were embedded in an Epon-Araldite mixture (Mollenhauer, 1964). Blocks were sectioned on a Porter-Blum MT2 Sorvall ultramicrotome and stained with 2% uranyl acetate in 50% acetone and Reynold's lead citrate (Reynolds, 1963). Sections were examined with a Zeiss EM 9S-2 electron microscope.

Lanthanium Oxide Staining.

Lanthanium staining was as described by Shea (1971). Only the Con A-treated cells were examined using this staining method. Cells were fixed in modified Karnovsky's fixative with 0.1% Alcian blue added to enhance staining of the glycocalyx. Fixation was carried out at room temperature for two hours.

Postfixation was carried out in 1% osmium tetroxide and 1% lanthanum nitrate in 0.1 M cacodylate buffer. The final pH of the fixative was adjusted by gradually adding sodium hydroxide (0.1 M) to the solution while on a magnetic stirrer. Sodium hydroxide was added just until a small amount of precipitate was formed (pH about 7.4). Fixation was carried out for two hours at room temperature. Dehydra-
tion was carried out in a graded acetone series, 1% lanthanum nitrate being added to the lower dilutions in the series. Embedding was as before.

Ruthenium Red Staining.

Ruthenium Red (RR) staining was performed as by Luft (1971). Only the manganese-treated cells and untreated cells were stained in order to enhance their glycocalyx.

A stock solution of RR was made by adding RR to distilled water (10mg/ml) and heating to a boil. This solution was cooled, placed in a centrifuge tube and spun in a clinical centrifuge for ten minutes at full speed. Supernatant was removed and used as a stock solution.

Staining was accomplished in the fixative by using a 1% solution of RR stock in 2.5% glutaraldehyde in 0.06 M sodium cacodylate buffer pH 7.3. The cells were fixed and stained for 4 hours at room temperature, then washed in 0.1 M cacodylate buffer followed by postfixation in 2% osmium tetroxide for 2 hours. Dehydration and embedding was as before. Sections were examined without any further staining.

Concanavalin A Agglutination.

Con A agglutination was performed according to the method of Oppenheimer and Odencrantz (1972). Equal numbers of washed cells were agglutinated with either a 500 or 1000 µg/ml solution of Con A in Hanks' BSS. The cells were suspended in 2 mls of the Con A solution or in Hanks' BSS, in screw-capped scintillation vials. The
vials were immediately placed on a rotary shaker having a 4-5/8" diameter of gyration and rotated at 65 rpm in a 37°C incubator for 15-60 minutes. At the end of this period, the cells were washed with several milliliters of Hanks' BSS and collected by slow centrifugation. The pellets were resuspended in a 1:1 mixture of Hanks' BSS containing 5 µg/ml of deoxyribonuclease (DNase), and Eagles Minimal Essential Media (MEM) without glutamine and buffered with 0.01 M Hepes, pH 7.4. The resuspended cells were then incubated in 25 mm plastic tissue culture dishes for one and four hours at 37°C. The cells were collected and fixed for electron microscopy with modified Karnovsky's fixative.

Factor Agglutination.

Ascites fluid from tumor bearing mice was collected as before. Cells were removed by centrifugation and the supernate was used as a crude "factor" preparation.

Washed cells were agglutinated in a 10% or 37% mixture of the crude factor preparation diluted with Hanks' BSS with DNase (5µg/ml) added and using a rotary shaker as with the Con A agglutination (Oppenheimer and Humphreys, 1971). After 15-60 minutes, the cells were washed with Hanks' BSS with DNase and pelleted by centrifugation or by settling. The pellets were resuspended in a 2:3 mixture of Hanks' BSS with DNase, and Eagles MEM without glutamine, incubated in 25 mm plastic tissue culture dishes for one or four hours, and fixed with modified Karnovsky's.
Manganese ion agglutination.

Washed cells were suspended in a large volume of Hanks' BSS. 4.5 mls of this suspension were placed in each of five 15 ml conical centrifuge tubes. To two of these tubes was added 0.5 ml of Hanks' BSS; these were control tubes. 0.5 ml of 0.1 M MnCl₂ in Hanks' BSS was added to the remaining three tubes and the tubes were immediately shaken; final Mn⁴⁺ concentration was 20 mM. Clumping occurred immediately. An equal volume of 2.5% glutaraldehyde fixative was added immediately after addition of Mn⁴⁺ to one Mn⁴⁺-treated and one of the control tubes. The cells were separated from the supernatant and re-suspended in fresh fixative. The other tubes were fixed in a similar manner at the 3 and 30 minutes fixations.

The cells cultured for four hours in Mn⁴⁺ were prepared somewhat differently. Washed cells were added to 50 mm plastic tissue culture dishes containing 7 mls of millipore filtered Eagles MEM with 20% fetal calf serum and with or without 20 mM MnCl₂. Cells were pipetted from the dishes at the end of four hours, spun, and then fixed with 2.5% glutaraldehyde fixative.
Results

Fine structure of S-180.

The diameter of S-180 cells is generally 15-20\(\mu\) but a variable percentage (about 5 - 15\%) of the population is found to be 25-30\(\mu\) (Plates 1A & B).

Plate 2 shows S-180's general appearance. Structurally the most prominent features are a large, highly fissured, multilobate nucleus (Plates 1A & 2) and a very active cell surface with numerous villus projections (Plate 2).

The nucleus characteristically has many nucleoli. Most are associated with the nuclear membrane at the distal ends of intranuclear caniculi (Plates 3A & B). Heterochromatic regions are normally perinuclear and closely associated with the nuclear membrane. Many fissures or deep invaginations of cytoplasm course through the nucleus (Plates 2 & 3). In addition, one occasionally sees several nuclear inclusions. In Plate 3 for instance, one sees clusters of granules, possibly viral cores, and what appears to be membrane bound vesicles.

The nucleoli are large. They distinctively have a large, light-staining pars amorpha, and extensive, dark-staining, granular pars granulosa. The pars fibrosa is small but characteristically is found between the pars granulosa and amorpha (Plate 3A) (Goessens and Lepoint, 1974). A region of chromatin (with caniculi) can be seen associated with the pars granulosa (Plate 3A).

