Intercellular Communication in Spheroids

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Biological Significance of Intercellular Communication

Although information can be exchanged between cells by several mechanisms, the development of direct junctional communication represents an important evolutionary step towards formation of highly differentiated multicellular organisms. For example, long before hormonal or neuronal communication is developed in an embryo, small molecules can move freely from cell to cell. This kind of information transfer is expressed even during early embryonic stages (for review see Griepp and Revel 1977) and may allow the formation of a morphogenetic field (Schaller and Bodenmüller 1981).

The first observation of intercellular molecule transfer dates back to 1925, when Schmidtmann described a method for measuring the intracellular proton concentration by dye indicators. He manipulated small crystals of phenol red, methyl red, or similar pH indicators into cells of different organs and tissues and determined the intracellular pH by the color of the dissolved indicator crystal. Especially in gastrointestinal epithelia he observed a rapid intercellular spreading of the dye into neighbors of the impaled cell (Schmidtmann 1925).

It was not until 1957, when Furshpan and Potter described an "electrical synapse" in abdominal nerve cords of the crayfish, that intercellular channels were detected by electrophysiological measurements (Furshpan and Potter 1957). The membrane structures that provide a path for this intercellular communication are fine protein channels which bridge the intercellular cleft of closely attached cells. In the electron microscope they can be made apparent by freeze-fracture techniques, when they appear as closely packed patches of protein particles called gap junctions or nexus (Fig. 7); they are, however, also identifiable on thin sections. The existence of these gap junctions is experimentally verified by electrophysiological techniques, i.e., injection of a pulsed current or a fluorescent dye in one cell and registration of the transfer of this signal into other cells (see section "Methodology").

During the last decade it has become apparent that gap junctions are also mediators of so-called metabolic cooperation (coupling) between cells. This phenomenon was first demonstrated by Subak-Sharpe and his associates, who found that a defective phenotype of certain mutant cells in tissue culture can be corrected by intimate contact with normal cells (Subak-Sharpe et al. 1969). They observed that mutant Chinese hamster fibroblasts deficient in the enzyme inosinic guanylic pyrophosphorylase were incapable of incorporating exogenous hypoxanthine into their nucleic acids when they grew alone, but that they did incorporate hypoxanthine when grown in contact with wild-type cells. This suggested that a low-molecular-weight compound, such as a nucleotide or a nucleotide derivative, is transferred from the wild-type cell to the mutant, thus bypassing the enzyme block in the

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mutants. This metabolic coupling is mediated by intercellular communication via gap junctions, as was subsequently shown by Gilula et al. (1972).
With respect to other biological functions, particularly concerning the nature of the intercellular messages, our present knowledge is very limited (see Loewenstein 1981 for review). Apparently, the individual channels of the gap junctions allow exchange of "small" molecules (mol.wt. $\leqq$ 900 daltons). It is hypothesized that regulatory molecules can be distributed in this way among the cells of a tissue. Alternatively, "morphogenic" gradients could be established via these junctions. These functions of the gap junctions are thought to induce differentiation in tissue cells. In addition, permeability of the channels can be controlled biochemically; this point will be treated in more detail in the sections "Biophysical and Biochemical Effects Associated with Intercellular Communication" and "Intercellular Communication and Radiosensitivity."
A particularly interesting property of the gap junctions is that they can become internalized, thus forming intracellular annuli (see Azarnia and Larsen 1977 for further details). Annular gap junctions are almost always found in tissues known to be targets of peptide hormones. From the experimental results it was suggested that gap junctions are clusters of hormone receptor-adenylate cyclase complexes (Albertini et al. 1975; Azarnia and Larsen 1977), which assigns to the gap junctions a regulatory function with respect to cAMP synthesis (see also section "Biophysical and Biochemical Effects Associated with Intercellular Communication").
Intercellular communication by gap junctions is a very widespread phenomenon observed in excitable and nonexcitable cells, both in vivo and in vitro. In this contribution we will concentrate on nonexcitable cells. (See Page and Shibata 1981; De Mello 1982 for review of cell communication in excitable tissues.) Particular interest in the phenomenon of intercellular communication has arisen from embryology and cancer research. There is evidence that regulation of growth and differentiation during embryogenesis is associated with variations of gap-junctional permeability and structure. This has been shown for mammals (Lo and Gilula 1979a, b), amphibians (Ito and Ikematsu 1980; Bennett et al. 1981), and insects (Weir and Lo 1982). An observation made with liver and hepatomas in situ, which revealed that the malignant cells had lost electrical coupling (Loewenstein and Kanno 1967), indicated the importance of intercellular communication in malignant transformation. However, these findings were contradicted by several authors who investigated malignant cells showing electrical coupling (e.g., Hülser and Webb 1973; Johnson et al. 1974). This controversy has stimulated this field of cancer research but it is still not known whether the absence of gap junctions is a necessary or sufficient condition for malignancy (see Weinstein et al. 1976 for review).
Obviously the spheroids represent a very attractive and realistic system for studying intercellular communication. They are easy to handle and can be obtained from a variety of cell lines. Compared with monolayers, they have the unique advantage of preserving the natural three-dimensional arrangement of tissue cells. In fact, as will be shown in the sections "Biophysical and Biochemical Effects Associated with Intercellular Communication" and "Intercellular Communication and Radiosensitivity," communication-dependent responses may well be different for spheroids and monolayers. Few experimental investigations on intercellular communication have so far been performed in spheroid systems. Thus one of the main purposes of this paper is to stimulate further experimentation in this domain. After describing the methodology for work on cell communication, we shall briefly outline the results and views on intercellular communication so far obtained from the spheroids. With reference to the terminology, the terms "cell coupling," "electrical coupling," "cell communication," will always

be used in this paper specifically to refer to gap-junctional intercellular communication.

Methodology

Microelectrodes

Gap junctions are usually identified by their permeability to ions and specific fluorescent dyes, which allows measurements of intercellular transfer of electrical signals or of dye spread. Signal recording and iontophoretic injection of currents or molecules are performed with glass microelectrodes. Capillaries of borosilicate glass (1 mm outer diameter, Hilgenberg) with inner filaments are pulled into micropipettes by means of a vertical (e.g., David Kopf Instruments) or a horizontal (Narishige) pipette puller. Prior to the experiment they are filled with the desired electrolyte fluid from the open end via stainless steel hypodermic tubing. The internal filament facilitates bubble-free filling down to the tip, which has a diameter of less than 0.5 µm. Electrodes filled with 3 *M* KCl are used for voltage recording and current injection. For iontophoretic injection of junction-permeable fluorescent dyes, e.g., Lucifer Yellow (Sigma), the tip is filled with a 4% dye solution overlayed by a 0.1 *M* LiCl solution. The electrical resistance of the KCl-filled electrode is about 20–40 MΩ, whereas that of the LiCl/Lucifer Yellow electrode amounts to 100–200 MΩ. The electrolyte-filled glass microelectrodes are then connected to Ag/AgCl or Au/AgCl electrodes in a plexiglass holder adapted to an appropriate high-impedance amplifier.

