LOW-RESISTANCE JUNCTIONS BETWEEN CANCER CELLS IN VARIOUS SOLID TUMORS

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ABSTRACT

Electrical coupling, which reveals the presence of specialized low-resistance intercellular junctions, has now been found in four types of tumors, Sarcoma 180, Novikoff hepatoma, and Morris hepatomas 3924-A and 7777. Coupled cancer cells were distinguished from coupled normal cells by intracellular marking techniques. Although the evidence suggests that coupling may be extensive in some cases, it is not possible to say that the coupling was normal. In particular, the results do not exclude less obvious defects in the specialized junctions, such as abnormal distribution or decreased permeability to molecules other than small inorganic ions. The results are discussed in relation to previous studies of coupling in Novikoff hepatomas and in cultures of S180I and II cell lines.

INTRODUCTION

Cells in a variety of tissues are linked to their neighbors by specialized junctions which allow small inorganic ions (3, 11 *a*, 21), and perhaps larger molecules (11 *a*, 16, 24), to pass directly between cytoplasms. Since these junctions offer a low electrical resistance between cell interiors, they are responsible for transmitting electrical signals when they connect excitable cells, such as cardiac (42) and smooth muscle (5), and, in limited cases, nerve cells (3, 11). More commonly they connect nonexcitable cells in adults (19–21, 24, 26, 30, 34) and embryos (13, 14, 31, 36, 37, 43), and here their function remains unknown; but an intriguing suggestion is that the junctions permit cells to exchange control substances, thereby influencing the activity, e.g. movement, division, or differentiation, of their neighbors (11 *a*, 20, 21, 24). A correlate of this suggestion is that defects in these junctions might lead to some observable disruption of coordinated cell behavior (11 *a*, 22, 23, 25, 26, 31).

Cancer cells characteristically demonstrate such lack of coordination in their growth, movement, differentiation (but see 27). Malignant tumors frequently grow rapidly and usually invade normal tissue, metastasize, and exhibit more or less severe disorganization of cytological and histological structure (4). Moreover, in cultures of certain cancer cells, interactions that require cell contact are impaired, i.e. the cells are poorly adhesive (6) and are little, if at all, "contact inhibited" by their neighbors (1, 2, 38). Thus, it is natural to ask if cancer cells have defective "communicating" junctions.

Electrophysiological methods for testing the presence of the junctions and the degree to which they pass small inorganic ions have been used in studies of certain solid tumors and cancer cell cultures. Loewenstein and his colleagues have investigated carcinomas originating from liver (25), thyroid (15), and stomach tissues (17), and have detected no junctions, i.e. no "electrical coupling," in any of these tumors, in contrast to the widespread coupling in the normal counterparts.

Furshpan and Potter, on the other hand, have found that virally transformed cancer cell lines have apparently unimpaired coupling, whereas the degree of coupling in two cell lines (S180I and II), derived from a mouse sarcoma (S180) (10), depends on the time elapsed since renewal of medium (11 *a*; unpublished observations).

These divergent results could suggest that cancer cells are coupled in tissue culture but not when growing in a solid tumor. The behavior of the S180I and II cells would be consistent with this idea provided the condition in vitro leading to uncoupling, i.e. renewal of medium, resembled the conditions in the solid tumor (11 *a*). However, the present experiments suggest that such a simplified generalization is inadequate since coupling could be detected in four different solid tumors, including a tumor grown in vivo from S180II cells.

METHODS

All experiments were performed on small tumor nodules which appeared growing on the mesentery 3–7 days after intraperitoneal injections of cells obtained from (*a*) cultures (Sarcoma 180II [10]) dissociated with trypsin; and (*b*) solid tumors, either dissociated with trypsin (S180, Novikoff hepatoma [29]) or crudely minced (Morris hepatomas 3924-A and 7777 [28]). Swiss-Webster mice (male weanlings) were used as hosts in the S180 studies. Charles River rats were used for the Novikoff studies, whereas pure-bred rats were used for the Morris hepatomas (ACI for 3924-A and Buffalo for 7777).