The nuclear envelop is similar to that of normal cells. The
The cytoplasm tends to be dense with large numbers of free ribosomes characteristic of tumor cells, and large numbers of mitochondria. The mitochondria are quite pleomorphic. They are most commonly tubular with a moderately dense and granular matrix, with typical cristae, though they may be club shaped or swollen in appearance (Plate 4). Less commonly, the mitochondria may have a vesiculated matrix having a lighter appearance but with typical cristae (Plate 2), and occasionally mitochondria may have a dense granular matrix and vesiculated cristae (Plates 5D & 7B). This pleomorphism is likely due to the energy state of the cell. The first and maybe the second types reflecting the "orthodox" or resting mitochondrion and the third type, with dense matrix and vesiculated cristae, reflecting the "condensed" or rapidly respiring mitochondrion. (Hackenbrock and Rehn, 1970). The mitochondria are generally distributed uniformly throughout the cytoplasm though it is not uncommon for them to be found somewhat more peripherally.

The centriole and Golgi complexes are found in the centrosphere (Plate 4). One to three Golgi complexes are commonly seen in a section, always associated with a large number of vesicles and vacuoles suggesting activity (Plate 5A). Often seen in the region of the Golgi are various darkly stained bodies (Plate 5A).

The amount of endoplasmic reticulum (ER) is somewhat variable, but usually is not extensive in these cells. Some smooth ER is seen,
but rough ER predominates. The ER is usually filled with a moderately staining material (Plate 5A). Annulate lamellae are seen but rarely.

Various types of lysosomal vacuoles are seen in these cells. Plate 5B shows what appears to be the fusion of residual bodies with a digestive vacuole. Autophagic vacuoles can likewise be seen (Plate 12D). In the presence of high concentrations of exogenous proteins (in this case ascitic fluid) numerous endocytic vesicles are seen (Plates 12B & C). These probably fuse with numerous lysosomal vesicles in this region, forming the numerous residual bodies which can be recognized by the halo between the contents of the residual body and the delimiting membrane (Novikoff, 1973). It was not uncommon to see large myelinoid-type residual bodies in these cells. These myelinoid figures appear to be readily exocytosed by the cells (Plate 12D).

Many large, spherical, lightly-stained bodies can be seen in these cells. They do not appear to have a delimiting membrane, and appear to be readily exocytosed (Plates 5C & D). These are probably lipid droplets as they appear similar to those described in other tumor cells (Chambers and Weiser, 1964; Bergstrand and Ringertz, 1960; Epstein, 1957).

In addition, there were other large spherical bodies with greater electron density. Some stained irregularly (Plate 5E) and others appeared to have a halo of densely stained particles surrounding them (Plate 5F). The latter type was frequently associated with myelin-like figures. No delimiting membrane could be seen around these bodies,
but the membrane may have been obscured by the density of the stain.
It was not uncommon to see the first type in clusters of two and
three. Apparently they coalesce together in the cytoplasm.

Rarely, one sees aggregates which appear to be viral capsids in
the cytoplasm (Plate 6A), and individual capsids in the ER. The cap-
sids are about 50 nm in diameter with isosahedral symmetry. Their
size and symmetry suggests they belong to the Papovavirus group (Davis,
et al., 1973).

The cell surface is quite variable. Most commonly the surface is
covered with numerous microvilli of varying size, shape and number.
These may appear thin and finger-like or thick and club shaped (Plate
6B). The distribution of microvilli on the cell is not always uniform.
Membrane loops are common, and endocytosis can be seen.

Concanavalin A agglutination.

After removal from shaker treatment with 500 or 1000 μg/ml of
Con A, cell aggregates were seen. These ranged from a few millimeters
across, down to two and three cell clumps in addition to unagglutin-
ated, single cells (Plates 1C & D). Within approximately four hours,
the large clumps had broken down into smaller aggregates of several
cells. Longer incubation (20 hours) resulted in low viability of the
cells. The decrease in aggregate size is probably not affected by
the dead or dying cells, as they were tightly associated with viable
cells (Plate 6C).

The agglutinated cells were examined ultrastructurally at one,
four and twenty hours after treatment. In general, the viability of the one and four material, as determined by dye exclusion, was high, but consistently too low in the twenty hour material to be meaningful. This loss in viability could have been due to the cytotoxicity of Con A, especially since it was used at possibly toxic levels in order to maximize agglutination for electron microscopy. Also, growth conditions may not have been optimal, as later work suggests these cells require a rich medium initially after transfer to "in vitro" cultures.

Three types of cell-cell interactions were seen between the Con A agglutinated cells. The interactions after four hours were similar to those seen after one hour. The interaction could be classified into: 1) Those mediated by microvilli-microvilli and microvilli-cryptic cell surface interactions; 2) intermediate associations usually of the focal type, in which the membranes are separated by about 100-250 Å and between which a lightly stained material is usually seen; and 3) close associations in which the membrane of adjacent cells are separated by less than 100 Å.

Plate 7A demonstrates the first type of interaction and compares Con A treated and untreated cell surfaces (Plate 7B). The Con A treated cells seem to have few projecting villi. Many villi which can be seen in cross section apparently have been agglutinated by the Con A to the parent cell surface and to the villi and cryptic surface of the adjacent cells (Plate 7A). Membrane loops can be seen on both Con A and untreated cells, but are accented on the Con A cells by the lack of projecting villi. The obvious projection of microvilli on the
control cells with few villi-villi or villi-cryptic surface interactions is quite apparent; in most instances no associations are seen between untreated cells (Plates 1A & B, 2).

How stable the microvilli mediated attachments are is unknown, but similar associations are seen in cells cultured four hours (Plate 8A). Plate 8B shows how extensive these associations can be, some villi actually seem to be pushing into the adjacent cell producing a very tight fit between surfaces. This situation was not seen very often, and only in the four hour preparations, though it may have been present at other times and not observed. The villi normally appear to be lying flat, apparently agglutinated to the surface of one or both adjacent cells, and many times lying between cells that are possibly being held together by areas of closer association (Plates 8A & C).

The intermediate associations are very common and characterized by the adjacent membranes being separated by greater than 100 Å. They usually have a lightly stained material in the intercellular space, and do not appear to be extensive in area (a focal type association) (Plate 9A). The adjacent membranes are approximately parallel. This type of association is most commonly seen in areas which hold the cells together in clumps (Plate 8A). Plates 9B and C show that multiple points of interaction can occur between adjacent cells. The common situation consists of large regions in which focal intermediate associations appear to be holding the cells together. In the intervals between these associations, the membrane appears irregular, commonly with villi projecting into the open spaces. The cell surface is
frequently characterized by amorphous debris possibly derived from the media.