We use WPI preamplifiers (model M750) for membrane potential measurements, and WPI M701 or List L/M-1 for current or dye injection and for membrane potential measurements. The microelectrodes are operated by means of micromanipulators (Leitz or Marzhauser) under a microscope with a fixed table and equipped with phase contrast, differential interference contrast, and epifluorescence illumination. Shock-absorbent mounting of the setup is recommended.

Three-Electrode Technique

Electrical coupling resulting from intercellular electrical signal transfer across the gap junctions, can be determined in different ways. For the three-electrode technique (Fig. 1), two high-impedance amplifiers and one pulse generator are used to measure the membrane potentials in two cells and to inject a pulsed hyperpolarizing current into one of them. In Fig. 1, a hyperpolarizing constant-current pulse injected into the left cell results in a pulsed hyperpolarization signal V1 across the cell membrane. This signal is superimposed onto a potential difference (PD1), mainly due to the negative membrane resting potential. In the neighboring cell (on the right in Fig. 1), a similar dc signal (PD2) occurs. When the two cells are electrically coupled a hyperpolarization signal is also observed (V2). The signals are either displayed on an oscilloscope or recorded by a suitable device. The ratio V2/V1 may be taken as an indicator for the amount of coupling, which gives comparable values, however, only when isolated cell pairs are investigated. The more cells couple together the smaller the values of V1 and V2 become, due to spreading of the constant current into the coupling cells. In principle, this method also allows determination of the cellular "input-resistance," which is the effective transmembrane resistance "seen" by the dc recording electrode (see section "One-Electrode Technique").

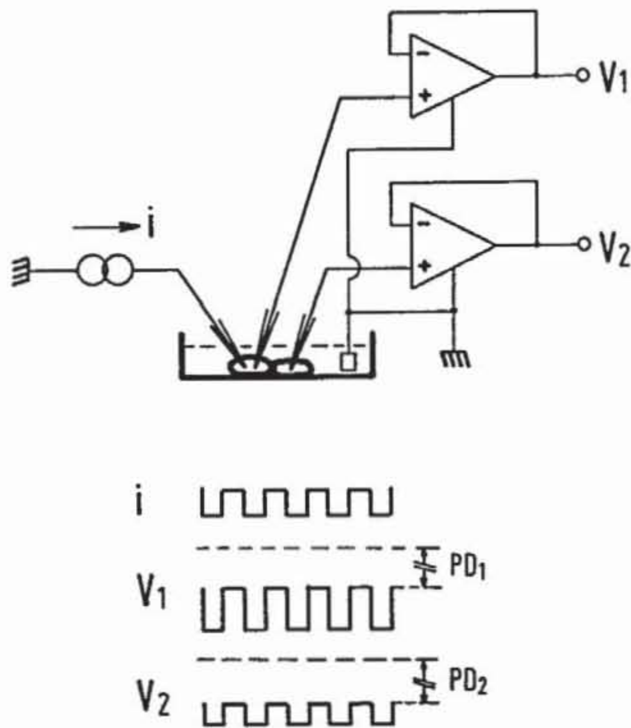


Fig. 1. Schematic representation of the three-electrode technique for measuring electrical cell-to-cell coupling. A rectangular constant-current pulse is fed into the left cell, where it causes a hyperpolarization signal *V1* superimposed on the negative membrane resting potential (*PD1*). When the cells in contact are electrically coupled a hyperpolarization signal (*V2*) is also observed, with the negative membrane resting potential (*P2*) in the neighboring cell

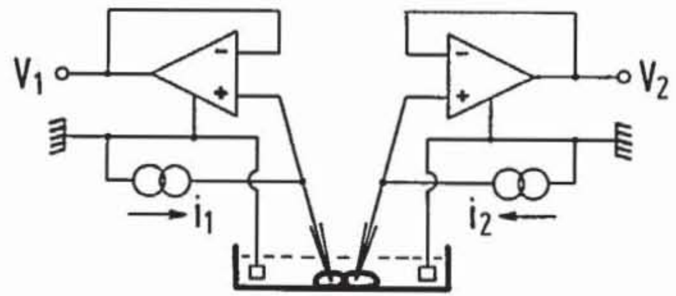
Manipulation of three electrodes is usually rather troublesome since it requires insertion of two electrodes into one cell. Not many cells survive this procedure for a sufficiently long time. Thus, other methods, requiring fewer than three electrodes, have been designed for detecting the low-resistance junctions.

Two-Electrode Technique

A separate current injection electrode is no longer required when the constant-current pulse is injected directly through the recording electrode. A typical setup is schematically shown in Fig. 2. In detail, this requires a bridge circuit, which allows for compensation of electrode resistance and leaves the gain characteristics unchanged during current injection. These amplifiers are commercially available (WPI M701 or List L/M-1). Use of two of these amplifiers provides the advantage of measuring membrane potentials (PD) in both cells and allows simultaneous passage of currents between adjacent cells in both directions without changing electrodes. However, the bridge adjustment is crucial and may easily lead to erroneous measurements of the coupling ratio since the electrode resistance can change noticeably upon impalement of a cell. Cautious interpretation of the signal of the impaled cell is required, and rebalancing of the bridge circuit with electrodes inserted will be necessary in most cases. This method is recommended when rather qualitative measurements of cell coupling are sufficient.

