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Intercellular Communication and Tissue Growth

VI. Failure of Exchange of Endogeneous Molecules between Cancer Cells with Defective Junctions and Noncancerous Cells

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Summary. Three cancer cell strains that fail to make permeable membrane junctions were tested for ability to transfer an endogenous hypoxanthine derivative from cell to cell. The cells of these strains, loaded with ^{3}H-hypoxanthine, were grown in contact with cells from a mutant line incapable of incorporating exogenous hypoxanthine. The transfer of the ^{3}H-hypoxanthine derivative to the mutant cells was determined by radioautography and, in the same preparations, the presence of permeable membrane junctions was determined by intercellular fluorescein tracer diffusion and electrical measurement. The cells of the three strains showed no transfer of hypoxanthine derivative to contiguous mutant cells; the cells that make permeable junctions did show such transfer, under the same conditions.

In contrast to this contact-requiring mode of transfer, a contact-independent transfer phenomenon was observed with these three cancer cell strains.

Three strains of cancer cells have been isolated in culture that, unlike normal cells, do not make permeable membrane junctions (Azarnia & Loewenstein, 1971). By means of intracellular injection of exogenous molecules, these cells were shown to be incapable of exchanging inorganic ions and fluorescein (330 mol wt) with each other or with normal cells. In the present work, it will be shown, by a technically entirely different approach, that these cells are also incapable of exchanging endogenous molecules involved in nucleic acid metabolism.

The approach is based on Subak-Sharpe, Bürk and Pitts' (1966, 1969) discovery that cultured cells exchange such molecules. These authors found that an enzyme (inosinic pyrophosphorylase)-deficient mutant cell that cannot incorporate exogenous hypoxanthine into nucleic acid when it grows alone, can do so when it grows in contact with a wild-type cell.

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Recent experiments indicate that probably a nucleotide or a nucleotide derivative is transferred from the wild-type cell to the mutant, bypassing the enzyme block in the latter (Cox, Krauss, Balis & Dancis, 1970; Pitts, 1971). The transfer is operative between cells of the same kind as well as between cells of different kind and different species (Stoker, 1967).

Here we pair the mutant with cells of the three cancerous strains, using it as an indicator of molecular transfer[1]. The cancer cells can incorporate hypoxanthine. Thus, the experiments consist simply of loading the cancer cells with tritiated hypoxanthine and counting the radioactive label in contiguous mutant cells in the radioautographs of the cocultures.

Materials and Methods

Cells and Media

The following cell types were used. *Cells lacking permeable junctions*[2]*: (i) A cell,* a cancerous epithelial cell, derived from Morris' (1965) H-5123 rat liver tumor, 2 to 12 weeks in culture (Azarnia & Loewenstein, 1971); *(ii) A′ cell,* a 3-year old cancerous epithelial cell line derived from the same liver tumor (Borek, Higashino & Loewenstein, 1969); *(iii) XD cell,* a cancerous epithelial cell line derived from X-irradiated embryonic hamster cells (Borek *et al.*, 1969). *Cells with permeable junctions: (iv) B cell,* normally growing fibroblast derived from the same tumor as the *A cell* (Azarnia & Loewenstein, 1971); *(v) liver cell,* an epithelial cell line derived from rat liver (Borek *et al.*, 1969); *(vi) PyY cell,* a polyoma virus-transformed hamster fibroblast line (Stoker & MacPherson, 1964); *(vii) mutant,* an inosinic pyrophosphorylase-deficient mutant of PyY (PyY/TG_1, Subak-Sharpe, 1965).

Cells *i* were cultured in Ham's (1965) F-12 medium containing a twofold concentration of amino acids; cells *ii–v,* in Eagle's medium as modified by Vogt and Dulbecco (1960); and cells *vi* and *vii,* in medium as modified by MacPherson and Stoker (1962). The media were supplemented with 10% fetal calf serum, except the medium for cells *vi* and *vii,* which was supplemented with 10% calf serum. Coculturing was done in the corresponding media of the cell partners (the medium of each partner was used); the same results were obtained in either medium.