There is no type of cytoplasmic specialization found in the intermediate type of association comparable to the classical zonula adherens type of junctional complex. But these may represent a modification of this junction, and it may form as a result of the cells being brought into close contact by the lectin during agglutination. It is not known if Con A is found between the cells in areas of these associations.

The last type of association seen, the close association (Plates 10 A-D), is characterized by the membranes of adjacent cells being separated by less than 100 Å. In Plate 10A, the gap between adjacent membranes can be estimated to be about 20 Å assuming the membrane thickness is about 70 Å. These may appear as either focal (Plates 10A & B) or zonula (Plates 10C & D) types of interactions. No cytoplasmic specialization is seen, and the outer leaflets of the opposing membranes do not fuse at any point. This type of association is fairly common; especially the focal type. Its appearance is very characteristic of the gap junction. Attempts to use the characteristic lanthanum oxide staining property of gap junctions failed to answer this question. It was felt this resulted from the failure of the stain to remain in the loose cell aggregates during washing and dehydration.

In no case were tight junctions observed in which the outer leaflets of the adjacent membranes would have fused, nor were desmosomes with their characteristic cytoplasmic tonofilaments seen in any of these
materials.

Factor agglutination.

Two dilutions of "factor" (Oppenheimer and Humphreys, 1971) were used: 10% and 37% in Eagles MEM. The results were dependent upon the concentration used. At the higher concentration after one hour (Plate 11) the cells actively phagocytosed a large amount of flocculent material from the medium. This material also appeared to be adhering to the cell surface (Plate 12A).

Using high factor concentrations, the cells aggregated to form large clumps, but there appeared to be no type of close cell associations. Moreover, it appeared that agglutination in this case was due to large amounts of material attached to the cell surface, which crosslinked the cells together (Plates 12B & C). However, it should be noted that the factor used in these experiments was relatively inactive, requiring high concentrations for cell binding activity.

The cells appear very vesiculated as a result of pinocytosis. Many lysosomal vesicles were obvious and the rough ER had become branched and slightly swollen with a moderately stained material (Plates 12B & C). The number of villi had decreased, possibly in response to the large amount of membrane involved in pinocytosis.

At the lower concentration of "factor" a much different situation was seen (Plate 13). The cells appeared normal with large numbers of villi and reduced amounts of rough ER.

In this case intermediate type associations similar to those
observed with Con A were seen between the cells (Plates 14A-C). A lightly stained material was obvious in some patchy areas (Plate 14D), and in many cases villi were absent from the intercellular spaces. No gap or tight junctions were apparent in these preparations.

The material in these associations could be the component(s) of the ascites which was responsible for agglutination and may be cross-linking the cells together. Shepard and Oppenheimer (unpublished observation) have shown some degree of specificity of the "factor" for these cells which may involve terminal galactose resides on the factor binding to cell surface receptor sites (Oppenheimer, 1975; Oppenheimer, personal communication).

With time in culture, the cell aggregates tend to breakdown. After four hours the number of associations decreased. Most cells appeared singly and looked like untreated cells with numerous villi and lobated surfaces. It could be that the components responsible for agglutination are removed from the medium and cell surface with time, possibly by pinocytosis and phagocytosis. Factor may have to be replenished to maintain the effect.

Plate 12D shows two cells, one of which is apparently exocytosing a large myelin figure. The complementarity of the cells' surfaces suggests they are being forced apart by the myelin figure. This could suggest that the strength of the association is not very great and can be broken by any projection from the cell surface. Also, since the cells have remained nearly complementary, cytoplasmic stabilization of surface shape may be an important aspect of adhesion.
Manganese ion agglutination.

Manganese ion produced a very rapid agglutination of S-180 into large clumps. The initial events in agglutination were examined by immediate fixation followed by additional fixation at 3 and 30 minutes. Light micrographs show clumping of the cells after fixation and infiltration with plastic (Plates 15 A-C).

Ultrastructurally no differences were seen in the association between the initial (Plates 16 A & B), and 3 (Plates 16 C & D) and 30 minute (Plate 17A) preparations. There did not appear to be any differences in the Ruthenium red staining properties of areas of interaction and noninteraction (Plate 17B). Plates 17 C & D are high magnification pictures of the interactions occurring between adjacent cell surfaces. The distance separating the membranes is very small, less than 60 Å and in many cases no distinct separation can be seen. While this suggests some type of primary interaction between the membrane surfaces, ionic bridging between components on the surface of the villi seems likely. These interactions appear static since no change is seen in them between the initial, 3 minute, and 30 minute cells. The strength of this interaction is not known, but probably requires the continuing presence of Mn^{++}.

After four hours in 20 mM Mn^{++} in serum containing medium, the cell aggregates were dissociated (Plate 15D). Most cells seem swollen (Plate 18A) and large numbers of vacuoles and invaginations are apparent. Plate 19A shows how deep the invaginations of the cells can be. The surfaces of the cells were quite variable. The cells appeared
completely smooth (Plate 18B), lobated (not shown), or with numerous villi (Plate 18C). The cytoplasm is denser around the nucleus in many cells and the mitochondria are condensed, deeply stained, and with swollen cristae.

The breakdown of the aggregates may, in part, be due to the loss of villi from the cell surface, but the morphology of these cells is quite unusual and may reflect a pathological situation as a result of the relatively high level of manganese ion in the medium.

The associations that occur with Mn\(^{++}\), while similar in some ways to those seen after Con A agglutination (Plate 7A), do not appear to lead to flattening of the cells' surfaces nor to more extensive interactions between cells. Perhaps the toxicity of the Mn\(^{++}\) produces a situation unsatisfactory for further involvement by the cell surface.

The relative staining intensity of the microfilaments within the microvilli is greater after Mn\(^{++}\) treatment (Plate 17D). This suggests Mn\(^{++}\) may be interacting with microfilament components.