One-Electrode Technique

Coupling can also be determined by simply measuring the input resistance. This requires only one electrode, which serves both as injecting and recording electrode. Constant-cur-



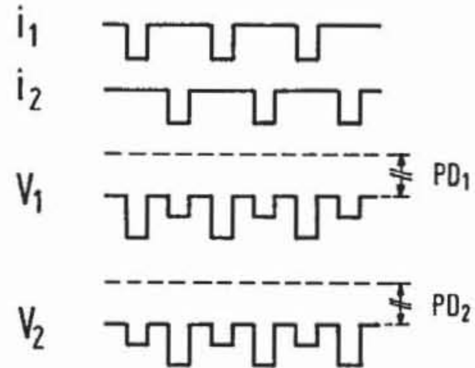


Fig. 2. Schematic representation of the two-electrode technique for measuring electrical coupling. Constant-current signals are intermittently fed into two cells in contact. If the cells are coupled the hyperpolarization signal of each cell (*V1, V2*) exhibits a contribution resulting from its own current pulse plus a smaller contribution from the current injected into the neighboring cell

rent pulses of 50 Hz applied to the electrode input of a high impedance amplifier (Fig. 3) cause a voltage change V according to the electrode-tip resistance, which can be determined by calibration with known resistors. With the tip inserted into a cell the effective membrane resistance (input resistance) is additive to the electrode resistance. The difference between the voltage change caused by an inserted electrode and that of the same electrode in the medium gives a fairly good estimate of the input resistance of the impaled cell (Fig. 3). Coupling can be inferred from measuring input resistances of both isolated cells (R') and cells in contact (R''), e.g., in monolayers or spheroids. Since the input resistance decreases with increasing effective membrane surface available for the passage of the current into the extracellular medium, R'' is smaller than R' if the cells are connected by low-resistance junctions, whose resistance is three orders of magnitude lower than the input resistance. Thus, the ratio R'/R'' can be used as a measure for the degree of coupling. $R'/R'' = 1$ indicates uncoupled cell lines; R'/R'' increases with rising quality of the coupling.

Because of its capacitance, the cell plasma membrane acts as a high pass filter. Therefore, with a sifficiently high frequency (> 1,000 cycles) of the current pulses, the resistance of an inserted electrode can be determined without interference with the input resistance. The one-electrode technique requires this additional checking of the unchanged resistance of an inserted electrode. Minor resistance changes of the inserted electrodes can be attributed to the different ionic composition of the cell plasma (Hülser 1974). However, this does not influence the results, since measurements in isolated and in adjoining cells of the same line are compared.

The one-electrode procedure tacitly assumes that the membrane resistance of the individual cells does not change with increasing cell density which would also influence the ratio R'/R''. Thus, it is recommended that the coupling be checked by at least one of the other methods discussed in this section.

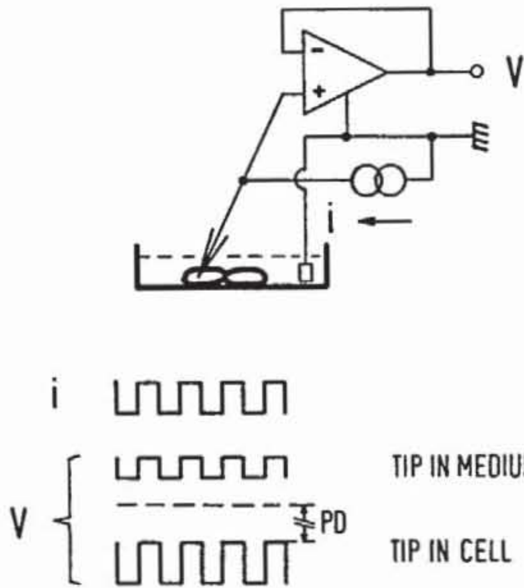


Fig. 3. Schematic representation of the one-electrode technique for measuring cellular input resistances. A constant current injected into a cell causes a signal V at the amplifier output, which is proportional to the sum of the electrode-tip resistance (measured with the tip immersed in the medium) and of the cellular input resistance

Dye Coupling

The existance of cell-to-cell channels can also be made apparent by injection of a fluorescent dye and subsequent observation of the dye spreading into neighboring cells. The injection of ionized dye molecules is easily performed by iontophoresis with glass microelectrodes and an appropriate current supply. The List L/M-1 amplifier provides a built-in iontophoresis device, which allows preselection of current settings for both "inject" and "retain" modes. In addition, the membrane potential of the injected cell is measured during this procedure with the same dye-filled glass microelectrode. For demonstration purposes this method is applied to monolayers of uncoupled mouse L-cells (Fig. 4) and coupled rat BICR/M1R-K cells (Fig. 5). Dye coupling between cells of small BICR/M1R-K spheroids is demonstrated in Fig. 6. Although Lucifer Yellow (Figs. 4–6) has become the most extensively used dye, other dyes can also be used. In particular, investigation with dyes of different molecular weight and diameter allows estimation of the channel bore size of the gap junctions (Rose 1980; Flagg-Newton 1980).

Electron Microscopy

The technique of freeze-fracturing biological material permits the analysis of large areas of plasma membrane fracture faces. Since gap junctions span the membranes of attached cells, their integrated protein complexes are visualized with a very high yield when this cryo-method is used. As long as only the existence of gap junctions between the cells of a spheroid is to be demonstrated, an advanced freezing techniques is not required. However, as soon as more detailed information is required, for example with respect to an open or closed status of the gap junction pores, a cryofixation method with a high cooling rate must be applied (see Raviola et al. 1980).

The "standard" freezing technique requires chemical fixation of the biological material. Spheroids may be transfered into 3% glutaraldehyde in phosphate-buffered saline and fixed for about 60 min at room temperature. After careful decanting of the glutaraldehyde, the spheroids are washed several times in phosphate-buffered saline. Finally, glycerol solutions in phosphate-buffered saline are repeatedly added, increasing concentrations of

