Intercellular Transfer of Toxic Components After Laser Irradiation [1,2]

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SUMMARY—The application of heat shock and ruby laser treatment of unstained cells, and argon laser treatment of acridine orange-photosensitized cells showed a loss of intact lysosomes, with the use of the Gomori acid-phosphatase staining reaction. Primary tumor cells, established within a 24-hour period in tissue culture, also were irradiated with an argon laser microbeam after acridine orange photosensitization. The irradiated cell demonstrated zeiosis and nuclear pycnosis which, in a few cases, showed a transmission of toxic products into adherent neighboring cells. The frequency of transmission ranged from 15–45% in most of the tumor cell types, but only 0–5% in nonmalignant cell types.—J Nat Cancer Inst 46: 655–663, 1971.

HELSPER *et al.* (*1*) and McGuff *et al.* (*2, 3*) suggested that ruby laser treatment of human tumors would lead to a progressive necrosis of the tumor cells at sites peripheral to the initial irradiation volume. Others, working with higher energy lasers, failed to observe this phenomenon (*4, 5*). Hence, its explanation has remained obscure until the present time. Recently, reports by Cone (*6*), Furshpan and Potter (*7*), Subak-Sharpe *et al.* (*8*), and by Kanno and Lowenstein (*9*) have suggested that chemical information can be distributed between cells. While these studies have suggested that the information is normally comprised of chemical agents which can enhance metabolism, it seems reasonable to assume that toxic products could be transferred as well. If so, the original observations of Helsper *et al.* (*1*) and McGuff *et al.* (*2, 3*) perhaps could be validated if a toxic substance derived from laser irradiation could be demonstrated to be transferred from an injured cell to its neighbor.

Many studies have indicated that a variety of forms of trauma can release the contents of lysosomes within the injured cell (*10*). This is particularly true of the interaction of photosensitizing dyes, such as neutral red (*11*) with light from the visible portion of the spectrum. It was further reported that acridine orange, when used as a vital dye, is concentrated selectively in lysosomes (*10, 12*). Because of the absorption characteristics of acridine orange, the wavelengths of the argon laser

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are readily absorbed (*13*). It was postulated, as a result of this information, that photosensitization of tumor cells with acridine orange followed by argon laser irradiation should result in the release of toxic products in the form of digestive enzymes normally residing in the lysosomes. In this study we wanted to determine whether laser energy could induce lysosomal lability and cellular injury and to establish whether the toxic components of the injured cell could be transferred to adjacent adhering cells.

MATERIALS AND METHODS

The ruby laser used in this study was able to produce an energy density range from 0–110 j within a 2.5 msec pulse width. The argon laser used to irradiate whole cell populations was operated as a pulsed laser. The pulses were 6 μsec in duration with a repetition rate of 60 pps. The average power output was 1.8 mw. The beam was diverged with a negative lens to cover an area of 1 cm^2. The monolayers of cells were exposed to 10^6 pulses to accumulate a total imposed energy of 0.288 mj/cm^2 or 2880 $ergs/cm^2$. The microbeam system also used a pulsed argon laser with a peak power of 1 w/pulse and a pulse width of 50 μsec. Single pulses of mixed frequencies were focused to an effective target diameter of 0.6–0.7 μ with a calculated energy density of 20 μj.

The cell type used to evaluate the effect of trauma on lysosomal lability was an established line derived from a human adenocarcinoma, designated CMP. This cell line was set up in Rose chambers, which were filled with Eagle's minimal essential medium supplemented with 10% fetal calf serum, and incubated for 48 hours at 37°C. Immediately before treatment, the complete medium was removed and the chambers were filled with Hanks' balanced salt solution (BSS). One set of cultures was heat shocked by placing the chambers in a water bath for 2 hours at 42°C. A second set of chambers was exposed to an unfocused beam from a ruby laser with an energy density of 50 j/cm^2. The cells of a third set were photosensitized with 0.001 mg acridine orange per ml of BSS for 10 minutes. At the end of this period, the dye was removed, and the cells were washed twice with BSS and treated with 2880 $ergs/cm^2$ from an unfocused, pulsed argon laser beam. Sets of control chambers were filled with either BSS or complete medium. After treatment the chambers were incubated at 37°C for 2 hours. At the end of this period, the coverslips containing the cells were removed and fixed in neutral formalin for 1 hour at 4°C and stained for acid phosphatase activity by the method of Gomori (*14*).