It is difficult to quantify the occurrence of each type of association in this study. However, using micrographs of situations which were judged to be typical, Table 1 was constructed to indicate the relative occurrence of each general type of association after treatment with each agglutinin. Use of the terms 'close', 'intermediate', and 'microvillous' does not imply that the agglutinins act in similar ways or that the fine structure of each is exactly identical.
Each association may occur alone or in combination with the other types. Some regions appear to have microvillous associations which are mediated by the other types of associations as for example in Plate 14A where two microvilli appear to be associated with the adjacent cell by an intermediate association. In the case of manganese, villi interact by associations which appear closer than the close associations seen with Con A.
Discussion

The fine structure of S-180 has been reported previously by Molnar and Bekesi (1972). This work expands on their observations and reports some differences.

S-180 shows the general structural characteristics of most tumor cells: The large, irregularly shaped, heterochromatic nucleus; the large number of surface villi; the large number of free ribosomes, and decreased amounts of ER (Molnar and Bekesi, 1972; Chambers and Weiser, 1964; Oberling and Bernhard, 1961; Bergstrand and Ringertz, 1960; Bernhard, 1958; Epstein, 1957; Dalton and Felix, 1956).

In addition, it shares some structural characteristics of other ascites tumors. Large lipid droplets were described in Sarcoma 1, Sarcoma 37 and MCLM tumors (Chambers and Weiser, 1964; Bergstrand and Ringertz, 1960; Epstein, 1957. These do not appear to be bound by membrane. They tend to show a light, uniform staining, and may also show a granular darkly stained periphery.

Similar lipid bodies were seen in S-180 (Plates 5C & D). As seen in Plate 5D these lipid bodies could be readily exocytosed. In addition to the bodies described above, there were other large, darkly stained bodies (Plates 5E & F). These did not appear to be membrane bound, but the membrane could have been obscured by the density of the stain. Whether these represent pleomorphic forms of the former type of lipid body, or a different type of vacuole was not determined. The large size and apparent lack of a delimiting membrane
might suggest they are lipid bodies. They may also represent some type of residual body. The association of myelin figures with one type (Plate 5F) may be suggestive of this, but residual bodies are bound by a tripartate membrane (Novikoff, 1973). A cytochemical analysis will be needed to characterize these bodies.

Viral capsids occasionally occurred in the cytoplasm of these cells. However, virus particles were not seen budding off the cell membrane and this suggests they are not an enveloped virus. Their size, symmetry and probable lack of envelop suggests they belong to the papovavirus group. Members of this group are commonly seen in a small percentage of cultured mouse cells and some tumor cell populations (Davis, et al., 1973; Sanders, 1973) and similar virus particles have been seen previously in S-180 (Molnar and Bekesi, 1972). C-type viruses were not seen.

The large number of microvilli on the cell surface is a common feature to ascites and also some solid tumors (Chambers and Weiser, 1964; Mercer and Easty, 1961; Bergstrand and Ringertz, 1960; Wessel and Bernhard, 1957). S-180 shows numerous villi of various lengths, number and shape. They could be irregularly, or uniformly distributed on the cell surface. While these appeared to be important in the cell-cell interactions described, regions of extensive interaction were not seen between untreated cells.

The S-180 cells used in this study characteristically have few microfilaments. On the other hand, bundles of fine filaments have been described in Sarcoma 1, Sarcoma 37, MC1M; and also in fibro-
blasts and as the tonofilaments of epithelial cells (Molnar and Bekesi, 1972; Chambers and Weiser, 1964; Journey and Amos, 1962; Ross and Benditt, 1961; Bergstrand and Ringertz, 1960; Epstein, 1957; Selby, 1955). The lack of cytoplasmic filaments in this study is in contrast to that of Molnar and Bekesi who observed them in the S-180 they used. This difference may relate to the particular line of S-180 used.

The ultrastructural features of the agglutinated S-180 cells differ depending on the agglutinin and this in turn on the action of the agglutinin on the cell surface.

In the Con A system, the cell-cell interactions were of three types: 1) Microvilli-mediated, 2) intermediate, and 3) close associations. These were seen after both one and four hours. It should be noted that aggregates, formed initially after treatment with Con A, were relatively large when examined by eye and these dissociated into smaller aggregates with further incubation. This would suggest that many of the interactions are weak, reversible, or subject to degradation possibly of the Con A or its receptor. In addition, further dissociations occurred as a result of the preparative steps for electron microscopy. Glutaraldehyde fixation did not stabilize the interactions between the cells. The smaller aggregates may represent only the stronger or more extensive associations that form and which are able to survive the technique.

Several workers have shown Con A receptor mobility (clustering) to be related to agglutination (Rutishauser and Sachs, 1974; Rosen-
blith, et al., 1973; Nicolson, 1972; Nicolson, 1971). The types of associations seen here may represent clustering of Con A receptors resulting in crosslinking of the cells, or some type of Con A initiated interaction between the cells. Most of the work on Con A receptor mobility and clustering involved examination of free surfaces of cells and not the interaction between cells. There does not appear to be any work examining the interactions between tumor cells. Nicolson (1972) has examined, by thin section, the agglutination of 3T3 cells after proteolysis, while paying heed to mobility of receptors on the free surface of these cells. Proteolysis stimulates agglutination between normal cells which agglutinate poorly at high lectin concentration. His work shows a greater concentration of ferritin conjugated Con A between the protease-treated 3T3 cells at the points of contact than on the free surfaces. Moreover, the ferritin Con A appeared in the region of contact to be clustered into patches crosslinking the cells. The agglutinated cell surfaces were separated by 200 - 400 Å. Cell aggregation could be reversed in this case by α-methyl-mannoside when added shortly after agglutination. Lee and Feldman (1964) reported a similar gap between human red cells agglutinated by ferritin conjugated group A antibody.

The first interaction, the microvilli-mediated association, could represent initial contacts between cells which then develop into the stronger types of associations. They may also be reversible and, therefore, can dissociate. There has been some work showing that the microvillus surface is chemically different from the inter-
villal membrane (Weiss and Subjeck, 1974). These differences may be reflected here but they are difficult to assess. The suggestion of villus interactions leading to the more extensive types of associations is difficult to reconcile with what is observed here. The villus interactions are seen in both the one and four hour material. It would be expected that many of these would have progressed into the other types of associations by four hours. Intermediate steps leading from villus interaction to the extensive close associations are not obvious in this material. The villi interact with cryptic cell surfaces and other villus tips by both intermediate and close associations. This may indicate that the interactions of the villous and cryptic cell surfaces are comparable to the interactions that occur in regions where membrane flattening has occurred. There did not appear to be any cytoplasmic or intercellular specializations occurring at the site of interaction. An increased staining density was observed at the site of microvilli interaction in Sarcoma 1, "in vivo" (Chamber and Weiser, 1964). One would expect the villi to interact extensively after treatment because of the large number of villi on S-180, and also because they would be involved in the initial contacts between the cells. It is difficult to discern whether this first type of association is independent in the agglutination process or plays a transitory role in the formation of the other types of associations.