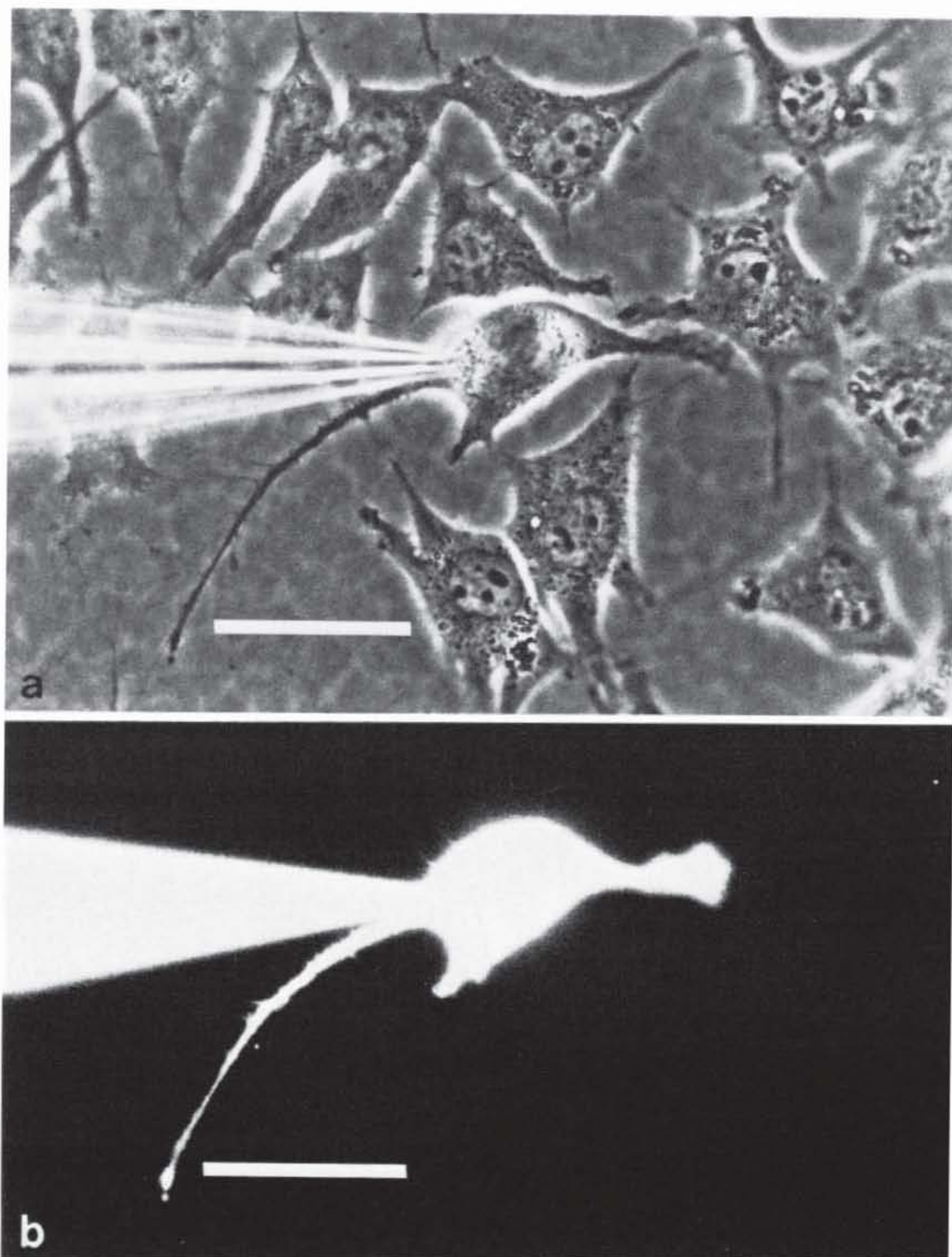


Fig. 4. a Mouse L cells grown two-dimensionally in a plastic petri dish (phase contrast). Capillary indicates cell where Lucifer Yellow was injected. *Bar length*, 50 μm; **b** Fluorescence epi-illumination of the same cells after Lucifer Yellow injection. No dye spreading can be observed: L cells are not coupled

glycerol being used up to a final concentration of 30%. After infiltration overnight at 4° C, the spheroids are freeze-fractured (without etching) and replicated in a Balzers BAF 301 instrument at a stage temperature of −150° C. The fractured membranes are shadowed with platinum/carbon and evaporated by a high-voltage electron gun, and the replicas are reinforced with carbon. The replicas are then cleaned with sulfuric acid and sodium

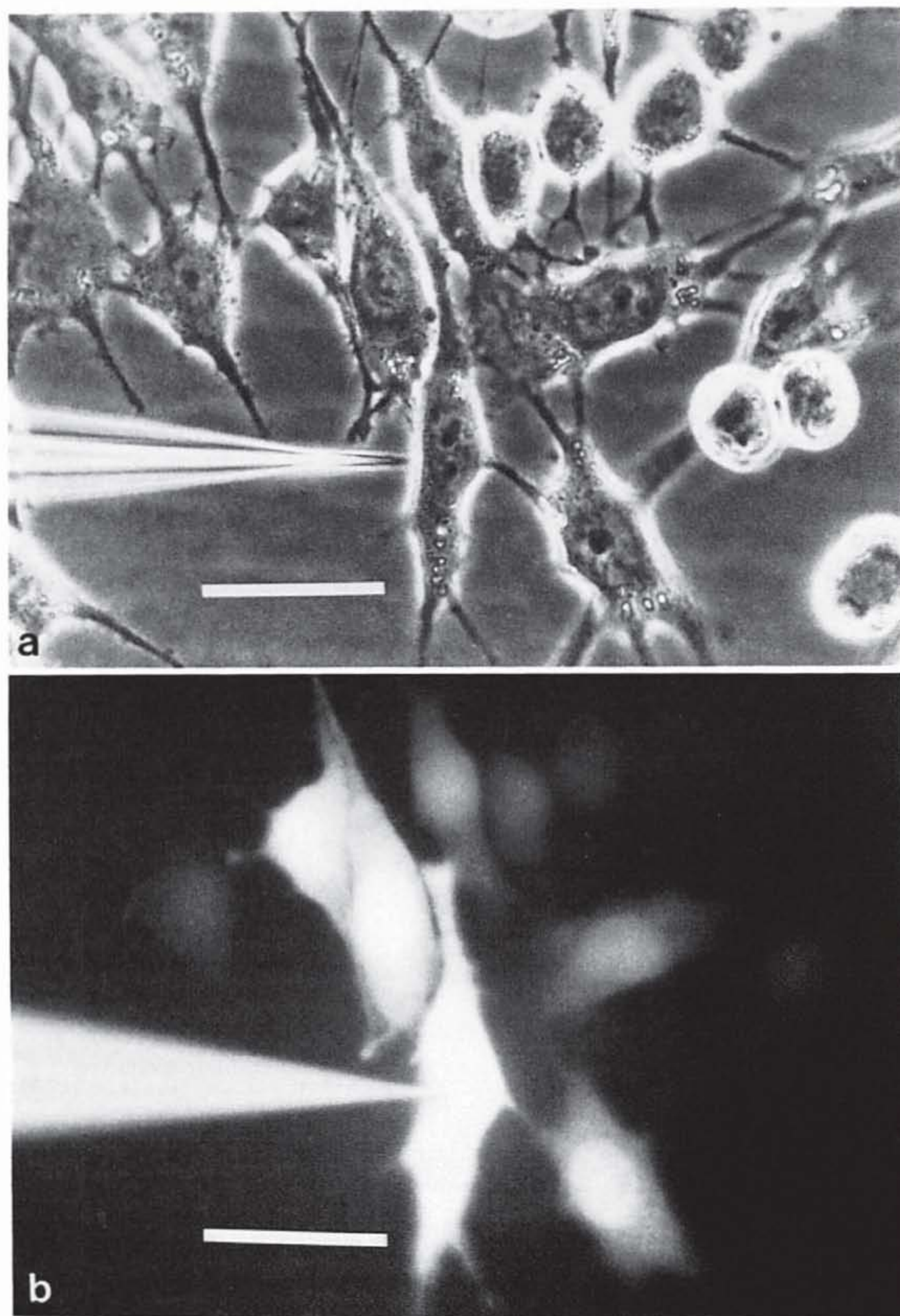


Fig. 5. a Rat BICR/M1R-k cells grown two-dimensionally in a plastic petri dish (phase contrast). Capillary indicates cell where Lucifer Yellow was injected. *Bar length* 50 μm; **b** Fluorescence epi-illumination of the same cells after Lucifer Yellow injection. Dye spreads into neighboring cells: BICR/M1R-k cells are coupled