Radioautography

Cell Loading with ^{3}H-Hypoxanthine and Coculture. The cells to be tested were mixed with the mutants, and the cell mixtures were grown at 37 °C on the bottom of plastic dishes (60 mm diameter) in medium equilibrated with a CO_2-air mixture containing tritiated hypoxanthine (Schwarz-Mann) 0.5 μC/ml with specific activities of 16 or 6,000 mC/mmole. The coculture periods were 6 to 22 hr in the medium with the high specific activity, and 24 to 48 hr in the medium with the lower one (*see also* p. 257). The minimum specific activity and coculture period in each case was found, in test runs with cells *iv, v* and *vi,* to be adequate for loading of the cells and for demonstrable cell-to-cell

1 The mutant was kindly provided to us by Dr. H. Subak-Sharpe.

2 Cells that fail to make permeable junctions, as determined by intracellular electrical measurement and fluorescein diffusion described by Azarnia and Loewenstein (1971).

transfer of the label. The cocultures were then washed twice in medium containing 10^{-4} M cold hypoxanthine, fixed with 2% glutaraldehyde in the dishes, rinsed in 10% trichloroacetic acid and water, and covered with Kodak NTB-2 emulsion for radioautography. Radioautography exposures were from 7 to 14 days.

Scoring of ^{3}H-Label Transfer. The radioautographic grains were counted in the cytoplasms and nuclei of the mutants for scoring of cell-to-cell transfer of ^{3}H-hypoxanthine label. (In the experiments with ^{3}H-thymidine pre-labelled mutants (*see below*), grains were counted only in the cytoplasm.) Transfer was scored negative when the mean grain count per unit area in the mutants did not differ significantly (99% confidence level) from the mean grain count per unit area of the background. The background counts were taken in the wide spaces between the cells. Control runs showed that this background did not differ significantly (99% confidence level) from the counts taken in mutant cells not in contact with other cells. The mean background counts ranged typically from 12 ± 3 to 17 ± 4 (SD) grains/1,000 μ^2. All data of grain counts in cell-covered area were corrected for background.

Cell Marking. The mutants were readily distinguishable from their A, A′ and XD cell partners by their lack of ^{3}H-hypoxanthine label. The mutants and the A cells were also clearly distinguishable by their morphology and clustering, independently of ^{3}H-hypoxanthine labelling (e.g., Figs. 1 and 2). Morphological distinction of A′ or XD cells from mutants was not possible. In some experiments with these cells, the nuclei were therefore tagged with ^{3}H-thymidine for identification independent of ^{3}H-hypoxanthine labelling. The mutants were exposed in these cases to 0.1 μC/ml tritiated thymidine (specific activity 600 mC/mmole) for 24 hr before cell mixing. The trichloroacetic acid treatment in the radioautographic preparation extracts the cytoplasmic ^{3}H-thymidine, which occurs as the triphosphate (Adams, 1969), leaving only the nuclei marked by the acid-insoluble ^{3}H-thymidine-containing material (Miller, Stone & Prescott, 1964) (e.g., Fig. 4).

Coupling Measurements. Junctional permeability was probed by electrical measurements with intracellular microelectrodes and by iontophoretic microinjection of fluorescein into the cells (Loewenstein & Kanno, 1964; Azarnia & Loewenstein, 1971). The measurements were done on the same cell groups on which the transfer of hypoxanthine-derived nucleotide was studied. Photographs of the live cell regions (phase-contrast or dark-field microscopy) were taken during the measurements. A double vernier built into the microscope stage provided a convenient coordinate system for cell localization in the dishes. The photographs could thus be readily matched with the radioautographs (e.g., Figs. 1, 2 and 5). All measurements were taken at 35 to 37 °C, while the medium was continuously flowed through the dishes by means of a vibration-free, thermoregulated perfusion system. It was necessary to do the measurements at this temperature, because at room temperature (23 °C) the mutant cells tended to retract.

Results and Discussion

Cell Contact-Dependent Transfer

Figs. 1 to 6 illustrate the results obtained with the various cell combinations. The results on contact-requiring intercellular transfer are simple: the cells that make permeable junctions transfer the radioactive hypoxanthine label to the mutant, the cells that fail to make such junctions do not transfer the label.

