of this system, or of the kinetochore placement and development system, could cause variable chromosome number and morphology without attendant genetic variability.

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References and Notes

1. The term mixoploidy is now used in place of heteroploidy to describe karyological states of interest to this paper. A recent terminology statement, sponsored by the Tissue Culture Association, is published in *Nat. Cancer Inst. Monogr.* **29**, 587 (1968).
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8. Monodisperse cells were prepared from monolayer cultures by dispersal with trypsin, ethylenediaminetetraacetic acid (EDTA), and deoxyribonuclease, neutralization with soybean trypsin inhibitor and bovine serum albumin, and fixation in formalin-saline G (Puck). Hydrolysis and Feulgen staining with auramine-O were done as previously described (*7*).
9. We further validated the flow microfluorometric method by analyzing DNA distributions of CHO cells during prolonged Colcemid block. Peaks emerged with modes in the relationship 2C, 4C, 8C, and 16C (*12*) as anticipated from E. Stubblefield's data [in *Cytogenetics of Cells in Culture*, R. J. C. Harris, Ed. (Academic Press, New York, 1964), pp. 223–248].
10. Chromosome counts were made on 50 metaphase cells, which were treated with Colcemid and hypotonic sucrose, fixed in methanol—acetic acid, spread by the air-dry method, and stained by the method of Klinger and Hammond [*Stain Technol.* **46**, 43 (1971)].
11. Sources of cell lines used in this study were as follows: WI-38, Tu Wi, and LLC-MK$_2$, from American Type Culture Collection; Rh (FRhL-3), from Division of Biologics Standards, NIH; HAT-1, LLB, and HeLa, from Charles James, University of New Mexico School of Medicine, Albuquerque; BHK-21-(C13), 3T3, SV3T3, PY3T3, and SVPY3T3, from Dr. Alexander Kisch, University of New Mexico School of Medicine, Albuquerque; PK-15, from the National Animal Disease Laboratory, Ames, Iowa; L5178Y, from Dr. Lionel Manson, Wistar Institute, Philadelphia; P183 [polyoma-transformed derivative of BHK-21(C13)], from Dr. Vittorio Defendi, Wistar Institute, Philadelphia; L-929, from Dr. Angus Graham, Wistar Institute, Philadelphia; WME (whole mouse embryo) and SHE (Syrian hamster embryo), from our laboratory. Cells were grown in F-10 medium or Eagle's BME medium (supplemented with 10 percent calf serum) in Blake bottles or plastic T-75 flasks (Falcon).
12. Abbreviations used are as follows: C, the amount of DNA in a set of haploid mammalian chromosomes; G_1, the period of interphase that precedes DNA synthesis; G_2, the period of interphase that follows DNA synthesis; S, the period of interphase when DNA is synthesized; M, mitotic phase.
13. Coefficient of variation (CV) in percent equals 100 times the standard deviation divided by the mean.
14. Analysis of 10^7 cells by a modified Schmidt-Thannhauser procedure indicated that proliferating diploid cells contained 8 pg of DNA per cell; "high heteroploid" lines contained 10 to 15 pg of DNA per cell.
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Junctions between Cancer Cells in Culture: Ultrastructure and Permeability

Abstract. *Cell junctions between Novikoff hepatoma cells (N1S1-67) growing as small clumps or chains in suspension culture have been studied with ultrastructural, electrophysiological, and dye-injection techniques. Cells within clumps are commonly electrically coupled and can exchange dyes with a molecular weight of 332 to 500. Gap junctions and intermediate junctions are present, whereas true tight junctions and desmosomes are absent or very rare. This system should provide a useful model for studying the properties of "communicating" junctions.*

Certain intercellular junctions involving close membrane apposition or fusion are possible sites for the direct exchange of small inorganic ions and larger molecules (*1, 2*). It has been suggested that such junctions might normally pass regulatory molecules from cell to cell (*1, 2*) and that alterations in the junctions might result in faulty coordination of cellular activity (*3–5*). Such considerations have prompted study of cancer cell junctions, but no clear picture has yet emerged. Electrophysiological studies indicate that some cancer cells lack low resistance junctions—that is, they are not "electrically coupled" (*5–7*). Other cancer cells remain electrically coupled (*2, 4, 7, 8*) and certain of these cells are capable of directly exchanging molecules larger than small ions (*2*). Electron microscopic studies indicate that still other cancer cells have junctions that are less frequent or extensive although they may retain a normal fine structure (*9, 10*). None of these studies fully defines the degree of abnormality or normality of cancer cell junctions partly because each system has been studied with only one or at best two of the available techniques. Thus the presence or absence of coupling has not been directly correlated with ultrastructural evidence and in only a few cases has the coupling been correlated with permeability to larger molecules.