A standard procedure was followed in all experiments. A piece of mesentery with adhering nodules was spread out and pinned to a soft plastic (Sylgard, Dow Corning Corp., Midland, Mich.) covering the bottom of a plastic culture dish. Locke's solution was used as a bathing medium (35). Two microelectrodes, filled with a blue dye, Niagara Sky Blue:6B, were used to test for coupling in the nodules: one electrode supplied current to the inside of one cell and the other electrode recorded the electrotonic potential change in a second cell (See Fig. 2 and legend). Dye was injected electrophoretically into each cell (generally during the test for coupling) (31, 36, 37). The tissue was processed for histological examination (31, 36, 37), and the stained cells were located in sections. Thus it was possible to determine the identity of the impaled cells (see Results for possible complications). In some experiments on S180II-induced sarcomas and Morris 7777 hepatomas, 3M KCl- or 0.5M K_2SO_4-filled electrodes (10–50 MΩ) were used. These experiments provided some information regarding the extent of coupling within the nodules, but the presence of heterogeneous cell types, especially the mesothelium that often covered the nodules, made it difficult to be certain that tumor cells rather than host cells were impaled.

Cells filled with Niagara Sky Blue:6B appear blue with transmitted light (bright-field or phase-contrast) but red with dark-field illumination. This red fluorescence provides the most sensitive indicator for the dye marks and thus dark-field illumination was used routinely to locate the cells (see Figs. 3–5).

RESULTS

The experimental results are summarized in Table I and are illustrated with one figure for each type of tumor studied. Since it was necessary to distinguish accurately between coupled tumor cells and coupled normal cells, only the pairs of cells

TABLE I

Summary of Coupling

Figure	Tumor type	Dye-confirmed coupling No. of pairs: Definite tumor cells	? Tumor cells	Definite mesothelium	No. of preps.
1 and 2	Sarcoma 180*	20 (3)	8 (1)	1	27 (3)
3	Novikoff hepatoma	9	5	1	8
4	Morris hepatoma 3924-A	8	5	2	6
5	Morris hepatoma 7777	3	1	1 Fibroblast	4

* Two sources of S180 cells were used in this series: (*a*) S180II cells grown in culture and (*b*) cells dissociated from S180 tumors carried in mice.
Results from both sources are pooled in the totals; numbers in ( ) refer to source (*b*).