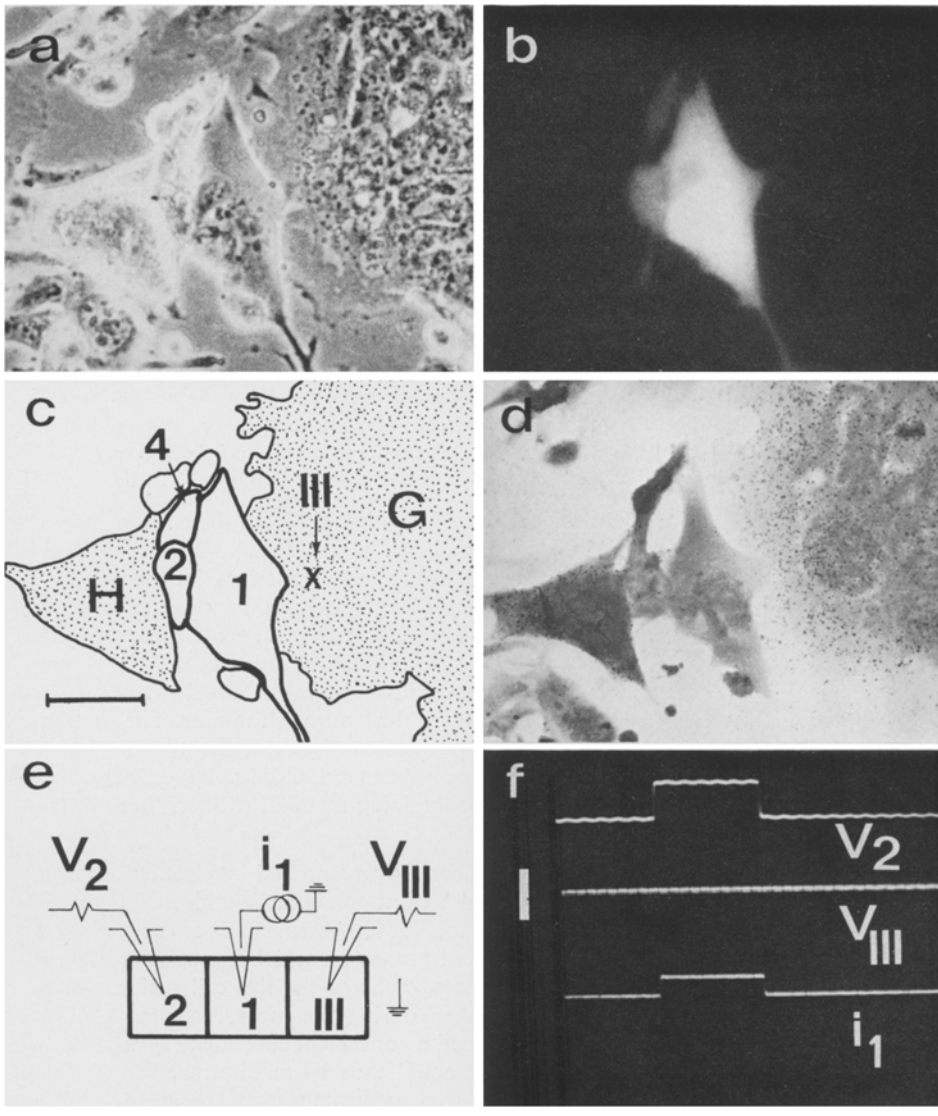

Fig. 1. Lack of molecular transfer between A and mutant cells. *a*, phasecontrast micrograph of the coculture. (For lettering see tracing in *c*.) *1*, *2* and *4* are mutant cells in contact with A cells; the A cells form islands (*G*, *H*). The coculture was exposed for 24 hr to ^{3}H-hypoxanthine, 16 mC/mmole specific activity. The radioautograph *d* shows the A cells heavily ^{3}H-labelled; the label of the mutants does not exceed that of the background. *c*, tracing of the outlines of the mutant cells and of the two A cell islands in contact with these cells (the outlines of many of the A cells in the densely packed interiors of the islands are not clearly visible). Scale for all micrographs, 50 μ. Just before fixation of the culture, junctional coupling was tested by injecting fluorescein into mutant cell *1*. The dark-field micrograph *b* shows the resulting cellular fluorescence after the injection. Fluorescein is seen to have spread from mutant cell *1* to mutant cells *2* and *4*, but not to any of the A cells. Simultaneously with the fluorescein injection, electrical measurements of coupling were taken between mutant cell *1* and contiguous A cell *III* (location of *III* on *G* island, marked by *x*). Current pulses ($i = 1 \times 10^{-8}$ amp; 100 msec duration) were passed between interior of cell *1* and the grounded exterior, as diagrammed in *e*, and the resulting voltages (V) were measured inside cells *2* and *III*. *f*, the corresponding oscilloscope records. Voltage calibration, 50 mV