We report here the results of our initial experiments on a cancer cell system that is particularly amenable to study by electrophysiological, dye-injection, and electron microscopical techniques. Using all three methods, we have shown (*11*) that these cells readily exchange small ions and certain dyes and possess one of the junctions, namely, the "gap" junction, thought to mediate such exchange in "normal" systems (*12*).

Our studies have been carried out on Novikoff hepatoma cells (N1S1-67) grown in suspension culture in accordance with reported procedures (*13*). In a few initial experiments, we confirmed the cancerous nature of these cells by injecting them intraperitoneally into randomly bred rats (*14*). In suspension culture, the N1S1-67 cells grow in clumps of 2 to 100 or more cells. Clumps are characterized by marked intercellular adhesion remaining intact during centrifugation, transfer by micropipette, and impalements with microelectrodes. These clumps were conveniently prepared for electron microscopy after being centrifuged into loose pellets. The pellets were fixed in glutaraldehyde-paraformaldehyde and osmium tetroxide either accompanied by lanthanum hydroxide (*15*) or followed by uranyl acetate in the block (*16*).

By electron microscopy we have identified two types of contact specializations. The most conspicuous type resembles the *zonula adhaerens* (or

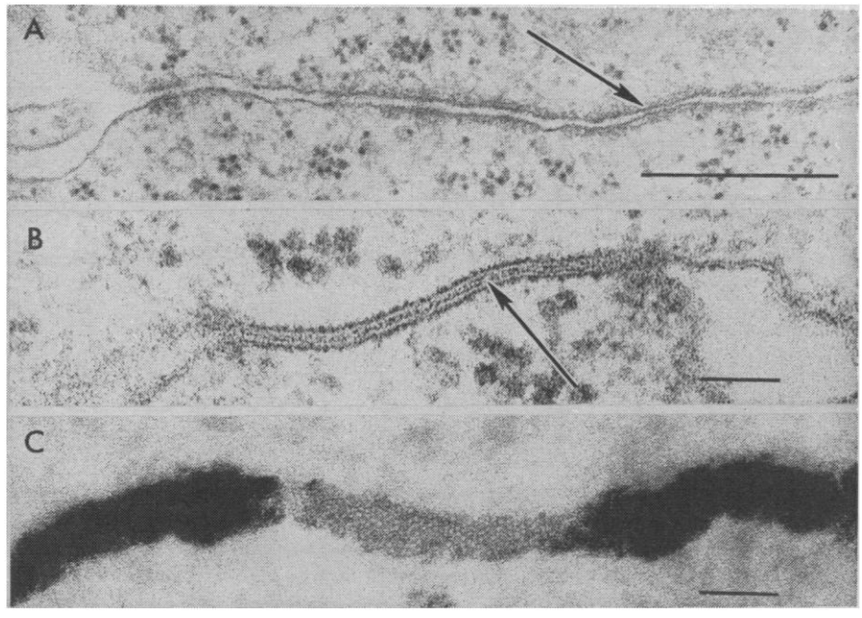

Fig. 1. Electron micrographs of Novikoff hepatoma cells grown in suspension culture. (A) Intermediate junction with the membranes of two cells possibly making contact at one point (arrow); clump surface at the left and intercellular space widens at far right (calibration, 0.5 μm). (B) Gap junction cut in cross section displaying the 20-Å separation of outer leaflets (arrow); stained with uranyl acetate en bloc (calibration, 0.1 μm). (C) Lanthanum preparation demonstrating the 90-Å center-to-center spacing of polygons in a tangentially cut gap junction (calibration, 0.1 μm).