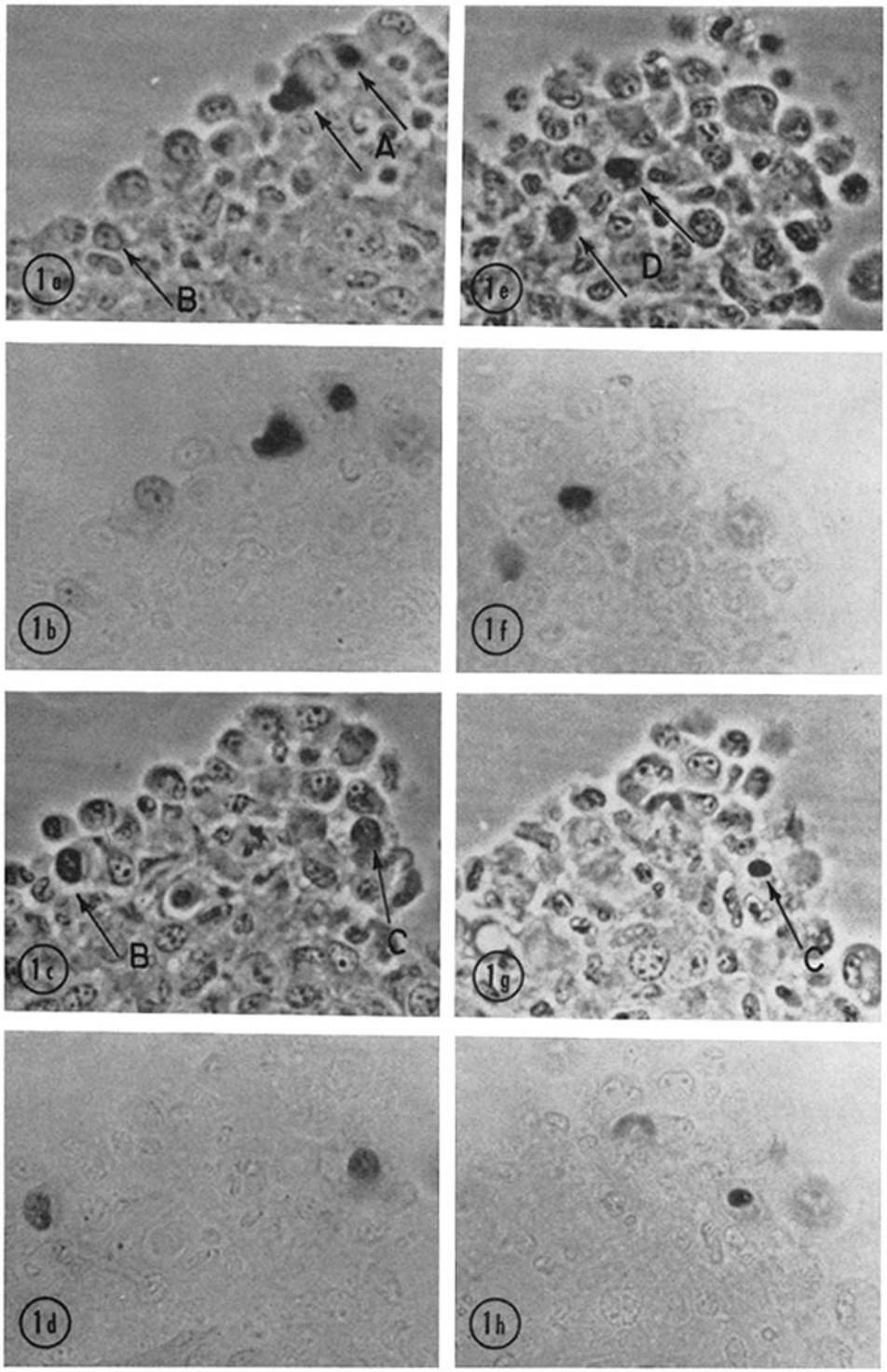

FIGURE 1 Photomicrographs of four sections through an S180 tumor nodule grown from S180II cells injected intraperitoneally. Each section photographed through both phase-contrast (1 *a*, 1 *c*, 1*e*, and 1 *g*) and bright-field (1 *b*, 1 *d*, 1 *f*, and 1 *h*) optics. The arrows indicate cancer cells marked by intracellular deposition of dye, Niagara Sky Blue:6B. The letters *A–D* refer to pairs of cells shown to be coupled; in two cases (*A* and *D*), both cells of a pair are seen in the same section (1 *a* and 1 *e*), but in the other cases (*B* and *C*), the two cells are in neighboring sections (1 *a*, 1 *c*, and 1 *g*). The upper case letters (*A–D*) are used in Fig. 2 to label the electrical evidence for coupling of each pair. × 600 phase; × 690 bright-field.