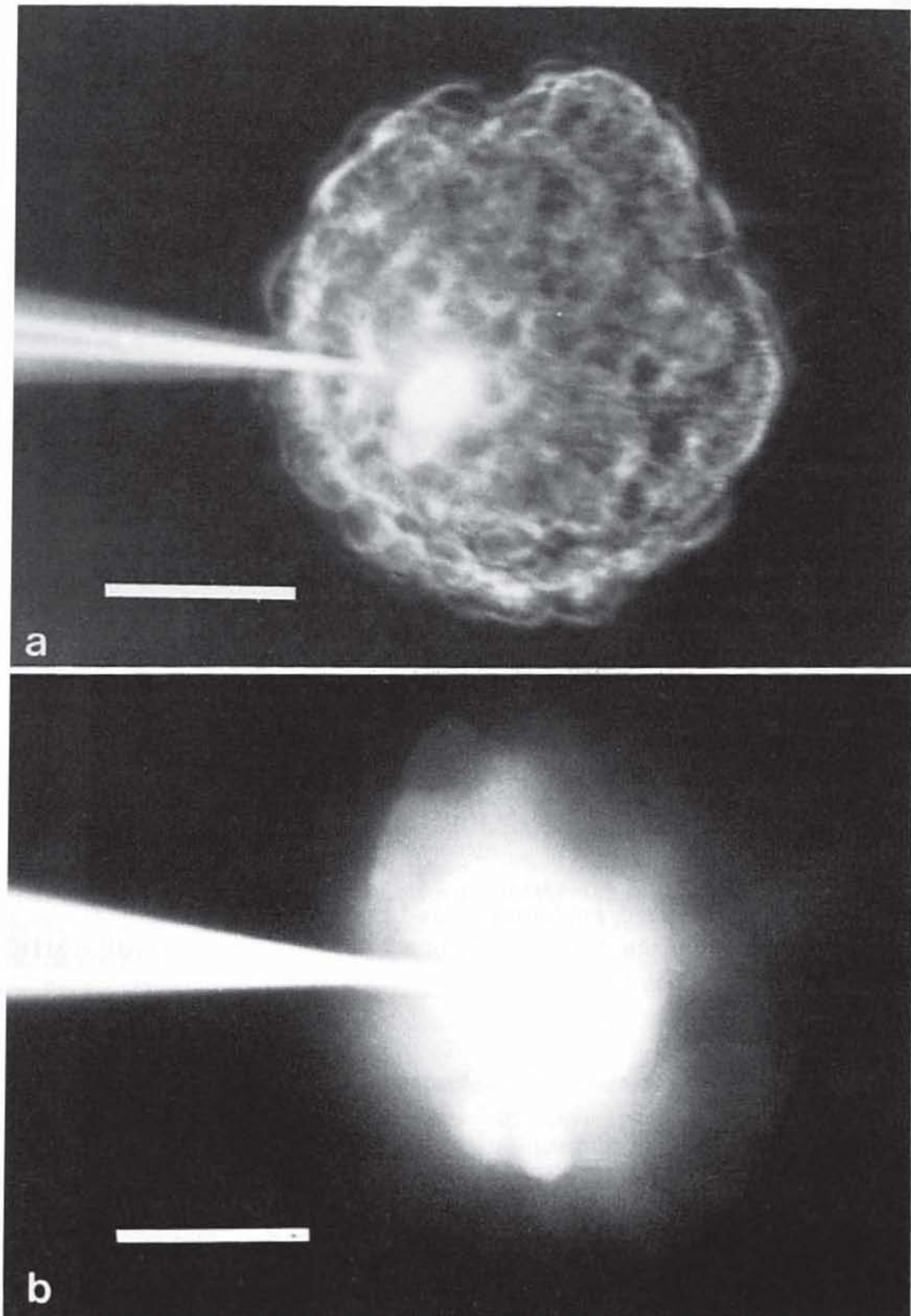


Fig. 6. a Rat BICR/M1R-k cells grown for 3 days as multicellular spheroid in a spinner flask (phase contrast). *Bar length* 50 µm; **b** Fluorescence epi-illumination of the BICR/M1R-k spheroid after Lucifer Yellow injection. Dye spreads into neighboring cells