The intermediate associations seen in this work resemble the interactions described by Nicolson between 3T3 cells. The associations
in this study are characterized by patches of a lightly stained, membrane associated material crosslinking the S-180 cells. This may very well be aggregates of Con A (Agrawal and Goldstein, 1968) agglutinating the cells together. The patchy nature of the interaction does suggest clustering of Con A receptors as a requirement for agglutination in this situation as in the 3T3 study.

Another interpretation of the intermediate association is that it represents some type of junctional complex which develops secondarily as a result of primary Con A agglutination. While these associations do appear similar in some ways to the zonula adherens junctions of normal cells, no cytoplasmic specialization is seen in S-180. The junctions would normally have actin-like fibers associated in a mat within the cortical cytoplasm under the plasma membrane. The absence of fibers and the general patchiness of juxtapositioning does suggest this association is not a zonula adherens, but represents the crosslinking of the cells by Con A. Atypical junctions cannot be ruled out by this work (McNutt and Weinstein, 1973; Pinto-daSilva and Gilula, 1972).

Close associations were not reported by Nicolson between agglutinated 3T3 cells. As seen in this study, these may represent regions of Con A crosslinking dissimilar to those described by Nicolson, but it is not likely that the close associations, especially the extensive ones, (Plate 10C), would represent areas held by Con A. Con A is a polymer in solution. The size of the basic protomer as determined by Edelman et al., (1972) is 42X40X39 Å. Above pH 7, Con A is
found in the form of tetrameric and larger polymeric aggregates of the protomer (McKenzie, et al., 1972; Agrawal and Goldstein, 1968; Kalb and Lustig, 1968). The gap in the close association is about 20 Å. Thus, the dimensions of the aggregated Con A agglutinating the cells (Con A tetramers and larger polymers) would be greater than that of the gap. This would argue against the direct involvement of Con A in the close association.

Rutishauser and Sachs (1974) reported some work using Con A derivatived nylon fibers which shows the cell-cell interactions between two lymphoma tumor cells after Con A agglutination is not reversible by α-methyl-mannoside. It was suggested by the authors that Con A acts to bring the cells into close contact at which time cellular components can interact to produce a mannoside-insensitive binding. These interactions were not examined ultrastructurally by the authors. The work here is suggestive of a comparable situation, though reversibility was not examined in this study. The extensive regions of close contact could represent regions in which strong cellular interactions have occurred; these associations being induced in some way by agglutination, but with cell surface components interacting.

The appearance and dimensions of the close association very nearly resembles the gap junction or nexus. An attempt to use lanthanum oxide staining of this association failed to produce any staining. It is possible the aggregates were too loosely associated to allow the stain to remain between the cells (Revel and Karnovsky, 1967).
question could be approached by freeze-cleavage techniques which should demonstrate the characteristic hexagonal array of membrane components in the gap junction.

Whatever the nature of the component(s) involved in the close association, they are present or inserted very quickly after treatment with Con A. This can be implied by the fact that close associations are seen after just one hour. If these components were being synthesized "de novo" one would expect the close association to develop later, possibly after four hours. Why these components do not interact "in vivo" is not known. Con A may allow the uncovering of cryptic sites, or the overcoming of physical restrictions.

A relationship has been demonstrated between lectin binding and cell behavior. Burger and Noonan (1970) used Con A to inhibit transformed cell growth, and Friberg and his group (1971) were able to show inhibition of migration. This type of "social" behavior is thought to be a form of cell-cell communication mediated by gap junctions (Bennett, 1973; Lowenstein, 1973). It might be speculated that what was seen by these groups represents a Con A induced coupling of cells through the gap junction-like close associations seen here, allowing for cell-cell communication.

Agglutination by the crude ascites "factor" at high concentration resulted in large amounts of amorphous material adhering to the cell surface resulting in clumping. The interaction of this material with the cell surface is probably nonspecific, binding in many areas of the membrane inducing pinocytosis of the material. The flocculent
material probably includes, in addition to "factor", other ascites proteins such as fibrin. The method of obtaining "factor" may in fact activate some of the clotting proteins resulting in their deposition on the cell surface. One would not expect to see large amounts of proteins adhering to the cell surface "in vivo". In fact, this material is not seen on cells fixed in ascites fluid immediately after removal from the peritoneum.

At lower concentration, the large amounts of flocculent material were not present and a situation develops similar to the intermediate association seen with Con A. These interactions appear to be mediated by small areas of cell surface in which a lightly stained material is obvious and, presumably, is the "factor" from the ascites. The patchy nature of these interactions and the lack of surface interactions in the regions without this material could suggest the involvement of membrane components or receptors. These may be associated as patches in a mosaic on the cell surface. Otherwise, the factor may promote the clustering of surface receptors in a manner similar to Con A and other lectins. The factor isolated from a teratoma cell line by Oppenheimer and Humphreys (1971) appears to be a glycoprotein, possibly synthesized by the mouse, the tumor cell, or both, and appears to possess terminal galactose residues which bind to the cell surface (Oppenheimer, 1975). Prefixed cells with 4% formaldehyde failed to inhibit agglutination suggesting that with teratoma "factor", active movement of membrane components is not necessary for agglutination to occur (Oppenheimer and Humphreys, 1971).
The S-180 ascites factor used in this study appears to possess different charge and size properties from the factor isolated from teratoma. There also appears to be a difference between the single cell form and the embryoid body form of the teratoma "factor" (Oppenheimer and Humphreys, 1971; Oppenheimer and Connally, unpublished results; Haskett and Oppenheimer, unpublished results). Whether the teratoma and S-180 "factors" are mechanistically similar is not known. If they are, the clustering of receptors by "factor" is probably not important in agglutination.