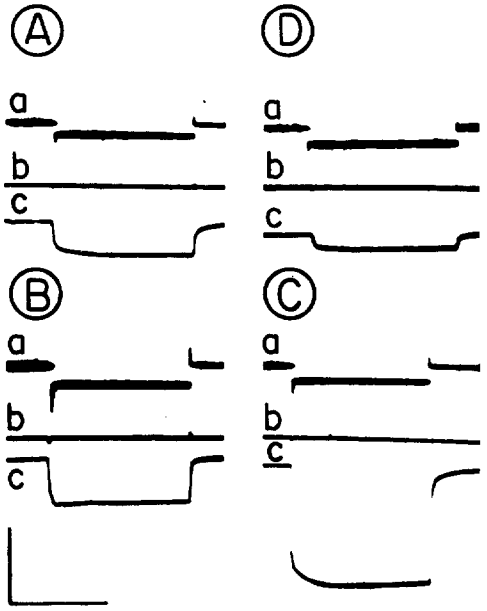

FIGURE 2 Electrical records for pairs of cells in Fig. 1. Each set of records for cell pairs *A–D* (note corresponding labels in Figs. 1 and 2) contains three traces: current injected in one cell (*a*) and membrane potential recorded inside (*c*), and just outside (*b*), second cell. Extracellular control insures that coupling depends on low-resistance junction(s) rather than high resistance external barrier or interstitial material (see references 31, 36, 37 dealing with similar circumstances). Vertical calibration: 1.8×10^{-8} A for current traces (*a*); 30 mv for voltage traces (*b* and *c*) in *A* – *C*; 39 mv for *D*. Horizontal calibration: 500 msec.

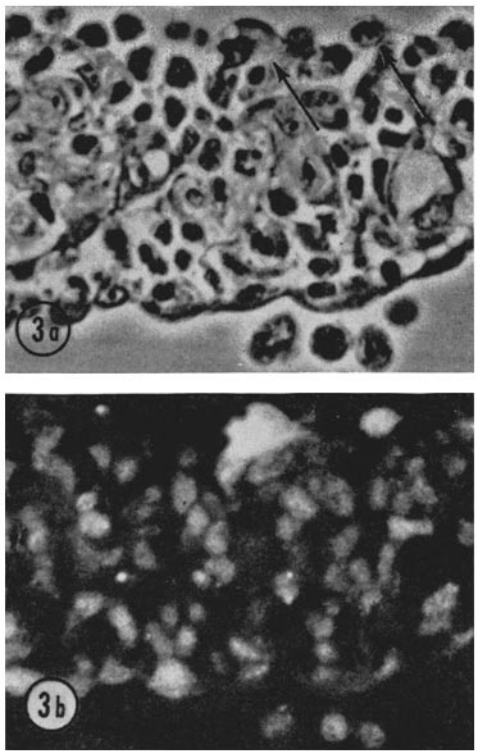

FIGURE 3 Section through Novikoff tumor nodule, photographed with phase-contrast (3 *a*) and dark-field (3 *b*) optics. Arrows point to pair of tumor cells each of which was found coupled to a third cell which was not adequately marked with dye. × 500.

marked with injected dye and located in histological sections are enumerated.

It is important to note at the outset that an absolute classification of individual marked cells into tumor or normal was often quite difficult. Attributes such as nucleolar configuration, cell size, and nuclear shape were used in the identification of the cells (e.g. Fig. 3, reference 29). Position of the cell was an additional guide, e.g. cells located below the surface of the nodule were unlikely to be mesothelial cells (Fig. 1). Sometimes the arrangement of the marked cells and their immediate neighbors, e.g. into clusters or tubules, was adequate for classification (Figs. 4 and 5). All of the cells listed in Table I as Definite Tumor Cells were identified by one or more of these means. Some cells on the surface of the nodule were obviously mesothelial cells (Definite Mesothelium). The remaining cells were possibly tumor cells, but could not be classified with any certainty (? Tumor Cells).