A number of freshly excised human malignant tumor biopsy specimens were obtained from surgery. The tissues were minced into 1–2 mm^2 fragments and treated in a 0.125% trypsin solution at 37°C for 10-minute intervals. The supernatant from the first trypsinization was discarded, but the supernatants from the subsequent 2–3 trypsinizations were collected. The isolated cells within the supernatants were centrifuged into pellets, the trypsin solution was removed, and the cells were resuspended in fresh culture medium. The harvested cells were pooled and injected into Rose multipurpose culture chambers.

When the single cells or small clusters of cells were partially spread on the glass surface over a 24-hour incubation period, the medium was withdrawn and replaced with acridine orange solution, as described above. After treatment for 10 minutes, the cells were washed twice with fresh BSS and left undisturbed for 30 minutes. After this short incubation period, fresh BSS was again introduced into the chamber. The argon laser microbeam system, first described by Berns *et al.* (*15*), was used to produce perforations in individual photosensitized lysosomes. The injured cells and their neighbors were observed for up to 2 hours after irradiation.

Cells from a human uterine fibroma were maintained in Rose multipurpose culture chambers until relatively confluent monolayers of cells were formed. After a week or 10 days, small sites of focal necrosis were formed. Some of these cultures were fixed in neutral formalin for 1 hour at 4°C in preparation for demonstrating the distribution of intact lysosomes, with Gomori's method of staining for acid phosphatase.

RESULTS

The acid-phosphatase staining reaction demonstrated the presence of lysosomes, as indicated by

dark-brown bodies within the cytoplasm. Cells that had been subjected to each of the forms of trauma showed a loss of intact lysosomes 2 hours after treatment (figs. 1a, b, c, d). Although these mild forms of trauma caused dispersion of the lysosomal contents, there was no evidence of zeiosis, nuclear pycnosis, or death of the cells.

Irradiation of individual acridine orange-stained cells with the argon laser microbeam showed perforation at the site of impact (figs. 2a, b). The cells were sufficiently traumatized to result in zeiotic blebbing around the cytoplasmic margin. This was followed by nuclear pycnosis and death within 15–30 minutes. In over half the microbeam irradiations, the irradiated cell was the only element affected by this treatment. However, in many other irradiation attempts, toxic products appeared to transfer from the injured cell to adherent neighboring cells, as evidenced by cytoplasmic blebbing, which progressed from the irradiated cell to engulf the entire margin of the adjacent cells within 1–1½ hours. This was followed by nuclear pycnosis and death. In others, no blebbing was evident. Instead, nuclear pycnosis followed cytoplasmic retraction (figs. 3a, b, c, d).

The frequency of this apparent progression of death peripheral to the injured cell was recorded from a total of 1,171 radiation experiences. These results are tabulated in table 1. It can be seen that, with the exception of the squamous cell carcinoma of the lung, the tumor types examined showed a frequency of transferral of toxic components ranging from 15–45%. The same evaluation for the normal tissues examined ranged from 0–5%. Interestingly, one case of endometrial adenocarcinoma and one case of an adenocarcinoma of the lung could be compared with their normal counterparts from the same sources. For the endometrium, the adenocarcinoma showed a 24.3% transmission of the toxic factors, whereas for its normal counterpart no transfer was observed. For the adenocarcinoma of the lung, a frequency of 21.4% of the cell pairs showed transmission of toxic materials, whereas the normal lung showed only 5% spreading of the toxic reaction.