Fig. 1 shows this for an example of an A cell/mutant combination. Several A cells containing radioactive hypoxanthine are in contact with three mutant cells (*1*, *2*, *4*). The A cells are heavily labelled in the radioautographs; the label in the mutants is merely that of the background (Fig. 1*d*). One of the mutants (*1*) was injected with fluorescein to probe junctional permeability. Fluorescein is seen to have spread to two contiguous mutants (*2*, *4*) but not to any of the A cells (Fig. 1*b*; *see* Fig. 2 for another example). A simultaneous electrical measurement shows the junctional permeability to inorganic ions between two mutants (*1*, *2*) and the

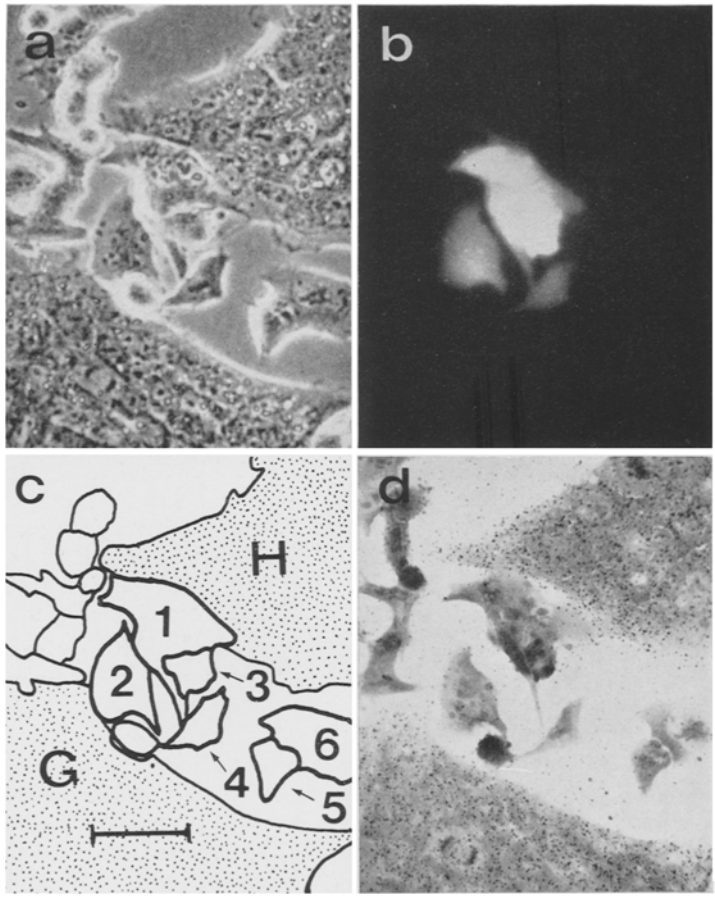

Fig. 2. Lack of molecular transfer between A and mutant cells. *1*, *2*, *3* and *4* are a group of mutants in contact with A cells of islands *H* and *G*. Coculture for 24 hr in ^{3}H-hypoxanthine, 16 mC/mmole specific activity. Before fixation, mutant *1* was injected with fluorescein to test coupling. *a*, phasecontrast photomicrograph; *b*, dark-field micrograph; *d*, radioautograph. Note that the label of the mutants in contact with A cells does not exceed that of the mutants (*5*, *6*) not in contact with A cells or that of the background. *c*, tracing. Scale, 50 μ