Although pairs of coupled tumor cells were found in nodules of each type of tumor, the question naturally arises whether or not these results are typical. A definitive answer cannot be given, but several observations suggest that coupling is widespread: (*a*) as many as four pairs of coupled tumor cells have been found in a single, small nodule (see Fig. 1); (*b*) in most cases the coupled cells were separated by at least one cell diameter, which indicates that the intervening cell, or cells, was coupled to both impaled cells (although it is possible that cell processes, too fine to be distinguished under the light microscope, connected the two cells directly); (*c*) in a few S180, Novikoff, and Morris 3924-A tumors, one cell was coupled to as many as five neighbors; (*d*) in some nodules (only S180 and Morris 7777 tumors) studied with salt-filled electrodes any two cells impaled were

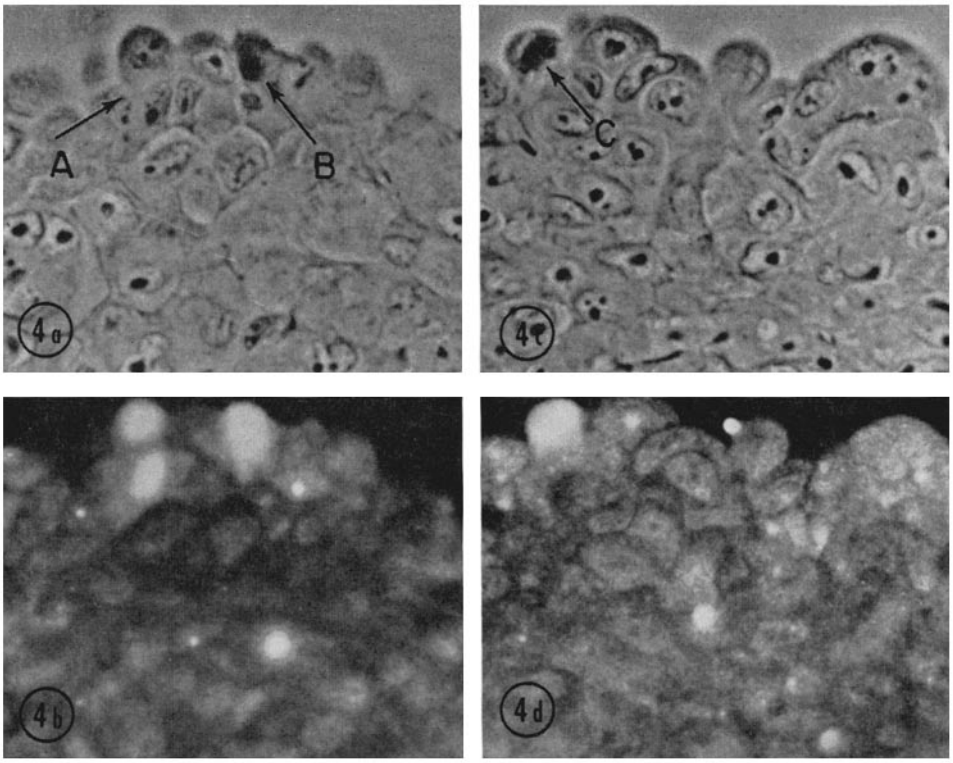

FIGURE 4 Two sections, photographed with phase-contrast (4 *a* and 4 *c*) and dark-field (4 *b* and 4 *d*) optics, at different levels through nodule of Morris hepatoma 3924-A. This nodule was free-floating in the peritoneal space and thus lacked obvious host cell contamination. Arrows indicate cells marked at three penetration sites (dye presumably passed between cells at *A*, see Fig. 4 *b*). Coupling was found between one cell at *A* and cells at *B* and *C*. × 600.

coupled, often over distances corresponding to many cell diameters. (Similar results were obtained with dye electrodes in the experiment illustrated in Fig. 4.)

Pairs of cells lacking coupling were found in most experiments, but these were most often correlated with an increased difficulty in cell penetrations. Thus, it is possible that the apparent lack of coupling was, in fact, the result of mechanical disturbance of the cells. Since, in addition, lack of coupling did not appear to be so frequent as coupling under optimal recording conditions, no attempt was made to list examples of lack of coupling. Instead, attention was focused on cases of coupling, and any information on extensiveness of coupling was derived from these cases.