The patchy nature of the interactions at low "factor" concentration can be explained by the presence of "factor" aggregates cross-linking the cells, these aggregates being observed as the lightly stained, amorphous material between the cells. Perhaps these aggregates are large enough to interact with several receptors without the need for clustering.

Recent work (Oppenheimer and Connally, unpublished observation) has shown that the ascites "factors" are inactivated by phosphate which was present in the Hanks' BSS. This may explain the relative inactivity of the material used in this study. Preparations have been partially purified in phosphate-free media which are stable and highly active at 0.5% V/V. Thus the results of this study can be considered only preliminary at this time. Until the active component of this factor is isolated and purified, it will be difficult to select a likely mechanism to explain these observations.

The last system studied involves the use of the divalent cation
manganese. It has been reported that this ion stimulated the adhesion of Sarcoma 1 to serum coated glass, Mn$^{++}$ having high specificity in this system (Rabinovitch and DeStefano, 1973). Its mode of action is not understood, but it was suggested that Mn$^{++}$ increases deformability of the cell. More cellular projections were seen; veils, villi, and cell flattening. It must be noted these cellular changes were seen at 1 mM Mn$^{++}$ and in fact 20 mM Mn$^{++}$ as used here was less efficient in stimulating adhesion to treated glass. It was suggested that microfilaments and microtubules may be involved since adhesion was sensitive to cytochalasin B and colchicine. What was seen in this study did not involve flattening of the cells nor formation of veils. In fact it appeared as if a stabilization occurred in the microvilli. Cell-cell interaction was not dependent on serum and interactions appeared to be mediated by the microvilli. Microfilament staining appeared to be enhanced somewhat by Mn$^{++}$. This was obvious at high magnification (Plate 17D). Interactions were very close and in some cases no gap was discernible between the cell surfaces. Ruthenium red shows these interactions involved the carbohydrate-rich glycocalyx of the cells and may reflect some type of ionic bridging or perturbation of the normal ionic environment in and around the plasma membrane. It has been reported that lanthanum ion can induce agglutination of Ehrlich ascites tumor and also results in K$^+$ efflux from the cell (Levinson, et al., 1972). Lanthanum can also be used to inhibit clustering of cell surface receptors (Sachs, et al., 1974). Zinc ion has been used to "fix" or stabilize mem-
branes (Brunette and Till, 1971). Oppenheimer (personal communication) has shown Zn\(^{++}\) and Mn\(^{++}\) to have a high specificity in the S-180 agglutination system, and perhaps the ions are interacting with surface glyco-protein and lipids producing a denaturation or ionic bridging between adjacent cells. Mn\(^{++}\) agglutination may be a phenomenon without an "in vivo" relevance though its mode of action may be of interest in determining the nature of the cell surface.

Junctional complexes are found between many types of tumor cells. It is difficult to assess whether or not these are functional. As mentioned previously, a positive correlation can be made between greater numbers of junctions being found between normal tissues than between tumor tissues. Also, the number of junctions correlates with the form of the tumor: for example, solid versus ascites. All types of classical junctions can be found in neoplasms, but tight junctions and gap junctions are usually not as common in comparison with normal tissues, (Trembley and Babai, 1972; Clarks, 1970; Martinez-Palomo, et al., 1969; McNutt and Weinstein, 1969).

The existence of junctions in solid forms of S-180 has been reported (Sheridan, 1970) and these appear to be able to transfer low molecular weight dyes and are electrically coupled. While the importance of the transfer of small molecules and electrical coupling has not been demonstrated, it has been suggested that these play a role in the "social" behavior of cells (Bennett, 1973; Lowenstein, 1973). Sheridan's work also demonstrated that the junctions involved in this coupling, usually considered to be the gap junction, were
able to form between S-180 cells. Heaysman and Pegrum (1973) examined the interactions of S-180 (an adherent explant of a solid form) with chick heart fibroblasts. Their results indicate their line of S-180 does not form specialized contacts with chick heart fibroblasts. One might ask whether such interactions would be expected to occur between mouse and chick cells. The S-180's used in these two studies were different from that used here, and one must question whether these observations are applicable in this study.

The work presented here suggests the possibility that Con A is able to induce cell-cell associations which appear similar to gap junctions. While these were not shown to be normal gap junctions with normal function, these associations could, nonetheless explain the return of "social" behavior of transformed cells after treatment with lectin. "Factor" produced interactions very similar to those reported by Nicolson between 3T3 cells with Con A after proteolysis, and similar to the intermediate association seen here with Con A. This suggests the possibility that "factor" works in a way similar to Con A; aggregates of factor crosslinking the cells. The mechanism of action of Mn\(^{++}\) is difficult to assess in this work. It produces very tight associations via the interaction of microvilli. The significance of this in physiological terms is not clear.
Bibliography


Inbar, M., Ben-Bassat, H. and Sachs, L. (1972) Inhibition of ascites tumor development by concanavalin A. Int. J. Cancer. 9:143.


Table 1. Relative Occurrence of Each Association After Agglutination.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Relative Percentage of Occurrence of Each Association</th>
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<tr>
<td></td>
<td>Microvillous</td>
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<tr>
<td>Con A</td>
<td></td>
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<tr>
<td>1 hour</td>
<td>60-80</td>
</tr>
<tr>
<td>4 hour</td>
<td>60-80</td>
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<tr>
<td>&quot;Factor&quot;</td>
<td></td>
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<tr>
<td>1 hour</td>
<td>10-25 b)</td>
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<tr>
<td>4 hours</td>
<td>10-25 b)</td>
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<tr>
<td>Manganese ion</td>
<td></td>
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<tr>
<td>0, 3, 30 minutes</td>
<td>100</td>
</tr>
<tr>
<td>4 hours</td>
<td>0</td>
</tr>
<tr>
<td>Untreated</td>
<td>0-10</td>
</tr>
</tbody>
</table>

a) The "Relative Percentage of Occurrence of Each Association" represents a rough estimate which encompasses both the number of times each association was seen and the relative area occupied by that association.

b) Microvilli appeared to be held by 'intermediate' type association.
Plates
Plate 1

A. Light micrographs of untreated S-180 after washing with Hanks' BSS and fixation with 2.5% glutaraldehyde and OsO4. Note the large nucleus (N) and dark staining body (\^{*}) in the cytoplasm. 4688X. Phase.