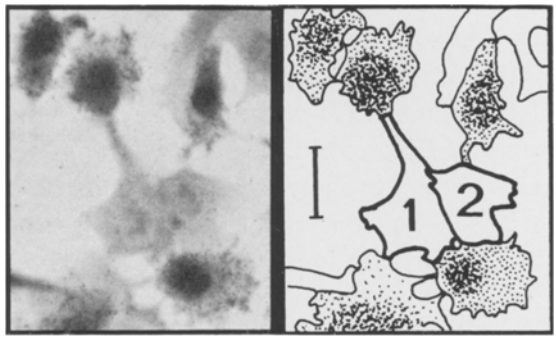

Fig. 3. Lack of transfer of ^{3}H-labelled material between A′ and mutant cells. Mutant cells *1* and *2* are in contact with four A′ cells (heavily labelled). Coculture 18 hr in ^{3}H-hypoxanthine, 6,000 mC/mmole specific activity. *a*, radioautograph; *b*, tracing of same. Scale, 40 μ

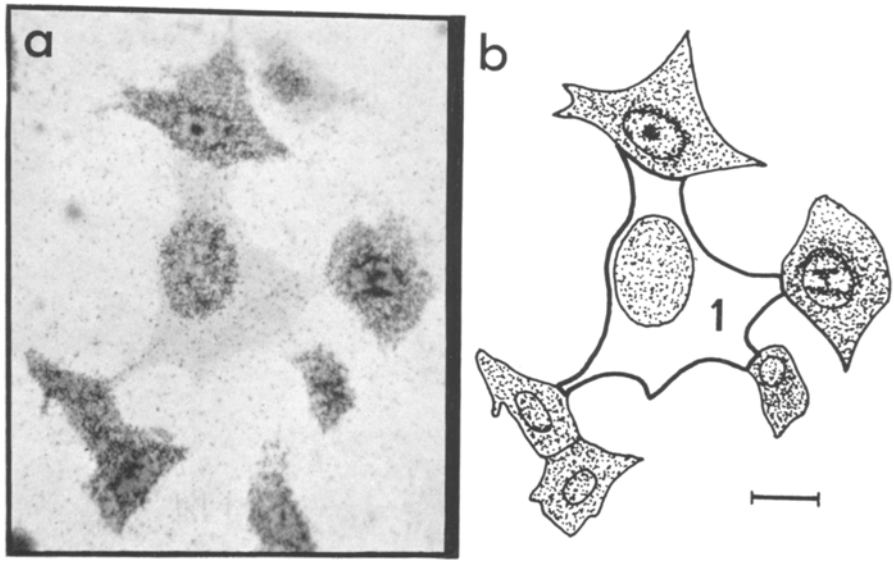

Fig. 4. Lack of transfer between XD and mutant cells. A mutant cell (*1*) in contact with four XD cells. Coculture 18 hr in ^{3}H-hypoxanthine, 6,000 mC/mmole specific activity. *a*, radioautograph. The XD cells (cytoplasms and nuclei) are heavily labelled with the ^{3}H-hypoxanthine-derived material. The nucleus of the mutant cells was tagged with ^{3}H-thymidine for cell identification (*see* Materials and Methods); the ^{3}H-label of its cytoplasm does not exceed that of the background. *a*, radioautograph. *b*, tracing. Scale, 25 μ

lack of such coupling between a mutant (*I*) and an A cell (*III*) (Fig. 1*e*,*f*). Figs. 3 and 4 illustrate similar ^{3}H-transfer failures in experiments in which A′ cells and XD cells were the partners of the mutant; both types of cells

