EFFECT OF MICROWAVES ON CELL FUNCTION AND VIRUS REPLICATION IN CELL CULTURES IRRADIATED *IN VITRO*

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Irradiation of cell suspensions *in vitro* with microwave energy results in functional disturbances at the cellular and subcellular level, which include inhibition of cell growth,[6] chromosomal damage,[1,6] increased cell membrane permeability,[2] depression of phagocytosis,[9] and formation of atypical cells.[11,14] In certain instances, small nonthermal doses of microwave radiation *in vitro* may stimulate human lymphocytes to divide and transform.[12] In view of the above observations, it seemed interesting to investigate the effect of microwave radiation on continuous cell lines growing *in vitro*. This system offers a very homogeneous cell population, distributed as a monolayer on a glass surface, and an excellent material for cytochemical and virologic studies.[5,7] Infections of cell cultures with myxoviruses results in well-established cytochemical and morphologic changes,[7,8,13] which include formation of large syncytia.

For the present study, WISH cells and virus parainfluenza 3 were chosen, because this system was used in our previous investigations[7,8,13] and has been tested under various conditions.

Materials and Methods

WISH cells 24 hr after passage were irradiated *in vitro* in plaque or Legroux flasks with 3 GHz (10 cm) electromagnetic waves. Morphologic and cytochemical examinations of the cells were performed 1, 24, and 48 hr after irradiation.

Irradiated and control cultures were infected with virulent virus parainfluenza 3 at 1 or 24 hr after irradiation. Morphologic observation and virus multiplication evaluation were performed 48 hr after infection.[7]

In the second group of experiments, the cultures were infected with virus parainfluenza 3 and irradiated with microwaves at 2, 8, or 16 hr after infection. In these cells, the morphologic and virologic observations were performed 48 hr after infection.

Irradiation

All the cultures used for the experiments were irradiated at the same conditions, with 3 GHz (10 cm) continuous wave electromagnetic energy generated by a 500-W magnetron. The cells were placed in free space at far-field conditions (more than 180 cm from a conical antenna with a 20 × 30-cm base and a 40-cm depth) in an anechoic chamber. Power density measurements expressed in mW/cm^2 were performed with a PO-1 MEDIK apparatus and a miniprobe in the form of an ele-

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mentary dipole (1 cm large) under the following conditions: in free space; flask surfaces, with cell cultures placed in the field; on posterior surfaces of empty bottles placed in the field; on posterior surfaces of flasks, with cell cultures placed in the field; inside empty flasks and flasks, with cell cultures placed in the field.

In view of the preliminary observations, 5 and 20 mW/cm^2 power densities, as measured on the antenna surface of the flasks, were used in further experiments; the irradiation time was 30 min. Under these conditions, no increase in culture medium temperature was detected.

Cell Cultures

All experiments employed WISH cells passaged once every 3 days growing on medium composed of 15% Hanks' balanced salt solution, 75% Parker's solution, 10% fetal calf serum, and kanamycin.

Virus

The parainfluenza 3 virus, strain EA-106, passaged in WISH cell cultures (infectious titer 10^6 $TCID_{50}$/ml, hemagglutination titer 1:16) was used for all experiments.

Morphologic Methods

Morphologic and cytochemical examinations were performed after trypsinization (1:400 trypsin) of cell cultures and suspension of the cells in Hanks' solution. The following techniques were employed:[3,5,10,13] supravital staining with 1:1000 nigrosine in saline; phase-contrast observations in the Zernicke system and supravital staining with 1:1000 Janus green B in saline or 1:10,000 neutral red in saline; May-Grünwald-Giemsa staining; glycogen staining, periodic acid-Schiff base technique; nitro blue tetrazolium reduction *in vitro*;[10] cytochemical activity of succinic dehydrogenase;[3] cytochemical activity of nonspecific acid phosphatase and aryl sulfatase, with 1-naphthyl esters used as substrates and diazonium fast blue B as the coupling salt.[3]

Virologic Methods

For determination of the infectious dose of viruses multiplied in irradiated cultures ($TCID_{50}$), serial 10-fold dilutions of the virus in medium TC 199 were prepared. Four tubes with WISH cells (10^5 cells/tube) were inoculated with each dilution. After 3 days of incubation at 37°C, readings were taken by microscopic examination for a cytopathic effect (CPE, syncytia). $TCID_{50}$/ml values were calculated according to Reed and Muench.[7,8]

The hemagglutination test was performed at 4°C with a 1:200 guinea pig erythrocyte suspension in saline.