To establish that 1) the toxic components act as lysosomal labilizers; 2) lysosomal labilization can involve several cells in sequence; and 3) the phenomenon can result from cellular degeneration from natural causes as well as from experimental induction, observations from uterine fibroma cultures are presented. The sites of focal necrosis within the fibroma monolayers always contained one or more dead cells which were characterized

Table 1.—Frequency of transmission of toxic factor(s) between adherent cell pairs

Tumor type	Source	Number pairs challenged	Toxic factor transmission		
			+	−	%+
Adenocarcinoma	Colon	48	15	33	31. 3
"	Colon	56	19	37	34. 0
"	Colon	49	18	31	36. 8
"	Appendix	59	22	37	37. 3
"	Parotid	72	31	41	43. 0
"	Breast (male)	50	18	32	36. 0
"	Breast (female)	32	5	27	15. 6
"	Endometrium	41	7	34	17. 1
"	Endometrium*	66	16	50	24. 3
"	Lung*	70	15	55	21. 4
"	Lung	54	8	46	14. 6
Lymphosarcoma	Lymph node	56	21	35	37. 5
Fibroma	Endometrium	81	37	44	45. 6
Epidermoid carcinoma	Phallus	69	27	42	39. 2
Undifferentiated carcinoma	Ocular orbit	56	20	36	35. 7
Squamous cell carcinoma	Lung	62	0	62	0
Nonmalignant	Lung*	60	3	57	5. 0
"	Skin	68	0	68	0
"	Endometrium*	59	0	59	0
"	Endometrium	63	0	63	0

* Normal and malignant cells came from the same organs.

by extensive cytoplasmic vacuolization and pycnotic nuclei (fig. 4, *arrow*). These cells were invariably surrounded by adherent cells showing small-diameter vacuoles scattered throughout their cytoplasm, but had a normal nuclear morphology. The absence of localized cytoplasmic deposits of lead sulfide reflected lysosomal labilization of these cells. On the margin of the zones showing lysosomal depletion were peripheral cells which showed lysosomal-rich cytoplasm at their distal poles, but an absence of intact lysosomes at their proximal poles (fig. 4).

DISCUSSION

An attempt was made to preserve *in vivo* functional capacities by challenge of primary human tumor cells *in vitro* within 24 hours of setup in tissue culture. Within this period, the cells demonstrated membrane mobility and a normal morphology, although optimum cytoplasmic spreading occurred at 48–96 hours of incubation. Despite this precaution, a visual comparison of the transmission of toxic products between 24-hour human uterine fibroma cell pairs with the same cells maintained for 7–10 days suggested that the older cultures had a greater frequency of cellular involvement than the younger ones. While the argon laser-irradiated cells showed a transmission frequency of 45.6% (table 1) to single neighboring cells, the monolayer cultures showed a loss of lysosomes in 5–10 adherent cells surrounding a degenerating cell (fig. 4). This difference could have been due to either the time required for the toxic products to diffuse from cell to cell, or the increased area of contact with the injured cell. If the total area of contact of a cell *in vivo* is considered, the frequency of transmission of toxic materials should be greater.

The lack of 100% transmission of toxic material was considered to be the property of the type of junction that existed between the cell pairs. Morphologic evaluation with the light microscope could not resolve them, but the work of Furshpan and Potter (*7*) and Martinez-Palomo (*16*) suggested that several kinds of junctions are formed between cells. These include desmosomes, nexuses, tight junctions, and simple appositions. Apparently, however, the toxic materials from the injured cell were not diffusing from that cell into the surrounding medium, but diffused only across the adherent cytoplasmic junctions. If the toxic products were diffusing through the culture medium, all cells surrounding the injured cell would show the toxic reaction. This was not true even of some cells which were in close apposition to the injured cell.