B. Similar to A. Note the lack of cellular aggregates. Cells appear to be mainly single cells with possibly a few pairs (\(-\rightarrow\)). 710X. Brightfield.

C. Light micrograph of Con A agglutinated S-180, unfixed. Note the large size of the aggregate. 3000X. Brightfield.

D. Similar to C, but after 4 hours in culture, unfixed. Note that the size of the aggregate has decreased in size compared to Plate 1C. 1875X. Brightfield.
Example of an untreated S-180 cell after removal from peritoneum and washing with Hanks' BSS. Fixation was with 2.5% glutaraldehyde and OsO₄. Note the irregular shape of the highly fissured nucleus. Also the apparent emperipolesis (✓). 7560X. Stained with lead citrate and uranyl acetate.
Plate 3

A. Cells were treated with Con A for 1 hour and fixed in modified Karnovsky's fixative and OsO$_4$. Micrographs shows a nuclear caniculus (c) associated with the nucleolus. Also note nuclear vesicles (→) and unusual aggregate of what could be viral cores (←). Pars amorpha, pa; Pars granulosa, pg; Pars fibrosa, pf; chromatin, Ch; nuclear pore (→). 24,600X. Stained with lead citrate and uranyl acetate.

B. Cells treated as in A. Note two large nucleoli, nuclear caniculi (c), and cytoplasmic channel (√) associated with one of the nucleoli. 17,100X. Stained with lead citrate and uranyl acetate.
Cells treated with "factor" for one hour and then fixed in modified Karnovsky's and OsO₄. Plate shows centrosphere region, note Golgi apparatus (G), and numerous vesicles and vacuoles in the cytoplasm. The centriole (Ct) is prominent and so is the large nucleolus (n). Nucleus (N), mitochondrion (M). 42,600X. Stained with lead citrate and uranyl acetate.
Plate 5

A. Cells treated with Con A for one hour and fixed in modified Karnovsky's and Os04. Note the Golgi apparatus (G) with large number of associated vesicles and vacuoles (v), the dense staining body (→), endoplasmic reticulum (E), and autophagic vacuole (av). 25,400X. Stained with lead citrate and uranyl acetate.

B. Cell treated as in A. Plate shows the condensation of several residual bodies with an autophagic vacuole. 49,000X. Stained with lead citrate and uranyl acetate.

C & D. Untreated cells fixed in modified Karnovsky's and Os04. Shows a large lipid droplet in the cytoplasm and the apparent exocytosis of a lipid droplet. Mitochondrion (M). C. 8560X. D. 8320X. Stained with lead citrate and uranyl acetate.

E. Cell treated as in A. Note the large dark and irregularly stained bodies, and the myelin figure (→). 13650X. Stained with lead citrate and uranyl acetate.

F. Cell treated with factor for one hour and fixed with modified Karnovsky's and Os04. Example of another type of dark staining body. 40,800X. Stained with lead citrate and uranyl acetate.
Plate 6

A. Cells treated with "factor" for one hour and fixed in modified Karnovsky's and Os04. High magnification of virus particles in cytoplasm. 114,660X. Stained with lead citrate and uranyl acetate.

B. Untreated S-180 cells after washing with Hanks' BSS and fixation with 2.5% glutaraldehyde and Os04. Note the variety of surface projections shown by the two cells and the lack of interaction between the cells. Apparent emperipolesis (√). 15,700X. Stained with lead citrate and uranyl acetate.

C. Cells treated with Con A for one hour then fixed with modified Karnovsky's and Os04. Shows the interactions between normal (Y) and an apparently dying cell (Z). The obliqueness of the section does not allow a clear determination of the intercellular area. 30,000X. Stained with lead citrate and uranyl acetate.
A. Cells treated with Con A for one hour and fixed with modified Karnovsky's and OsO₄. Note the large number of microvilli which have been agglutinated to the surface of the parent cell and also the number of cell-cell interactions between the microvilli and other membrane projections. 18,100X. Stained with lead citrate and uranyl acetate.

B. Untreated cells fixed with modified Karnovsky's and OsO₄. Compared with A, the microvilli tend to project from the cells and are not flattened to the cell surface; also the number of cell-cell contacts are reduced. Mitochondrion (M). 18,100X. Stained with lead citrate and uranyl acetate.
A. Cells treated with Con A for 4 hours and fixed with modified Karnovsky's and Os04. Plate shows an aggregate of five cells. Several types of associations appear to be involved. Note that the microvilli in the free regions are apparently projecting out from the cell, and are no longer agglutinated to the surface. Intermediate association (—), close association (—). 15,650 X. Stained with lead citrate and uranyl acetate.

B. Cells treated as in A. An extreme example of the extensive interactions which can occur between villi of adjacent cells. Note how adjacent cell villi are pushing into the cell surface of the other cell (~) and that some of the villi appear to branch (+). 35,400X. Stained with lead citrate and uranyl acetate.

C. Cells treated with Con A for one hour and fixed with modified Karnovsky's and Os04. A region in which villi are pinned between adjacent cells. Note some villi are agglutinated to both cells. A close association is also seen in this region (—). 10,300X. Stained with lead citrate and uranyl acetate.
A. Cells treated with Con A for one hour and fixed in modified Karnovsky's and Os04. Note lightly stained material occurs only in region where intermediate association (—) occur. 68,000X. Stained with lead citrate and uranyl acetate.

B. Cells treated with Con A for 4 hours and fixed with modified Karnovsky's and Os04. Region of interaction between two cells in which intermediate associations (bars) and microvilli interactions (→) occur. 21,900X. Stained with lead citrate and uranyl acetate.

C. Cells treated as in B. Two cells held by several small intermediate associations (—). 22,300X. Stained with lead citrate and uranyl acetate.
A. Cells treated with Con A for one hour and fixed in modified Karnovsky's and Os04. An example of the close association seen after agglutination. 203,000X. Stained with lead citrate and uranyl acetate.

B. Cells treated as in A. An example of a common type of association, probably a close association cut obliquely in several regions (—). Note villi between cells. 37,300X. Stained lead citrate and uranyl acetate.

C. Cells treated as in A. This shows adjacent cells held by an extensive close association. 68,000X. Stained with lead citrate and uranyl acetate.