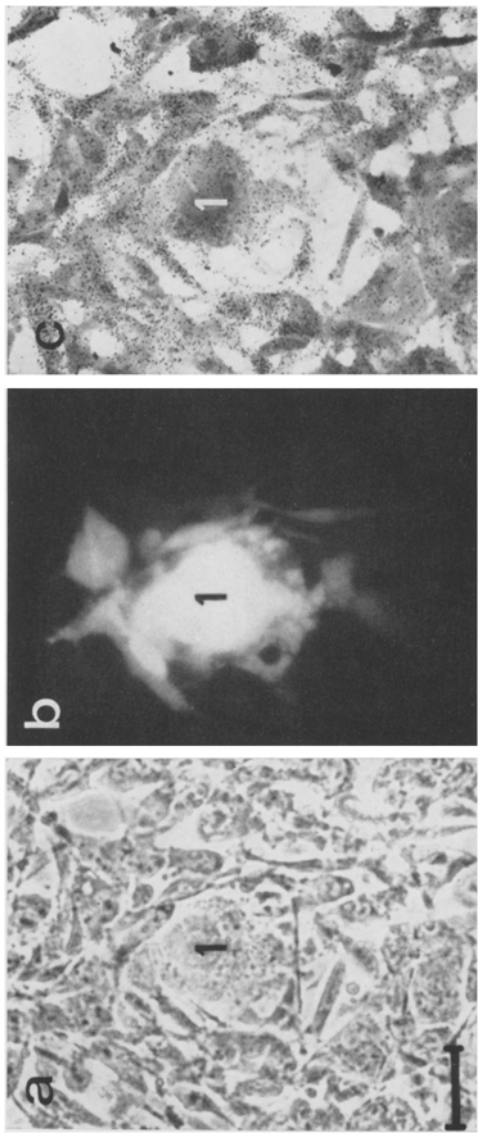

Fig. 5. Molecular transfer between B and mutant cells. Coculture 24 hr in ^{3}H-hypoxanthine, 16 mC/mmole specific activity. *a*, phasecontrast micrograph of the coculture; mutant cell (*1*) is in contact with several B cells. Before fixation, mutant cell *1* was injected with fluorescein which, in the dark-field micrograph *b*, is seen to have spread to many of the B cells in contact. *c*, radioautograph. Scale, 50 μ

also fail to make permeable junctions (Borek *et al.*, 1969; Azarnia & Loewenstein, 1971).

The contrasting behavior of the cells that make permeable junctions is illustrated in Figs. 5 and 6. Mutants in contact with ^{3}H-hypoxanthine-loaded B or PyY cells are clearly labelled above background; and in chain-like cell configurations of the mutants, as in the example of Fig. 6, the label density often diminishes noticeably from cell to cell from the first mutant in contact with the loaded test cell to the last of the chain. Presumably,

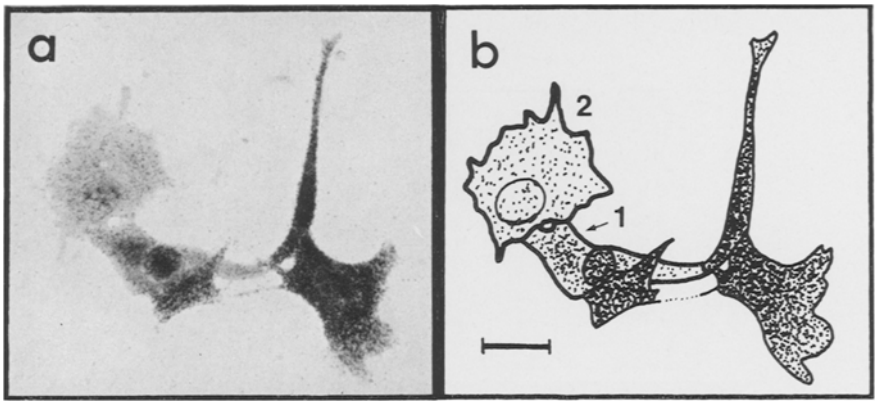

Fig. 6. Transfer of ^{3}H-labelled material between PyY and mutant cells. A string of mutants (*1*, *2*) in contact with a set of three PyY cells. Coculture 18 hr in ^{3}H-hypoxanthine, 6,000 mC/mmole specific activity. *a*, radioautograph. *b*, tracing. Note that the label density of mutant *1* in direct contact with PyY cells is greater than that in mutant *2*. Scale, 40 μ

as in the experiments of Subak-Sharpe *et al.* (1969), the ^{3}H-hypoxanthine-containing material in the labelled mutants came from the loaded test cells in contact. (Mutant cells not in contact do not acquire label.) In the example of Fig. 5, a mutant cell (*1*) was injected with fluorescein. The spread of this tracer shows that the B cells make permeable junctions with the mutant and that the mutants make such junctions among each other (Fig. 5*b*) (for an electrical demonstration of the coupling capability of these cells, *see* Azarnia & Loewenstein, 1971). The PyY cells are also capable of making permeable junctions with the mutants (Michalke & Loewenstein, 1971).

Entirely analogous results were obtained in experiments in which liver cells – another cell capable of junctional coupling (Borek *et al.*, 1969) – were the partners of the mutant.