Interestingly, the ruby laser could induce a diffusion of acid phosphatase from lysosomes. Since there was no effective chromophore present to absorb the 6943 A wavelength, it was assumed that the laser irradiation produced a mild temperature elevation that resulted in lysosomal labilization of the same type resulting from heat shock.

The precise nature of the toxic product(s) is unknown. It is reasonable to assume that one or more of the enzymes normally contained within the lysosomes were capable of producing the cytotoxic reaction. Studies by Kanno and Lowenstein (*9*), with fluorescence tagging methods, have suggested that small molecular weight substances can be transferred between cells relatively easily, but diffusion in adjacent cells of serum albumin with a molecular weight of 69,000 was limited. However, Subak-Sharpe *et al.* (*8*), using a variant subline of the PyY cells unable to incorporate hypoxanthine because of a deficiency in inosinic pyrophosphorylase, found incorporation of hypoxanthine-H3 when these cells were placed in conjoint culture with the nonvariant parent strain having the normal enzyme. These results suggested that the variant strain showed incorporation of the hypoxanthine because of the transfer of the missing enzyme from an adherent parent cell.

This morphologic demonstration of transmission of toxic material suggests an explanation for the results reported by Helsper *et al.* (*1*) and by McGuff *et al.* (*2*, *3*). The relatively low doses of laser energy could act as a lysosomal labilizer in the same form as heat shock, releasing digestive enzymes to result in death within the irradiated cell. However, at this energy level the injured and dying cells could also release toxic products into adjacent cells which could result in the progressive necrosis described. In contrast, higher energy levels from ruby lasers would produce enzyme inactivation through heat denaturation with a loss of peripheral biologic activity. Therefore,

a reproduction of this experimental result could only be obtained within an optimal laser energy density range.

The preferential transmission of toxic products in the malignant cell suggests a novel approach to the treatment of cancer. The ruby laser is a relatively inefficient system to produce lysosomal labililzation in comparison with various chemical agents. The results of this study suggest that tumors injected with a lysosomal labilizer might induce a progressive necrosis analogous to that described by Helsper *et al.* (*1*) and McGuff *et al.* (*2*, *3*) under economical and practical clinical conditions.