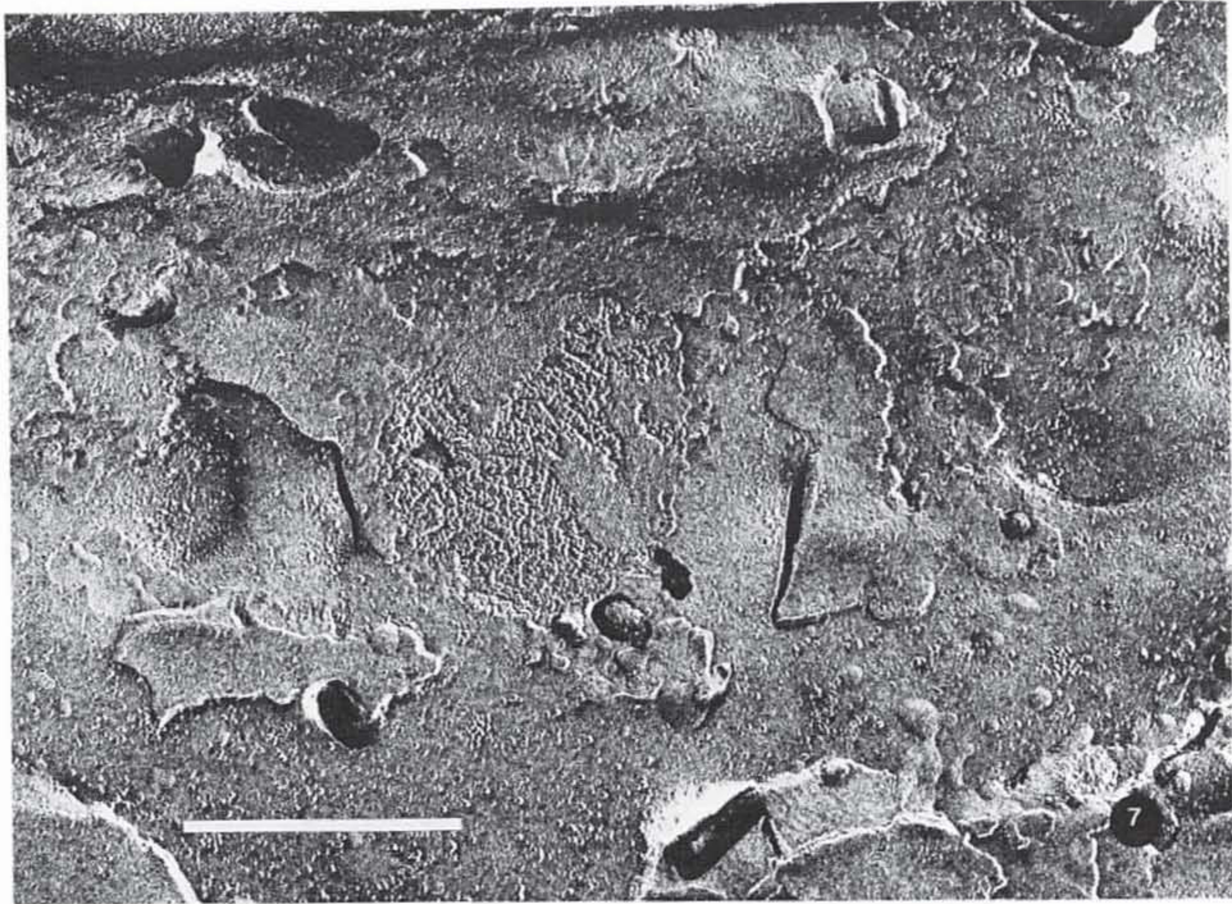


Fig. 7. Gap junction betwen BICR/M1R-k cells grown as spheroid for 2 days (freeze-fracture replica). *Bar length* 0.05 μm

hyposulfite and mounted on Formvar- and carbon-coated 50-mesh copper grids. An example of a gap junction between cells of a 2-day-old spheroid of BICR/M1R-k cells is shown in Fig. 7.

Biophysical and Biochemical Effects Associated with Intercellular Communication

Junctional intercellular communication, in terms of exchange of biochemical and biophysical signals, is generally accepted to be a prerequisite for homogeneous and specific functioning of a tissue. On the other hand, specific functioning of a tissue requires certain modifications in the behavior of the individual cells, which could themselves be induced by the processes associated with junctional communication. We present evidence here for such physiological changes ("differentiation") occurring in spheroids of coupled cells which are not seen in coupled monolayers or in noncoupling cells.

Cyclic AMP and Gap Junctions

Convincing evidence showing that a particular biochemical signal is involved in the functioning of the gap junction has been obtained by various authors. There is general agreement that exogenous cAMP or intracellular pulses of cAMP produced by the

Table 1. Cyclic AMP content[a] of cells from spheroids (diameter 0.3 mm) and from exponential monolayers of two solid human tumor cell lines: NA 11 (melanoma) and HT 29 (colic adenocarcinoma)

	NA 11	HT 29
Monolayer	0.20 ± 0.01	0.31 ± 0.03
Spheroids	0.23 ± 0.01	0.21 ± 0.03
Electrical coupling (monolayer)	No	Yes

[a] pmol/µg DNA; competitive binding assay kindly provided by F. Laval, IGR, Villejuif, France

interaction of certain hormones with plasma membrane receptors may change the junctional permeability. For example, studies on salivary glands of larvae of *Drosophila hydei*, in which intracellular cAMP levels are elevated by incubation of the gland in a medium containing either dibutyryl-cAMP, theophylline, or ecdysterone, have shown that increases in intracellular cAMP are accompanied by increases in gap-junctional permeability (Hax et al. 1974). Recently, Radu et al. (1982) described upregulation of gap-junctional permeability by exogenous dibutyryl-cAMP and also by prostaglandin E_1 or isoproterenol. Even the formation of gap junctions can be induced by cAMP, as has been shown by Amsterdam et al. (1981). Using cultured rat granulosa cells, these authors found that exposure to follicle-stimulating hormone induced the cells to become highly aggregated and to develop gap junctions and luteinizing hormone receptors. 8-Bromo-cAMP or cholera toxin, a potent activator of adenylate cyclase, was also able to induce this type of differentiation, which was not seen, however, in untreated granulosa cells primarily growing as monolayers.

The fact that cAMP may be a key substance for understanding of the function of the gap junction prompted us to measure cAMP synthesis in cultured cells of known capacity for gap-junctional interaction and with special reference to the influence of three-dimensional growth conditions (Dertinger et al. 1982). The results obtained with monolayers of four different mammalian cell lines followed the usual pattern of cAMP synthesis with the maximum level in the plateau phase.

However, an unexpected result was obtained when spheroids 0.3 mm in diameter were assayed. Whereas in spheroids of noncoupling cells the activity of adenylate cyclase increased relative to monolayers, a drastic fall in enzyme activity was observed in the coupled spheroids. In fact, the enzyme activity was much lower than under any other culture condition (single cells and monolayers, respectively).

Table 1 shows the levels of intracellular cAMP for monolayers and spheroids of two human solid tumor lines: the melanoma NA11 (not coupled) and the colic adenocarcinoma HT29 (coupled; see Dertinger et al. 1983 for coupling results). As expected from the above results, there is a slight increase of cAMP (monolayer vs spheroid) in NA11, but a fall in HT29. The reduction of cAMP in spheroids of coupled cells sharply contradicts the common view that the plateau phase is characterized (and even induced) by an increase in cAMP synthesis (e.g., Rudland et al.1974). In fact, spheroids contain a certain fraction of noncycling cells (Dertinger and Hülser 1981) which, for the spheroids investigated here, may amount to roughly half the spheroid cell population. As in the two-dimensional case, therefore spheroid cells should never contain less cAMP than cells taken from proliferating monolayers.

This shows that concepts established from experiments with monolayers may not necessarily be valid for the more realistic situation of a three-dimensional cell matrix. From the findings discussed above it may be expected that certain cAMP-dependent processes (e.g., protein phosphorylation) are changed in spheroids of coupled cells. In biological terms this could lead to a functional differentiation of cells not seen in monolayers or in spheroids composed of noncommunicating cells.

Closing of Gap Junctions in Spheroids

From the observation that gap-junctional permeability can be upregulated by cAMP one could expect that the reduced cAMP synthesis in spheroids might be correlated with a decrease in intercellular communication. In fact, a decrease in coupling with increasing spheroid age (size) was demonstrated by Hülser and Brümmer (1982) and by Dertinger et al. (1982), using spheroid of different cell lines. Hülser and Brümmer, who besides electrical coupling and dye-exchange measurements performed electron-microscopic investigations, presented evidence that the loss of gap-junctional permeability is due rather to closing of the gap junction channels than to a decrease in frequency of the gap junctions. In contrast to spheroids, monolayers showed stable coupling, even at confluence (Hülser and Brümmer 1982).