Results

Irradiation Conditions

The flasks with cell cultures were placed 230 cm from the antenna (far-field conditions began at 180 cm) and were irradiated at 20 mW/cm² at the surface, as seen in FIGURE 1. Under this condition, the power densities 5 cm in front of the flask were 29 mW/cm² for empty flasks and 31 mW/cm² for those with WISH cell monolayers. Inside the flasks, the values were 14.5 and 12.5 mW/cm² for empty and culture-containing flasks, respectively (FIGURE 1). On the posterior surface of the flasks, 7.5 and 6.5 mW/cm² power densities were found for the empty and cell-containing bottles, respectively. In view of the above measurements, the following lowerings of power density were calculated:

$$L_{\mathrm{dB(glass\ of\ the\ flask)}} = 10 \times \log \frac{20}{14.5} = 1.35\ \mathrm{dB},$$

$$L_{\mathrm{dB}} = 10 \times \log \frac{20}{7.5} = 4.24\ \mathrm{dB},$$

$$L_{\mathrm{dB(flask\ with\ cell\ culture)}} = 10 \times \log \frac{20}{6.5} = 4.88\ \mathrm{dB}.$$

Power density measurements on the inside surfaces of the flasks demonstrated the existence of field distribution homogeneity, with maximal deviations of ± 10%.

Morphologic Investigations

In control cultures, only a few percent of the cells stained with nigrosine during the entire observation period (TABLE 1). In the phase-contrast system, viable cells without any symptoms of degeneration were noted (FIGURE 2,a). No diffuse

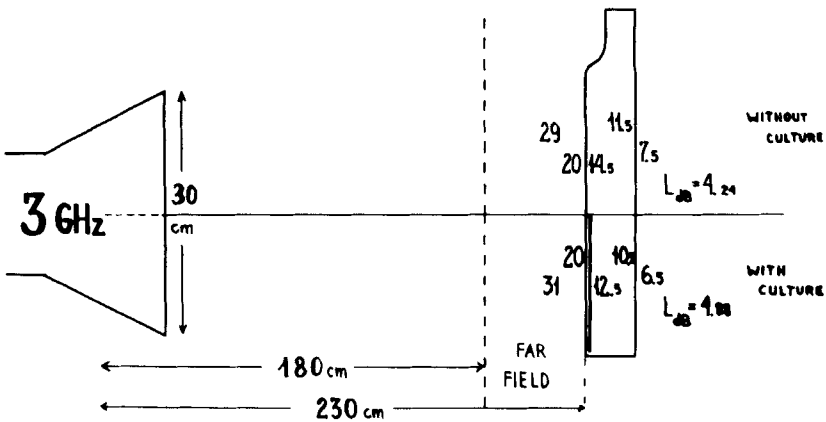
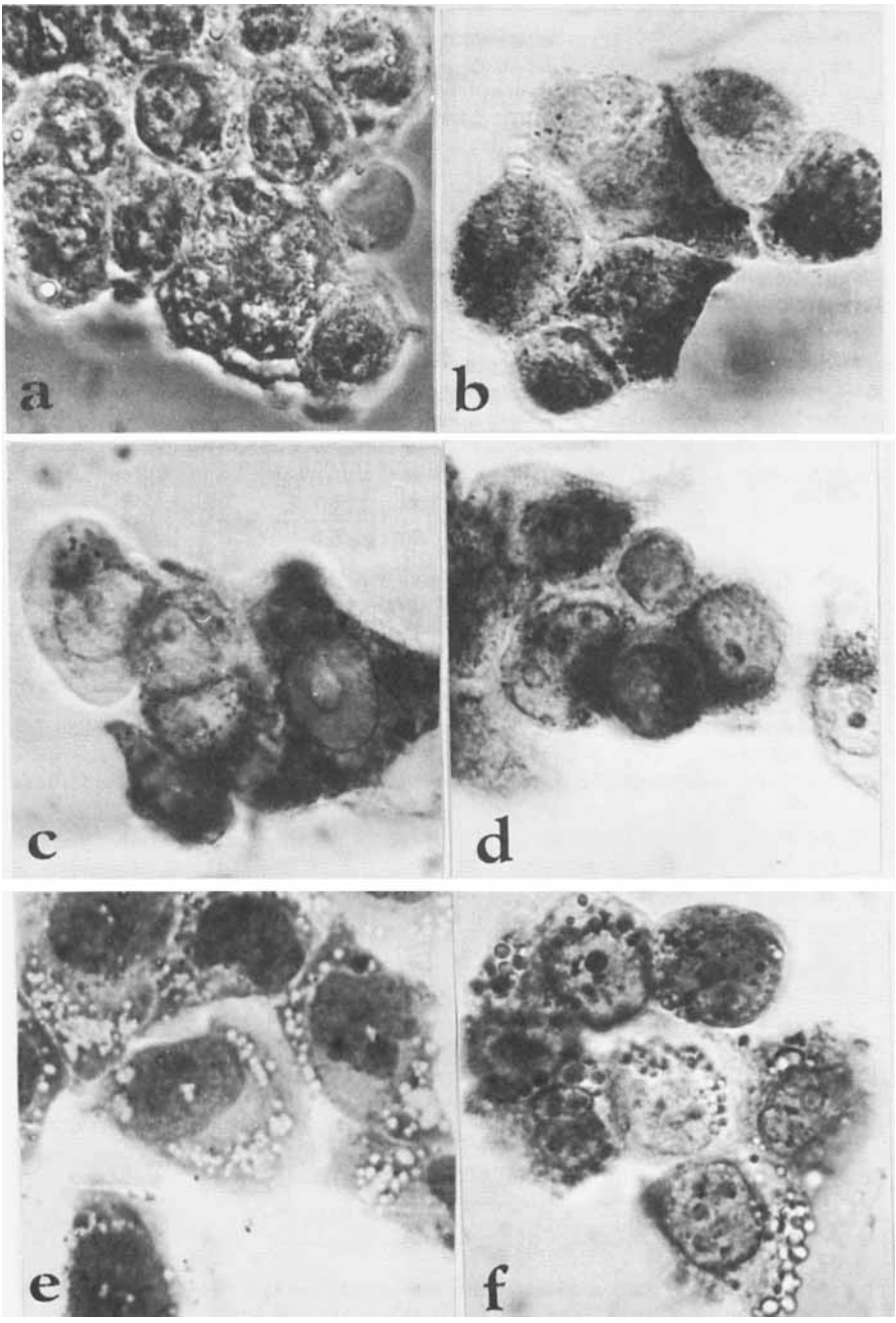