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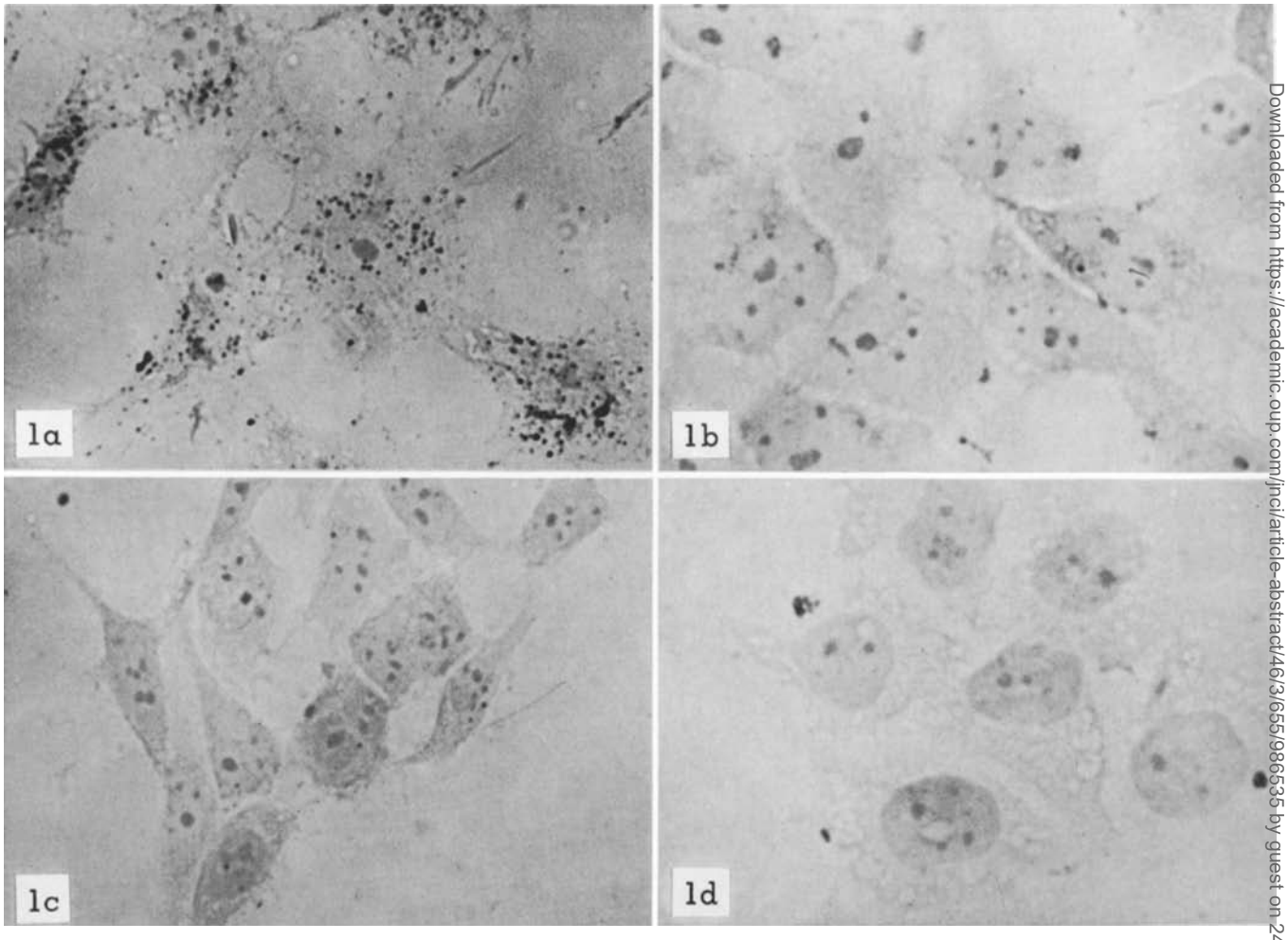

FIGURE 1.—a) Cells of an established line of human adenocarcinoma of the colon (CMP), fixed and stained for acid phosphatase 2 hours after incubation in BSS.

b) CMP cells stained for acid phosphatase activity after 2 hours of incubation at 42°C. Loss of discrete, stained particles indicates lysomal lability.

c) CMP cells after exposure to 50 j/cm^2 from a ruby laser.

d) CMP cells photosensitized with 0.001 mg/ml acridine orange and exposed to 2880 ergs/cm^2 from a pulsed argon laser.