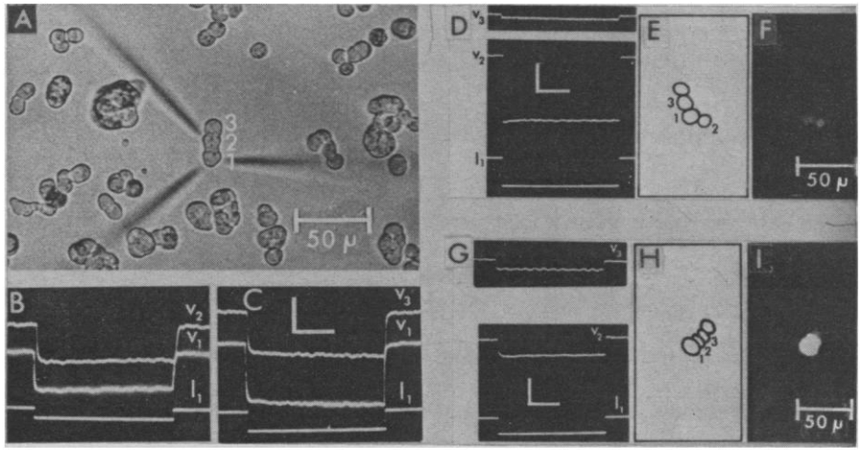

Fig. 2. (A to C) Illustration of a three-microelectrode study of electrical coupling in a three-cell chain. (A) Bright-field micrograph of cells and microelectrodes just prior to impalement. (B) Transmembrane voltage changes in cell 1 (V_1) and cell 2 (V_2) produced by pulse of current (I_1, 1.25 $\times$ 10^{-9} ampere) supplied to cell 1. The attenuation factor [(AF, V_1/V_2) was 1.1]. (C) Same as (B) but showing coupling between cells 1 and 3. The current (I_1) was 2.5 $\times$ 10^{-9} ampere; AF, 1.5 (D to F) Demonstration of the transfer of Procion yellow between two coupled cells in a four-cell chain. (D) Electrical records showing much greater voltage change in cell 2 (V_2) than in cell 3 (V_3) when current pulse (I_1, 3.75 $\times$ 10^{-9} ampere) was supplied to cell 1 (measured first in cell 3, then cell 2). (E) Drawing shows relative positions of cells 1, 2, and 3. (F) Procion yellow has transferred to cell 2 but not to cell 3 within 4 minutes of injection time [pulses as shown in (D) repeated once every 2 seconds]. Dark-field illumination with mercury lamp, blue filter, 10-second exposure on Tri-X, developed with Acufine developer. (G–I) As in previous series but with fluorescein as a tracer. (G) Electrical records showing voltage change in cell 3 (V_3) about half that in cell 2 (V_2) when current pulse (I_1, 2.5 $\times$ 10^{-9} ampere) was supplied to cell 1 (measured first in cell 2 and then in cell 3). (H) Drawing shows relative positions of cells 1, 2, and 3. (I) Fluorescein has transferred to cells 2 and 3, which appear as bright as cell 1, within only 3 minutes of injection time; same illumination and photographic details as (F) (calibration for electrical records: vertical, 10 mv for B, C, D, and G; horizontal, 20 msec for B and C; and 200 msec for D and G).

"intermediate junction") (*17*). The junctional membranes are separated by a space of about 200 Å and have dense, amorphous material on the cytoplasmic surfaces (Fig. 1A). These junctions occur routinely at the exposed surfaces of peripheral cells and sometimes deeper in the clumps.

The second type of contact resembles the "gap" junction described by Revel and Karnovsky (*15, 18–20*). Figure 1, B and C, illustrates two distinctive features which serve to characterize this junction: a gap of 20 to 30 Å between the outer leaflets of the junctional membranes cut in cross section and a polygonal substructure (with 90-Å center-to-center spacing) as revealed by lanthanum in tangential sections of the junction. The junctions vary in length from 0.1 to 1 μm, and, although their frequency is difficult to quantitate, one or two are commonly found in each filled grid square of a 300-mesh grid.

We have looked for regions of fusion of apposed membranes, that is, true tight junctions (*17–19, 21*). Although we have seen infrequently sites of apparent membrane contact (Fig. 1A), we have not found any convincing instances of fusion—places where the combined width of the junction is less than that of two unit membranes.

The observation of gap junctions between the N1S1-67 cells led to a study of the ability of adjacent cells to exchange inorganic ions and larger molecules. The ease with which inorganic ions can be exchanged is reflected in the electrical resistance between adjacent cell interiors and can thus be detected and quantitated by conventional microelectrode techniques. Two or three micropipettes filled with 3*M* KCl (resistance 20 to 50 Mohm) and arranged for passing current or recording potential changes (or both) were used to impale cells within small clumps that had settled out in a plastic culture dish. Experiments with one current passing and one recording electrode were sufficient for qualitative demonstration of the presence and distribution of electrical coupling (*22*). In general we detected coupling whenever two cells within a given clump were impaled. In more extensive experiments, the degree of coupling was assessed with the use of three microelectrodes. For these experiments, isolated pairs or short chains of cells were studied because outlines of the impaled cells could be observed more distinctly (Fig. 2A). By dividing

the voltage produced in the cell supplied with current by that in an adjacent cell, an "attenuation factor" (AF) (*23*) was obtained. The mean AF from 48 pairs of cells was 1.96, a value comparable to those found for other cultured cells, both normal and cancerous (*2, 7*).