FIGURE 1. Field distribution measurements with cell culture flask placed in the field (with and without cell monolayers). Power densities are expressed in mW/cm².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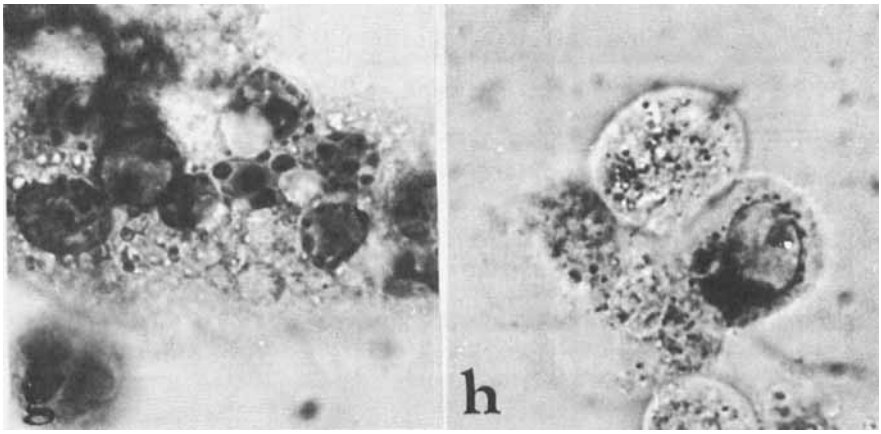


FIGURE 2. Morphology and cytochemistry of WISH cells exposed to microwave radiation. a, Normal WISH cells, phase-contrast system, × 1000; b, nitro blue tetrazolium reduction in WISH cells. Note the cells with large granules and deposits, cells with numerous small granules, and negative cells. × 800; c, succinic dehydrogenase in WISH cells. Note differences in enzyme activity in single cells. × 800; d, acid phosphatase in WISH cells. All cells strongly positive. × 800; e, vacuolization of cytoplasm in WISH cells 1 hr after exposure to microwaves at 20 mW/cm², MGG staining, × 1000; f, same as e, phase-contrast system, × 1000; g, diffuse staining with neutral red, WISH cells 1 hr after exposure to microwaves at 20 mW/cm², × 1000; h, lower reduction rate of nitro blue tetrazolium in WISH cells 1 hr after microwave exposure at 20 mW/cm², × 1000.