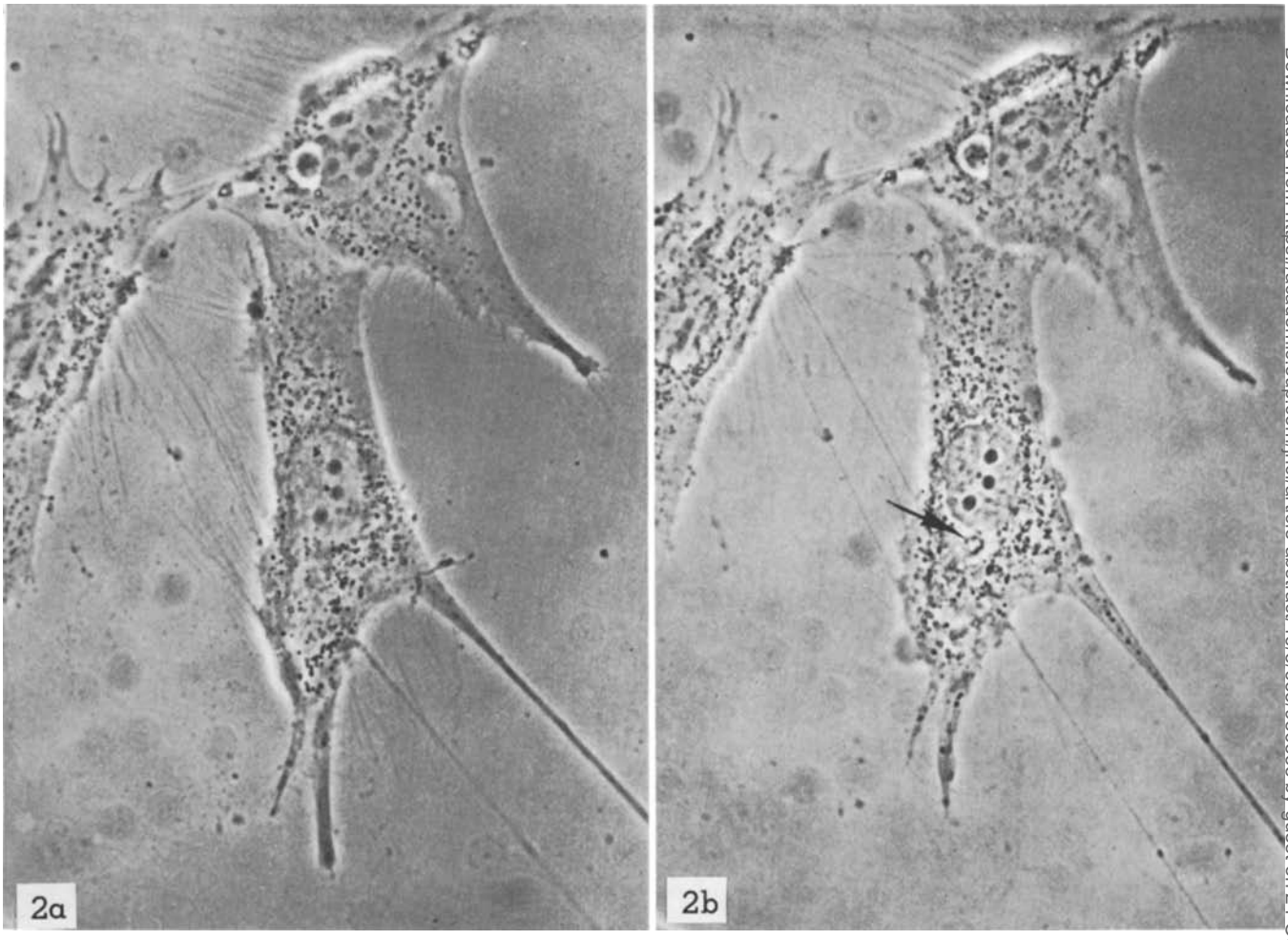

FIGURE 2.—a) Human glioblastoma cell photosensitized with 0.001 mg/ml acridine orange.
b) Same cell 30 minutes after argon laser microbeam perforation (*arrow*). Cellular death followed zeiosis and nuclear pycnosis.