The methods used in these experiments permitted no easy means of assessing the degree of coupling since there was no measurement of the change in membrane potential produced in the cell supplied with current. Values obtained by dividing the potential change in the second cell by the delivered current (only carried out for the S180 series) ranged from $2 \times 10^5\ \Omega$ to $6 \times 10^6\ \Omega$ as compared with values of $5 \times 10^6\ \Omega$ to $8 \times 10^6\ \Omega$ obtained in two cases in which the same cell was impaled with the current and recording electrodes. This range of values is also similar to that obtained by Furshpan and Potter when studying normal and transformed fibroblasts (as well as S180I and II cells) in culture (11 *a*; unpublished observations).

DISCUSSION

Electrical coupling has been observed in four types of cancer tumors, S180 (generated either from S180II cells or cells dissociated from S180 tumors), Novikoff hepatoma, and Morris hepatomas, 3924-A and 7777. The occasional lack of coupling, not investigated for reasons discussed above, must

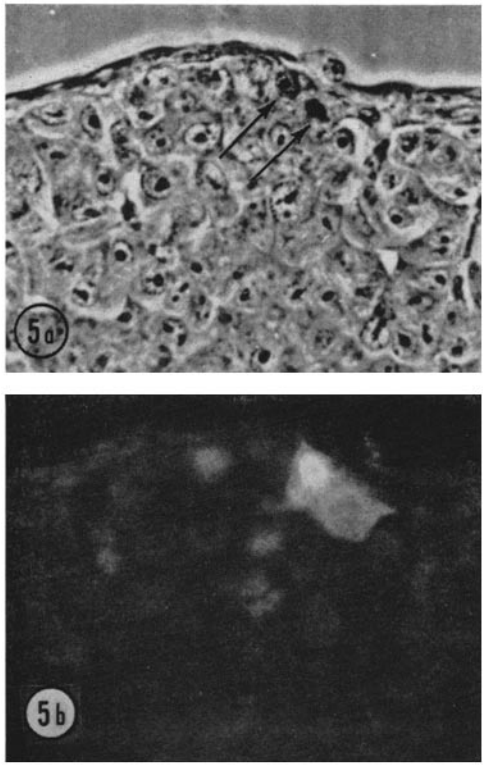

FIGURE 5 Same format as Fig. 3, but with Morris hepatoma 7777. Two adjacent, coupled cells indicated by arrows. × 500.

remain as one important reservation in any attempts to generalize from these results. Furthermore, there may be other defects in the junctions, such as abnormal distribution or impaired permeability to molecules other than small ions (11 *a*, 15, 26).

The present study suggests that the junctions can occur widely, but no other conclusions can be drawn about their distribution. Furthermore, the normal pattern of coupling is known only for the hepatoma counterpart (30), liver; fibroblasts, the counterparts of sarcoma 180 cells, are extensively coupled in culture (11 *a*, 31), but their behavior in vivo is untested.

The electron microscope provides another means for comparison of the distributions of cell junctions in normal and cancerous tissue. There is strong circumstantial evidence that special points of close membrane apposition or fusion are the sites of the low-resistance connections in normal tissues (3, 11 *a*, 21, and 8, 9, 18, 32–34, 40, 41), and these junctions occur between normal liver cells in vivo (32), and between normal fibroblasts in vitro (7). There is now electron microscopical evidence that these junctions are deficient in certain tumors (12, 26 *a*, 27 *a*). Hruban et al. (12) report that Novikoff cells lack tight junctions; however, their study did not relate specifically to junctions, and the micrographs shown were of relatively low magnification and resolution. Martinez-Palomo and coworkers (27 *a*) report that certain transformed fibroblast strains have greatly reduced numbers of tight junctions. Furthermore, McNutt and Weinstein (26 *a*) show that cells in cancerous cervical epithelium make very few specialized junctions ("nexuses" or "gap" junctions) with one another, in contrast to the extensive junctions joining normal cells. In some areas in the tumors, the authors calculated that there could be as many as four junctions per cell which could still couple cells extensively; in other areas, however, no junctions were found. The possibility of such variability is an important factor to consider in interpreting the present and previous electrophysiological results (see below).