absorption of Janus green B or neutral red was found. The nitro blue tetrazolium test revealed three easily distinguishable classes of cells: those with large granules and deposits of blue-stained formazan (++ cells), cells with small distinct granules without diffuse staining (+ cells), and unstained cells (− cells) (FIGURE 2,b). The relative percentages of these cells in control cultures are presented in TABLE 2. Succinic dehydrogenase staining gave the same three classes of reactivity (++ cells,

TABLE 1

STAINING OF WISH CELLS WITH NIGROSINE (PER 100 CELLS)

	Control	Irradiated Cultures	
		5 mW/cm²	20 mW/cm²
After Microwave Irradiation			
1 hr	8	10	34
24 hr	6	7	13
48 hr	12	14	11
Virus Infection After Irradiation			
1 hr	46	21	82
24 hr	39	24	73
Virus Infection Before Irradiation			
2 hr	52	28	—
8 hr	47	31	—
16 hr	42	37	—

TABLE 2

REDUCTION OF NITRO BLUE TETRAZOLIUM IN WISH CELLS (PER 100 CELLS)

	Control	Irradiated Cultures	
		5 mW/cm^2	20 mW/cm^2
After Microwave Irradiation			
1 hr	60 (++)*	55 (++)	10 (++)
	30 (+)†	45 (+)	50 (+)
	10 (−)‡		40 (−)
24 hr	65 (++)	85 (++)	20 (++)
	35 (+)	15 (+)	40 (+)
			40 (−)
48 hr	50 (++)	40 (++)	30 (++)
	40 (+)	50 (+)	60 (+)
	10 (−)	10 (−)	15 (−)
Virus Infected After Irradiation			
1 hr	25 (++)	40 (++)	5 (++)
	10 (+)	40 (+)	20 (+)
	65 (−)	20 (−)	75 (−)
24 hr	20 (++)	20 (++)	10 (++)
	10 (+)	30 (+)	30 (+)
	70 (−)	50 (−)	60 (−)
Virus Infected Before Irradiation			
2 hr	15 (++)	80 (++)	
	20 (+)	10 (+)	
	65 (−)	10 (−)	
8 hr	20 (++)	85 (++)	
	20 (+)	5 (+)	
	60 (−)	10 (−)	
16 hr	15 (++)	30 (++)	
	10 (+)	10 (+)	
	75 (−)	60 (−)	

*Diffuse staining, large granules and/or formazan deposits.
†Small distinct granules, without diffuse staining.
‡Without formazan.

+ cells, and − cells) (FIGURE 2,c). Acid phosphatase and sulfatase activities were found in all control cells, as revealed by distinct granules and diffuse staining (FIGURE 2,d). Only in certain lone cells were dye deposits or vacuoles with enzyme activity found.

Irradiated Cultures

20 mW/cm^2

One hour after irradiation, 30–38% of the WISH cells were stained with nigrosine. At this time in the May-Grünwald-Giemsa-stained slides and in the phase-contrast system, the majority of the cells exhibited numerous small round vacuoles (FIGURE 2, e & f). Most of these organelles stained supravitally with Janus green B, whereas only a single vacuole retained the neutral red dye. Under these conditions, approximately 50% of the cells displayed diffuse staining with neutral red and Janus green B of both the cytoplasm and the nucleus, with heavily stained nucleoli (FIGURE