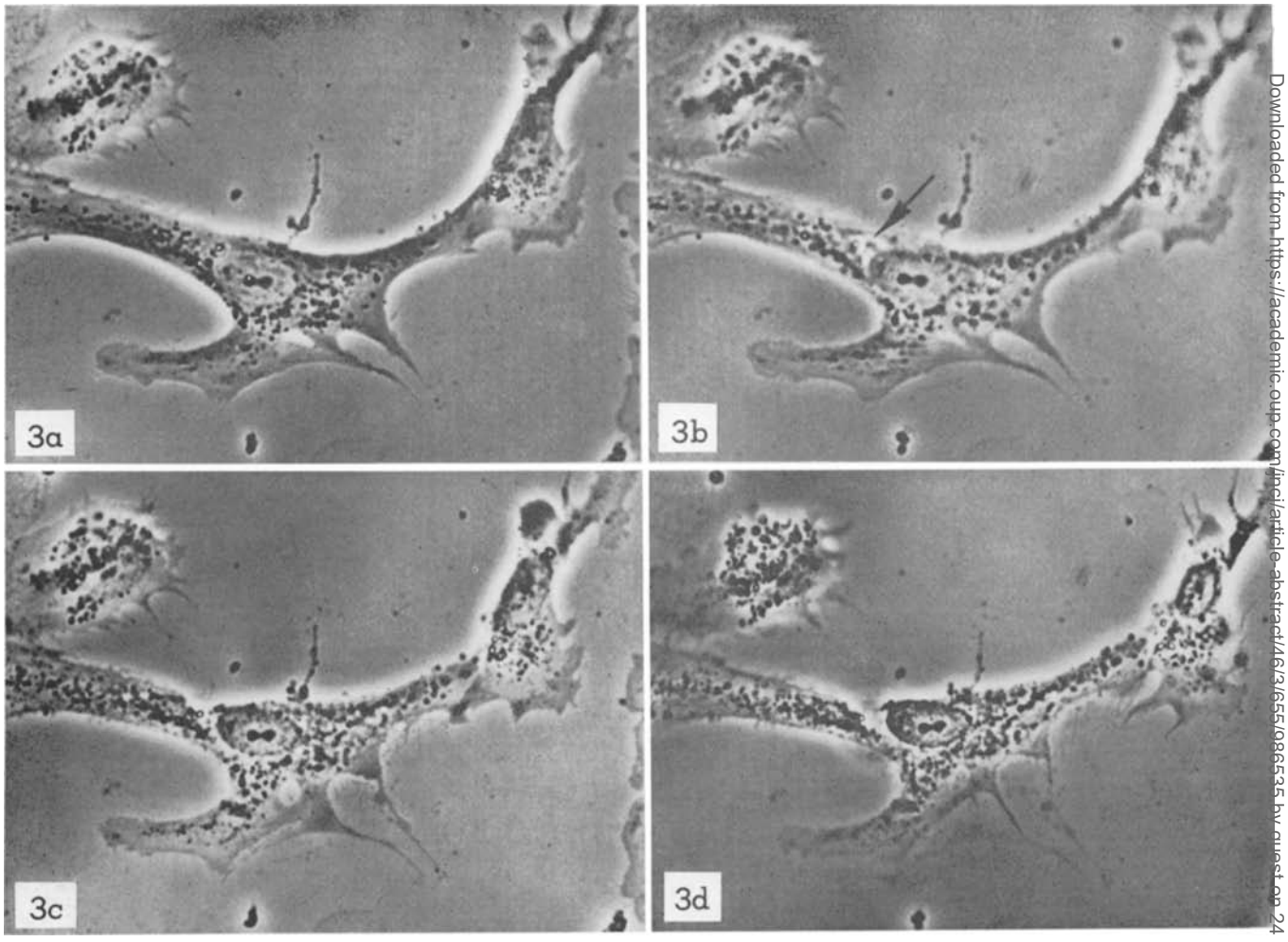

FIGURE 3.—a) Preirradiation image of 2 connected adenocarcinoma cells derived from human endometrial tumor.
b) *Arrow* indicates localized lesion in the central cell's juxtanuclear cytoplasm.
c) Irradiated cell shows cytoplasmic retraction and nuclear pycnosis 15 minutes after laser treatment. Adherent cell to *right* shows no effect.
d) Both cells show nuclear pycnosis as evidence of death 50 minutes after irradiation.