Normal cells can exchange molecules the size of small metabolites or larger (11 *a*, 16, 39), perhaps via their low-resistance junctions. Complete absence of coupling, as reported for other tumors by showing that the movement of small ions between cells is blocked, implies that the exchange of large molecules is impeded as well (26). However, the presence of coupling, even if distributed normally (see above), is not as easily interpreted. Cancer cell junctions, for example, might allow small inorganic ions to move easily but might restrain larger molecules. Then coupling, which depends on ion movement, would be intact while cell interactions, which depend on the larger molecules, would still be impaired. This consideration indicates the limitations of electrophysiologic methods for the study of the junctional properties. A solution to this problem must await development of appropriate tracer molecules that must first be used to determine the normal permeability of low-resistance junctions.

The apparent dye movement between two cells shown in Fig. 4 is difficult to interpret; this dye is a poor tracer due to its binding properties and toxicity (31, 36).

Additional comments should be made concerning first, the conflict with results of previous experiments on Novikoff hepatomas and second, the

relation between the coupling in S180 tumors and that in cultures of S180I and II cells. In an earlier study of Novikoff tumors, Loewenstein and Kanno (25) detected no coupling in 13 cases (see their Table I), whereas, by contrast, nine pairs of coupled Novikoff cells were observed in the present study (see Table I and Fig. 3). There are several possible reasons for this apparent discrepancy. The tumors may have been intrinsically different since they were obtained from different immediate sources, although from the same original tumor. The results may have been influenced by the tumor sites which differed in the two cases (tumors in the earlier study were growing on the liver surface). There may be variations in the number of junctions in different areas of a given tumor (26 *a*). Gross changes in tumor organization may have produced an apparent lack of coupling. In the normal liver, parenchymal cells are organized into interconnected plates; coupling between adjacent cells leads to homogeneous coupling throughout the tissue (26, 30). However, the Novikoff tumor cells are often found in clusters isolated by stroma; under such a condition, even with extensive coupling between adjacent tumor cells, the clusters would not be connected, and the coupling in the tumor might be harder to detect. The degree of disorganization of tumor cells can vary from preparation to preparation and could, therefore, be an important factor in coupling patterns observed. Another possibility is that uncoupling represents an early phase in cell death, although this seems unlikely since both studies deal primarily with surface cells, whereas necrosis is more likely to occur in the center of nodules. Finally, coupling between cancer cells may be abnormally sensitive to mechanical stress (15). If so, impaling one or both cells of a given pair with two microelectrodes (as was done in the previous study) might lead to uncoupling. Such sensitivity could be intrinsic to the low-resistance junctions in the tumors or could merely reflect the decreased adhesiveness characteristic of many cancer cells (6).

Furshpan and Potter recently reported that, after a renewal of medium, S180I and II cells in culture undergo a cycle of uncoupling followed by recoupling (11 *a*; unpublished observations). These studies posed the question of whether daughter cells, growing in vivo as a solid tumor, would be predominantly coupled or uncoupled. The uncoupled state, according to one line of reasoning, might be favored since the in vivo equivalent to the "medium" would continuously be renewed. This suggestion would be consistent with the conclusions of studies on other solid tumors by Loewenstein and colleagues (15, 17, 25). The present results, however, show that coupling can be quite extensive in an S180 tumor (see Fig. 1) and it is, therefore, unlikely that the uncoupled state of S180 cells is favored. Whether or not there is any in vivo expression of the effect of renewal of the medium on coupling between S180 cells remains unknown.

In conclusion, these results show that coupling is present, sometimes extensively, in certain solid tumors. However, it remains to be shown whether all aspects of cell coupling are normal in these tumors.

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