The mechanisms promoting closing of the gap junction channels in spheroids are not fully understood. In principle, an increased intracellular Ca^{2+} level and/or a lowering of intracellular pH could account for the observed uncoupling (see Loewenstein 1981, or De Mello 1982 for a review of the pertinent results). However, since most of the evidence for the uncoupling capacity of Ca^{2+} and pH stems from rather unphysiological treatments of the cells (injection of Ca^{2+} or exposure to 100% CO_2) it must be questioned whether these results also describe the situation in spheroids. Here uncoupling occurs "spontaneously" under entirely physiological conditions and between the fully intact cells of the outer spheroid cell layer. More work is required to demonstrate that physiological changes in Ca^{2+} or pH could trigger gap-junctional uncoupling. With regard to pH, this requires detailed information as to whether physiological elevation of intracellular acid concentration, such as lactate, in otherwise unaffected cells would be sufficient to interrupt intercellular communication.

Nevertheless, closing and opening of gap junction channels appears to be a widespread phenomenon among multicellular, in particular differentiation-competent, systems. For example, Lo and Gilula (1979b), using mouse embryos, observed partial segregation of cell communication, as indicated by a limitation of dye spread with increasing embryonic differentiation. Although coupled spheroids are basically aggregates of communicating cells, they are not comparable to early embryos with respect to their potential for differentiation. However, it cannot be ruled out that the decreased junctional coupling in spheroids also reflects some level of differentiation that cannot occur in monolayers.

Intercellular Communication and Radiosensitivity

The "Contact Effect"

One of the earliest observations made with Chinese hamster V 79 cells when cultured and γ-irradiated as spheroids was an apparently increased radioresistance over that in

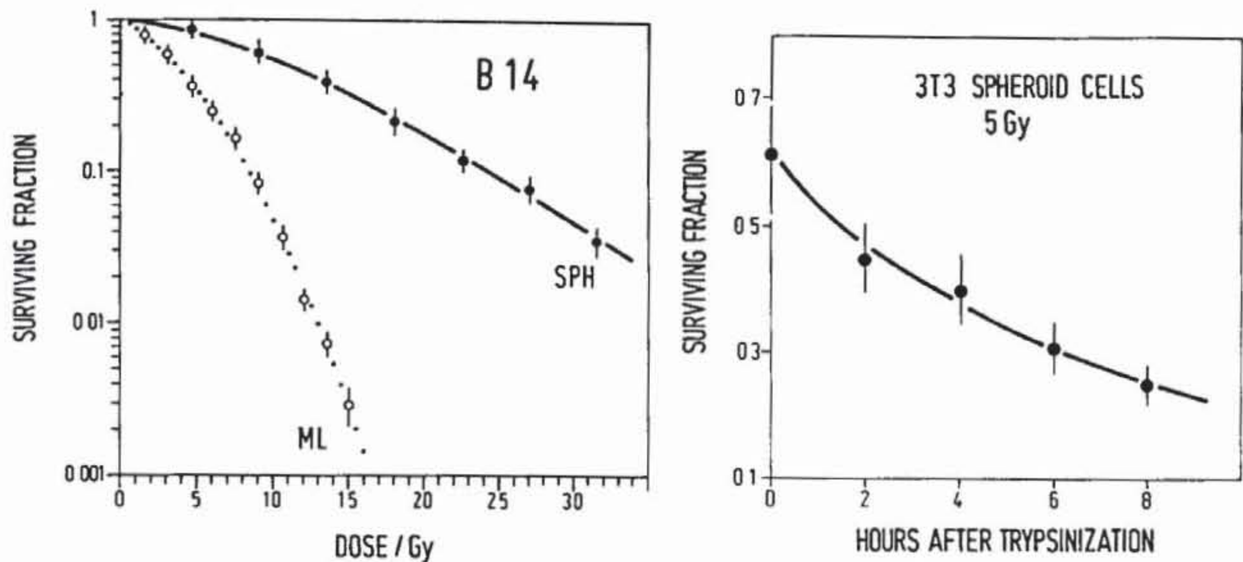


Fig. 8 *(left)*. Survival curves after γ-irradiation of *B 14* FAF 28 monolayer (*ML*) and spheroid (*SPH*) cells, based on the colony-forming criterion. Due to CE, the spheroid cells (diameter of spheroids 0.3 mm) are more radioresistant than the monolayers. (Dertinger et al. 1982)

Fig. 9 *(right)*. Decay of CE in 3T3 spheroids following destruction of cell contact by trypsinization. The fraction of colony forming cells after a dose of 5 Gy is plotted versus time between trypsinization and irradiation; t = 0 represents the survival of intact spheroids (diameter 0.25 mm). Survival level of 3T3 monolayers after 5 Gy: 0.16

monolayers of the same clone (Durand and Sutherland 1972, 1973; see also Fig. 8). This phenonemon was termed contact resistance or contact effect (CE), and it was hypothesized that it might be due to an improved repair of radiation damage resulting from extensive intercellular contact of the cells within the tightly packed spheroid matrix. The first indication that CE is a specific property of electrically coupled cells came from the work of Dertinger and Hülser (1981), who found that cell lines existed that did not exhibit CE. Interestingly, these cell lines were not electrically coupled, whereas those showing a CE were coupled. Quantitative determination of coupling in terms of input resistances (see section "Methodology") revealed that the magnitude of CE could be correlated with the strength of coupling. (Note that in this investigation electrical coupling was tested in monolayers, thus ensuring that the maximum coupling capacity was determined and that the results were not influenced by the uncoupling phenomenon described in the section "Closing of Gap Junctions in Spheroids.") Meanwhile, the correlation between CE and cell coupling has been established for additional cell lines (Dertinger et al. 1983) and even for human tumor xenografts (Guichard et al. 1983; Dertinger et al. 1984).
More detailed investigations revealed that CE did not depend on the proliferative status of the spheroid cells: the outer, proliferating spheroid cells were nearly as radioresistant as the inner nonproliferating spheroid cells (Hinz and Dertinger 1983). Moreover, CE could not only be demonstrated with respect to cell viability (clonogenicity), but also "protected" cells against several types of cytogenetic damage: in the spheroid cells, irradiation produced less chromosome damage, fewer mutations, and less cell-cycle disturbance than in monolayer cells (Hinz and Dertinger 1983). However, monolayer and spheroid cells did not differ in their capacity to repair DNA strand breaks, as was also found by other authors (Durand and Olive 1979). When the spheroids were trypsinized before irradiation, CE did not disappear immediately. By varying the time interval between trypsinization and

irradiation, a "half-life" of CE amounting to 3–4 h became apparent (Hinz and Dertinger 1983; see also Fig. 9), which is in line with earlier results (Durand and Sutherland 1972). A particularly interesting observation was that very small spheroids were not yet contact-resistant but showed a response to irradiation similar to that of monolayers (Hinz and Dertinger 1983). Only beyond a critical diameter of 0.2 mm did the spheroids become contact-resistant, with no further change in radioresistance as they grew larger. This "switch" to CE also took place in the outer cells (Dertinger et al. 1983).