Transfer of ^{3}H-label was noticeable in nearly all cases where a mutant was in contact with a cell capable of junctional coupling. The transfer could also be demonstrated independently of radioautographic identification of the mutants. Mixtures containing equal numbers of mutants and B cells were seeded 10^6 cells per dish to form confluent cocultures. Essentially, all cells were ^{3}H-hypoxanthine-labelled upon exposure to ^{3}H-hypoxanthine, under these conditions: the cocultures contained cells with grain counts of the same high order as the pure B cell cultures, and cells with somewhat lower grain counts (presumably the coupled mutants), but virtually none with the background count of pure mutant cultures (Fig. 7*a*, *b*, *c*)[3].

3 Pitts (1971) has shown that there is no selective loss or death of the mutant cells under these conditions.

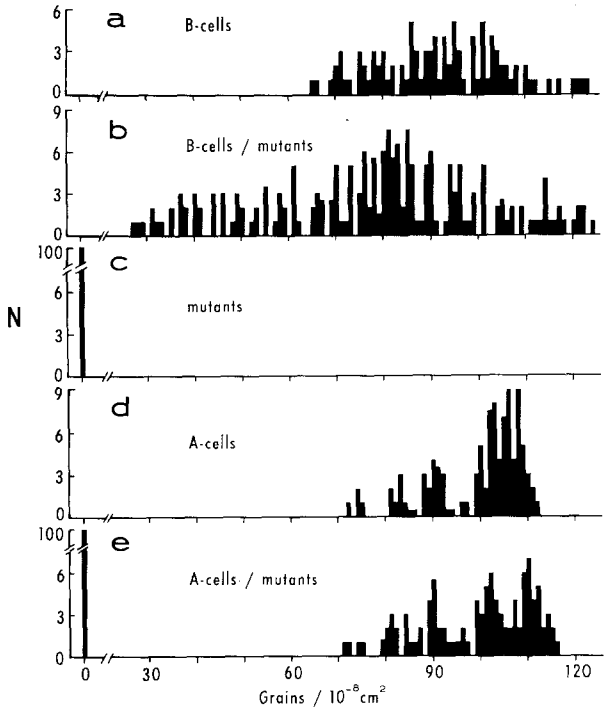

Fig. 7. Histogram of grain counts per 1,000 μ^2 of cell-covered surface. *a*, B cell culture alone; *b*, B cell/mutant coculture; *c*, mutants alone; *d*, A cells alone; *e*, A cells/mutant coculture. All cultures 24 hr in ^{3}H-hypoxanthine, 16 mC/mmole specific activity. The two cell types in each coculture were seeded in equal proportions. Grain counts were corrected for background

The corresponding grain count distribution in the cocultures with the cancerous A cells, that are incapable of junctional coupling, was quite different. These cocultures had cells with as high counts as the corresponding pure cancer cell colonies, as well as cells with counts not above background (Fig. 7*d*, *e*).

The results show a correlation between defective cell-to-cell transfer of ^{3}H-hypoxanthine-derived material and defective junctional transfer of inorganic ions and fluorescein in these three cancer cells. It is not clear whether the two defects correspond to one and the same membrane alteration; i.e., whether the transfer of the hypoxanthine-derived material in the normal counterparts is via the same junctional route as the transfer of inorganic ions and fluorescein. But this would seem likely if the hypoxanthine-derived material is a nucleotide; inosinic acid and other nucleotides have about the same molecular weight as fluorescein. Be this as

it may, it is interesting that the three cancer cell types have defects in membrane interaction demonstrable in different ways.

Contact-Independent Transfer

Under certain conditions, a form of molecular transfer was observable in combinations with A, A′ or XD cells, in which the culture medium appeared to be the vehicle of the transfer. This kind of transfer was shown by coculturing the mutants with 10^6 A, A′ or XD cells for 19 hr in 4 ml of medium (unchanged) containing 0.5 μC/ml of ^{3}H-hypoxanthine with specific activity of 6,000 mC/mmole, and by transferring the medium at the end of this period to a dish with mutants only. After 6 hr of incubation, the grain counts in the mutants of this dish were above background. Also the mutants in the coculture were labelled above background, and this was so regardless of whether the mutants were in contact with A, A′ or XD cells or widely separated from these cells. No such labelling was detectable in coculture when the medium was changed every 3 hr. All this suggests that the A, A′ and XD cells may lose some material involved in nucleic acid metabolism, which may be taken up from the medium by the mutants.