We investigated next the ability of these coupled cells to exchange dye molecules, such as fluorescein, molecular weight (M.W.) 332; dansyl-L-glutamate (DG; that is, the dimethylaminonaphthalenesulfonamide of L-glutamic acid), M.W. 380; dansyl-DL-aspartate (DA), M.W. 366; or Procion yellow M4RAN, M.W. about 500. Each of these compounds is fluorescent and has a net negative charge (*24*). One cell at the end of a short chain was impaled with a micropipette filled with dye. Another cell in the chain, either adjacent to the first or more distant, was impaled with a KCl-filled micropipette. Dye was iontophoretically injected into the first cell with negative-going, rectangular current pulses of about 5×10^{-9} ampere and 500-msec duration passed once every 2 seconds. During the injection, the potential changes signifying coupling to the adjacent or more distant cell were monitored by the second electrode. Movement of dye was observed directly under ultraviolet illumination, and photographs were taken for permanent records (*25*).

We observed movement of dye to adjacent, coupled cells in 7 out of 7 cases for fluorescein (Fig. 2, G–I), 8 out of 8 cases for DG, 5 out of 5 cases for DA, and 31 out of 39 cases for Procion yellow (Fig. 2, D–F). In most experiments with fluorescein, and with DG and DA as tracers, transfer was first evident after less than 1 minute of injection. Procion yellow is substantially less fluorescent than the other dyes, which might explain the finding that transfer of Procion yellow was first observed 1 to 3 minutes after injection in six cases, and after 3 to 10 minutes in the rest.

In five of the Procion yellow injections and all of the DG and DA injections, the coupling was monitored in the cells at the end of the chain opposite the site of injection, and thus dye moved to an unimpaled cell. This indicates that the movement did not depend on damage by impalement. Any impairment of coupling incurred during impalements severely restricted dye transfer, and cells that were definitely uncoupled never exchanged dye. When cells were immersed in fluorescein, DG, or Procion yellow, there was no detectable accumulation of dye, an indication that the nonjunctional membranes have limited permeability to the dyes (*26*).

Thus, our evidence from electron microscopic, electrophysiological, and dye-injection experiments demonstrates the presence of cell junctions that have numerous features in common with those of various "normal" systems. There are intermediate junctions that probably are responsible in part for the strong adherence of cells within clumps. There also are gap junctions identified in cross sections of conventionally stained material as well as in tangential sections after lanthanum treatment. Since gap junctions appear to provide intercytoplasmic channels of high permeability to ions and larger molecules in other systems (*12*), they are likely to be responsible for the electrical coupling and dye exchange we have demonstrated. Furthermore, the permeability of the junctions reflected in the degree of coupling and size range of tracers which are transferred is comparable to that found in other systems (*2, 7, 12*). We cannot exclude the possible contribution of true tight junctions to cell adhesion and molecular exchange (*18, 19, 21*), but our present evidence suggests that this contribution is probably small at best.

Although our studies have not revealed any obvious qualitative alterations in the junctions in the system or in the properties thought to depend on these junctions, we cannot rule out significant quantitative deviations from normal, such as smaller junctional area, slower rate of transfer of dye molecules, and greater restriction on size of permeant molecules. Furthermore, the absence of desmosomes and the paucity or absence of true tight junctions may indicate a significant departure from normal (*10*). Nevertheless, the qualitative "normality" of the existing junctions as well as the feasibility of applying a variety of techniques to the same system or even the same cells suggest that this system will provide useful information about "communicating" junctions in general.

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14. Tumors were produced either at the site of injection or on the mesentery and abdominal organs after injections of as few as 0.5×10^6 cells (20 animals were injected); three of the five animals injected with 10×10^6 cells and one of the five animals injected with 5×10^6 cells died 11 days after injection, and all these animals had extensive intraperitoneal tumor growth and and ascites.
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25. Illumination was provided by a mercury lamp with blue excitation filter (BG-12, Leitz). Photographs were taken on Tri-X, 35-mm film developed with Acufine developer to push the ASA to 1200. With the resultant film sensitivity, 10-second exposures were adequate to record low levels of fluorescence.
26. The concentrations of dye used to test nonjunctional membrane permeability were chosen arbitrarily and were 1 to 2 m*M* in Hanks salt solution. Cells were left in the dye solution for 5 minutes, then centrifuged once for 3 minutes, washed and centrifuged twice more, and resuspended in Hanks salt solution. This method is a better test for exclusion of Procion yellow, which slowly binds to cell constituents, than for the other dyes which bind much less extensively.
27. We thank Dr. Peter Plagemann and Dr. Richard Estensen for supplying cells, Dr. William Herman for reading the manuscript, and Mrs. Marie Hammer and Mrs. Virginia Ellis for technical support. Supported by grants from the Minnesota Division American Cancer Society, from the University of Minnesota Graduate School, and from National Cancer Institute (R01-CA11114).

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