Although the mechanisms of CE are not yet fully understood, the results presently available may give some hints as to the possible role of the gap junctions in expression of CE. The most elementary function of the gap junctions is probably equilibration of concentration differences of channel-permeant molecules between tissue cells, which creates the basis for tissue homeostasis (Loewenstein 1981). That the coupled spheroids, with respect to CE, behave like a homogeneous tissue is reflected in the uniform switch to CE which occurs within a short time interval of probably only a few hours (Hinz and Dertinger 1983), and in the fact that all cells of the spheroids become contact resistant (Dertinger and Hülser 1981).

However, the present results suggest that extensive junctional communication is only required to induce CE. In fact, CE, once induced, seems to be a single-cell property not requiring strong intercellular communication for its maintenance. The latter conclusion is supported by the decoupling phenomenon in contact-resistant spheroids (Dertinger et al. 1982; see also section "Biophysical and Biochemical Effects Associated with Intercellular Communication"), but also by the preirradiation trypsinization experiment (see Fig. 9 and above): CE is still observed when single cells originating from contact-resistant spheroids are irradiated instead of intact spheroids.

On the other hand, the requirement of strong junctional communication for induction of CE is best demonstrated by the response to prostaglandin E_1 of very small spheroids that are not yet contact-resistant. Hinz and Dertinger (1983) were able to induce CE – although only transitorily – in the small aggregates by a preirradiation exposure to prostaglandin E_1. Since prostaglandin E_1 also stimulates cAMP synthesis, the relevant channel-mediated message for induction of CE could be cAMP (see also sections "Cyclic AMP and Gap Junctions" for discussion of the relationship between cAMP and channel permeability).

From these results, and in particular from the properties of CE, it is tempting to conclude that it may arise from some functional differentiation occurring in coupled spheroid cells. However, as long as a more detailed biochemical characterization of this state of differentiation is lacking, this conclusion remains hypothetical.

Uncoupling Drugs

Further insight into the role of the gap junctions in expression of CE was gained from substances that interfere with the process of differentiation or with the relevant function of the gap junctions, thus eventually eliminating CE. Apart from this scientific interest, chemical inhibition of CE would be of practical significance, for example for improvement of the response of contact-resistant solid human tumors to radiotherapy. Drugs with the capacity to uncouple the gap junctions are of primary interest in this context.

In a first approach to this problem, we have tested two compounds with an established capacity for uncoupling: the tumor promoter TPA (Enomoto et al. 1981), and ouabain (De Mello 1977). We also included procain hydrochloride (novocain), which has also been

Table 2. Survival of B 14 FAF 28 monolayers and spheriods after irradiation with 10 Gy

Drugs	Monolayers		Spheroids	
	Untreated	Treated	Untreated	Treated
Procain HCl (10 m*M*; 6 h)	0.053 ± 0.009	0.088 ± 0.009	0.25 ± 0.03	0.031 ± 0.001
Ouabain (1 m*M*; 6 h)	0.046 ± 0.009	0.040 ± 0.01	0.30 ± 0.05	0.081 ± 0.018
TPA (20 ng/ml; 24 h)	0.035 ± 0.002	0.040 ± 0.001	0.20 ± 0.02	0.046 ± 0.004

The cells were pretreated with various drugs; the survival data of the untreated reference cultures (irradiation only) were measured simultanously and are given for comparison. Standard errors refer to 2–4 different experiments

found to uncouple cells. Monolayers and contact-resistant spheroids of B 14 FAF 28 cells were preincubated with the drugs and then subjected to γ-irradiation with 10 Gy. Uncoupling was monitored by means of microelectrode techniques. The results given in Table 2 refer to exposure protocols for maximum uncoupling but avoiding cytotoxicity. Evidently, as inferred from the survival levels, the contact-resistant spheroids are sensitized towards the monolayer level, whereas the survival of the monolayers is largely unchanged by the uncoupling treatment.

Uncoupling drugs and treatments induce significant alterations in gap junction structure (Délèze and Hervé 1983), but the biochemical mechanisms promoting these changes are not well understood. Possibly a recent observation could augment our present knowledge. Evidence has been presented that anti-calmodulin drugs are potent channel uncouplers (Lees-Miller and Caveney 1982) and that the gap-junction proteins provide binding sites for this important regulatory protein (Welsh et al. 1982). Since calmodulin is the major cellular Ca^{2+} receptor, and since binding of Ca^{2+} is mandatory for the regulatory function of this protein (Means et al. 1982), this again suggest that Ca^{2+} could be involved, directly or indirectly, in structural and functional changes of the gap junctions, such as inducing uncoupling (see also section "Closing of Gap Junctions in Spheroids"). At the same time, the involvement of calmodulin, which is known to mediate the regulation of a large number of fundamental intracellular enzyme systems, lends additional support to the view that some cell-regulatory function is associated with the gap junctions.

Conclusions

This chapter has shown that the response of spheroid cells to gap-junctional communication may lead to certain metabolic and cell physiological changes. It has also become apparent that the functions of the gap junctions are very complex. They may, for example, be related to the fundamental effects of cAMP and/or Ca^{2+}. These lines of evidence should be pursued further. However, further insight into these functions may also be gained from a study of the structure and function of the gap-junctional proteins, as well as from a genetic approach (e.g., Willecke et al. 1982, 1983). In this context, the spheroids are of particular importance as test systems, since they perfectly simulate the three-dimensional arrangement of cells encountered in a tissue. Indeed, the results presented in the sections "Biophysical and Biochemical Effects Associated with Intercellular Communications" and "Intercellular Communication and Radiosensitivity" have revealed clear-cut differences

between cells growing as spheroids or as monolayers in response to communication-dependent processes, which indicate that the response of the monolayers could be somewhat trivial. The advantage of multicellular spheroid systems with three-dimensional growth over monolayer cultures is unquestionable. Cells growing in three-dimensional multicell spheroids may re-establish their regulatory activities and, therefore, match the in vivo conditions more closely. Multicell spheroids allow in vitro investigations on differentiating systems and on interactions between normal and malignant cells, thus substituting costly in vivo experiments.

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