This phenomenon was absent when liver or PyY cells were used in combination with the mutants. The phenomenon is thus distinct from the cell contact-requiring transfer of ^{3}H-hypoxanthine derivatives between cells with permeable junctions, dealt with in the preceding section. We report the phenomenon here, because it may indicate another membrane defect in these cancer cells. We have as yet no information on the nature of the defect beyond the indications that the A, A′ and XD cells lose something, except that this is not simply caused by a general leakiness of their cell membranes; the membrane conductance, at least in the A′ cells, is not very different from that in normal cells (Borek *et al.*, 1969).

The main distinctive feature of the phenomenon is its independence of cell contact. Besides, several other properties, relating presumably to the mode of transfer via the medium, set the phenomenon apart from the contact-dependent transfer of ^{3}H-hypoxanthine material in cells with permeable junctions: the phenomenon depends on the number of ^{3}H-hypoxanthine-loaded cells in the cocultures. It was detectable at A′ or XD cell densities $\geqq 10^6$/dish; the contact-dependent transfer was detectable in cocultures containing only a few loaded liver or B cells as well as when many such cells were present. Detection of the phenomenon required also much higher levels of ^{3}H-hypoxanthine loadings and longer coculture periods than detection of contact-dependent transfer in the coupling cells.

The highest amount of label incorporated in the mutants due to this phenomenon was 42 grains/1,000 μ^2 under the experimental conditions specified above. This is little compared to the amounts due to contact-dependent transfer of ^{3}H-hypoxanthine-derived material from the cells that make permeable junctions (where in all cases the counts were well above 400 grains/1,000 μ^2, the upper limit for resolution of single grains in our radioautographs), but sufficient to mask the results on such transfer from the A, A′ and XD cells, under these conditions. This difficulty did not arise (a) when the coculture medium was changed every 3 hr, (b) when the ^{3}H-hypoxanthine specific activity was lowered to 16 mC/mmole, (c) when the coculture time was lowered to 6 to 8 hr, or (d) when the number of A′ or XD cells in the coculture dishes was reduced to 10^4.[4] Under any one of these conditions, the phenomenon was no longer detectable. (The contact-dependent transfer of ^{3}H-hypoxanthine derivative between the cells that make permeable junctions was clearly detectable under any of these conditions; and the liver and B cells remained electrically coupled to the mutants after changes of medium as in condition (a).) All of the tests on contact-dependent transfer between the three noncoupling cancer cells and the mutants described in the preceding section were therefore carried out under one of these conditions.

The presence of this phenomenon complicates the interpretation of the results on contact-dependent transfer of ^{3}H-hypoxanthine-derived material in the noncoupling cells. If the material lost by these cells is the same ^{3}H-hypoxanthine derivative transferred between coupling cells, it is possible that the lack of such transfer between noncoupling cells reflects this loss rather than a regional difference in membrane permeability at the cell contacts. There is no such uncertainty, of course, in regard to the findings of junctional coupling obtained by the electrical measurements and fluorescein diffusion; the input resistance (and in cells not in contact, the membrane resistance) is high in all the noncoupling cells (Borek *et al.*, 1969), and the injected fluorescein stays in the interior of these cells as well as in those of the coupling cells.

We are greatly indebted to Prof. H. Subak-Sharpe, Glasgow, for providing us with the mutant cells, and to Mrs. Carol Quigley for unfailing assistance with the tissue cultures. Dr. Michalke was a Fellow of the Deutsche Forschungsgemeinschaft. This work was supported by research grants from the U.S. National Institutes of Health and the National Science Foundation.

Note Added in Proof: This work was begun in the winter of 1969 at the Cell Physics Laboratory, Department of Physiology, Columbia University College of Physicians and

4 Condition (a) was tested with all cell types except PyY cells; condition (b) with the A and B cells; conditions (c) and (d) with A′, XD and liver cells.

Surgeons. It was interrupted in 1971 and completed in 1972 after the authors moved to the University of Miami. In the meantime, an interesting paper by N. Gilula, U. R. Reeves and A. Steinbach has appeared (*Nature* **235**: 262, 1972) showing that, in certain fibroblast cultures, the presence of "gap" junctions correlates with electrical coupling and with transfer of ^{3}H-hypoxanthine derivative.

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