D. Cells treated as in A. Another example of an extensive close association. 54,600X. Inset illustrates the close apposition of adjacent membranes and also shows an unusual thickening of the membrane which appears to be a third bilayer (++) 221,000X. Region enlarged. Stained with lead citrate and uranyl acetate.
Cells treated with a high concentration of "factor" for one hour, then fixed with modified Karnovsky's and Os04. Shows the extensive pinocytosis and phagocytosis of the flocculent material on the cell surface. About 7500X. Stained with lead citrate and uranyl acetate.
A. Cells treated with a high concentration of "factor" for one hour then fixed with modified Karnovsky's and Os04. Higher magnification of the flocculent material on the cell surface. 60,000X. Stained with lead citrate and uranyl acetate.

B. Cells treated as in A. Note the large amount of material adhering to the cells' surface. Interactions seem to be the result of the material on the cells' surface (→). Also seen are numerous smaller lysosomal vesicles (√) possibly GERL (Novikoff, 1973) which appear to fuse (←) with the larger residual body (Rb). 13,700X. Stained with lead citrate and uranyl acetate.

C. Cells treated as in A. Shows the large amount of material sticking to the surface of the cells. Interactions appear to be due to the material on the cell surfaces. Again numerous lysosomal vesicles are present (√). Note the swollen ER (E) filled with a moderately stained material. 20,000X. Stained with lead citrate and uranyl acetate.

D. Cells treated with a low concentration of factor for one hour then fixed with modified Karnovsky's and Os04. Shows the exocytosis of two large myelin figures. It appears as if the cells are being forced apart by the vacuoles. Note the surfaces of the adjacent cells appear to be complementary. Several autophagic vacuoles (av) can be seen in the cells. 20,000X. Stained with lead citrate and uranyl acetate.
Cells treated with low concentration of "factor" for one hour then fixed with modified Karnovsky's and Os04. Note the numerous villi and lack of vacuolization as seen at higher concentrations of "factor". Also note viral aggregates (V) in the cytoplasm. About 7000X. Stained with lead citrate and uranyl acetate.
Plate 14

A. Cells treated for one hour with "factor" at low concentration, then fixed with modified Karnovsky's and OsO₄. Shows region of intermediate association (~); note microvilli involved (→). 23,600X. Stained with lead citrate and uranyl acetate.

B. Cells treated as in A. Shows patchy nature of the interaction between these cells. Bars indicate regions in which patchy material occurs. 23,700X. Stained with lead citrate and uranyl acetate.

C. Cells treated as in A. Shows region of intermediate interaction. 81,000X. Stained with lead citrate and uranyl acetate.

D. Higher magnification of A. Note the interaction appears to be mediated by patches of staining material (~). 65,400X. Stained with lead citrate and uranyl acetate.
Plate 15

A. Cells were fixed immediately after addition of Mn\(^{++}\) with 2.5% glutaraldehyde. Note the lack of large aggregates. 600X. Phase microscopy. Postfixed with OsO\(_4\).

B. Cells were fixed three minutes after addition of Mn\(^{++}\) with 2.5% glutaraldehyde. Shows a large aggregate of cells. 1500X. Phase microscopy. Postfixed with OsO\(_4\).

C. Cells were fixed 30 minutes after addition of Mn\(^{++}\) with 2.5% glutaraldehyde. High magnification of a small aggregate. Microvilli can be made out in areas. 3750X. Phase microscopy. Postfixed with OsO\(_4\).

D. Cells were fixed four hours after addition of Mn\(^{++}\) with 2.5% glutaraldehyde. Note vacuolization of cells, also no aggregates are seen. 1500X. Phase microscopy. Postfixed with OsO\(_4\).
A. Cells were treated with 20 mM Mn$^{++}$. 2.5% glutaraldehyde was added immediately after addition of Mn$^{++}$. Shows the interactions between the cells are mediated by surface projections. Note that interactions are not very extensive. 7000X. Postfixed with OsO$_4$. Stained with lead citrate and uranyl acetate.

B. Cells treated as in A. Shows interactions can occur between several types of surface projections. 12,500X. Postfixed with OsO$_4$. Stained with lead citrate and uranyl acetate.

C. Cells were treated with 20 mM Mn$^{++}$. 2.5% glutaraldehyde was added after three minutes. Shows that the number of villi involved in interaction has increased and some cell flattening has occurred. 14,100X. Postfixed with OsO$_4$. Stained with lead citrate and uranyl acetate.

D. Cells treated as in C. Villi interact at point contacts. The cell surface does not smooth out in the regions of interaction. 27,300X. Postfixed with OsO$_4$. Stained with lead citrate and uranyl acetate.
Plate 17

A. Cells treated with 20 mM Mn\(^{++}\) for 30 minutes then fixed with 2.5% glutaraldehyde. After extended incubation, interactions are still similar to those of shorter duration. 17,100X. Post-fixed with OsO\(_4\). Stained with lead citrate and uranyl acetate.

B. Cells treated as in A, but fixed using the ruthenium red staining method. There appears to be no differences in staining properties between those areas of interaction and those not involved in agglutination. 38,200X.

C. Cells treated with 20 mM Mn\(^{++}\). 2.5% glutaraldehyde was added immediately after addition of Mn\(^{++}\). Plate shows the interactions are tight between villus membranes. 168,000X. Postfixed with OsO\(_4\). Stained with lead citrate and uranyl acetate.

D. Cells treated with 20 mM Mn\(^{++}\) for three minutes then fixed with 2.5% glutaraldehyde. Again villus interactions are very tight; note also obvious microfilaments in the villi (→). 226,000X. Postfixed with OsO\(_4\). Stained with lead citrate and uranyl acetate.
A. Cells were treated with 20 mM Mn²⁺ for four hours, then fixed with 2.5% glutaraldehyde. Cells have become very vacuolated. Cytoplasm seems to have two distinct areas, one staining darker (d) than the other (l). Mitochondria stain darkly. Villi are much reduced in number. 7000X. Postfixed with OsO₄. Stained with lead citrate and uranyl acetate.

B. Cells treated as in A. Unusual cell with large number of vacuoles and completely smooth surface. 7800X. Postfixed with OsO₄. Stained with lead citrate and uranyl acetate.

C. Cells treated as in A. Cell has villi and is not vacuolated. Cytoplasm appears to have a dark and a light staining region. 3510X. Postfixed with OsO₄. Stained with lead citrate and uranyl acetate.