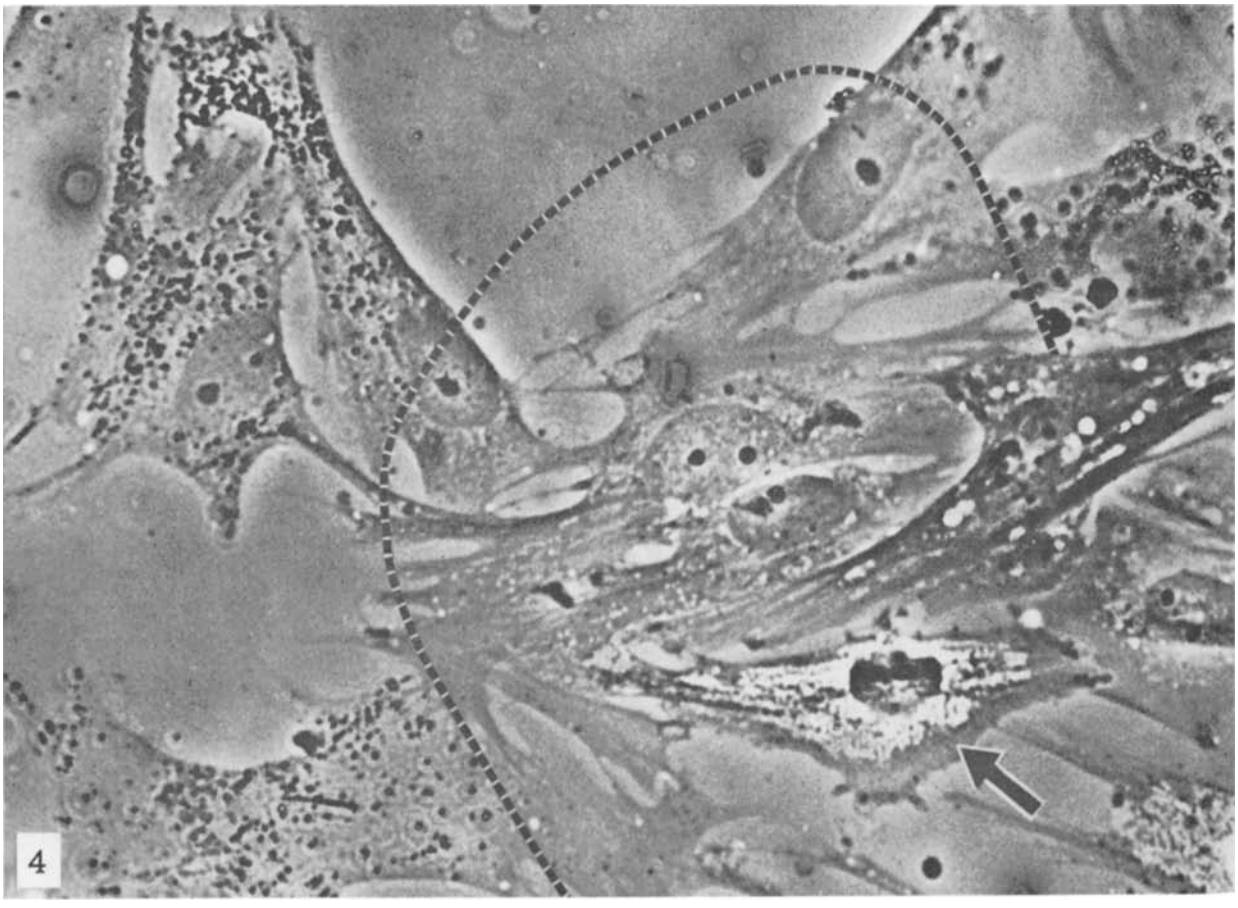

FIGURE 4.—Phase-contrast microscopy of uterine fibroma cells in tissue culture, after fixation and staining with the Gomori method to demonstrate acid phosphatase deposition in intact lysosomes. Dead cell (*arrow*) is surrounded by a zone occupied by cells showing a loss of intact lysosomes. At margin of this zone, cells show an absence of intact lysosomes at their proximal poles, but lysosome-rich cytoplasm at their distal poles.