2,g). The reduction rate of nitro blue tetrazolium in WISH cells 1 hr after irradiation at 20 mW/cm² was markedly lowered (FIGURE 2,h; TABLE 2). Only 10–15% of the cells showed large granules and deposits of formazan and diffuse staining, as compared to 50–70% of the control culture cells. The percentage of cells with succinic dehydrogenase activity markedly declined in irradiated cultures (TABLE 3). Acid phosphatase and sulfatase activities were visible in all cells, both in irradiated and in control cultures. Twenty-four hours after irradiation at 20 mW/cm², cell function had partially returned to normal, as compared to the picture 1 hr after irradiation. The percentage of cells stained with nigrosine decreased to 13, a figure very similar to that observed in control cultures (TABLE 1). Vacuoli stained supravitally with Janus green B were still evident in most of the cells, whereas diffuse staining with the same dye and with neutral red was observed only in a few cells. The reduction rate of nitro blue tetrazolium was still lower in irradiated than in control cultures 24 and 48 hr after treatment, although it was distinctly higher at these times than 1 hr after irradiation (TABLE 2; FIGURE 3,a). The percentage of cells that demonstrated succinic dehydrogenase activity was low during the entire observation period (TABLE 3).

TABLE 3

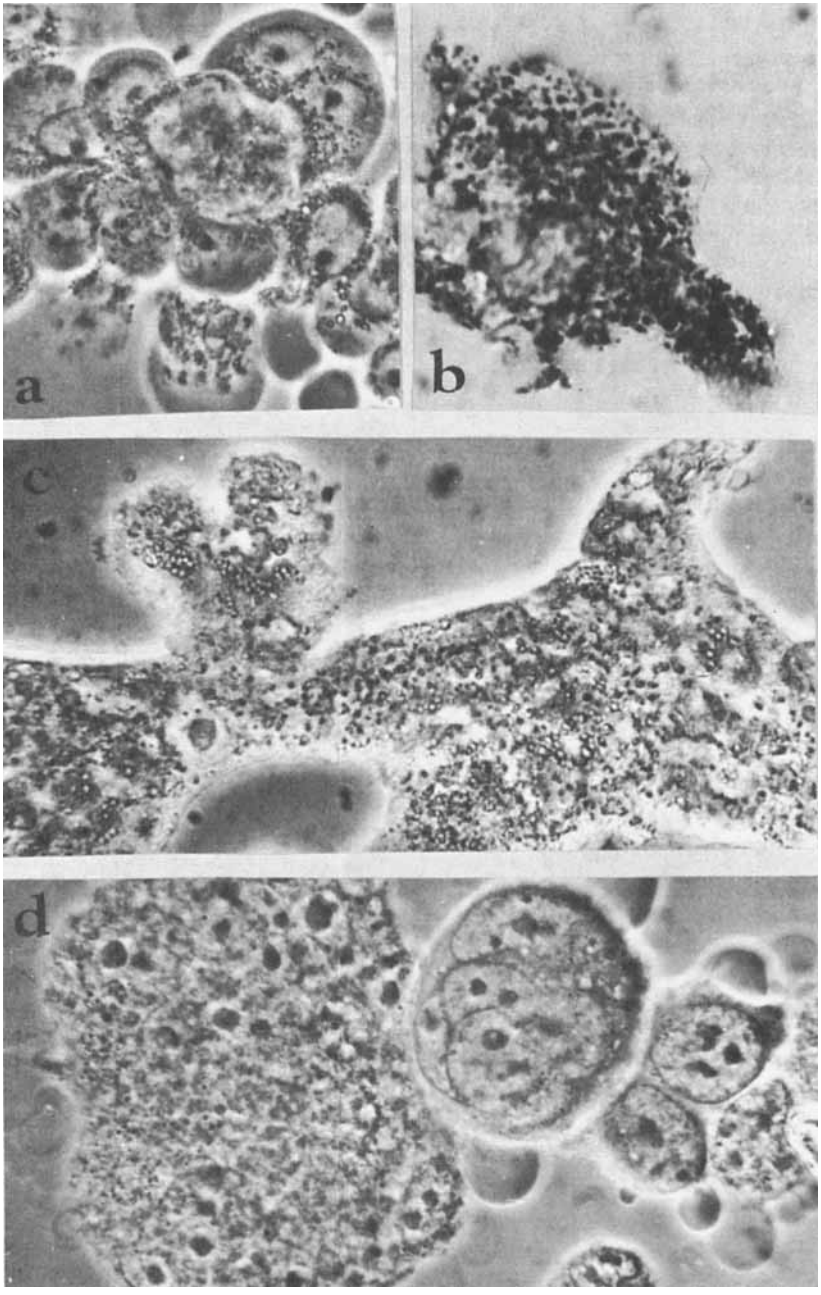
SUCCINIC DEHYDROGENASE ACTIVITY IN WISH CELLS (PER 100 CELLS)

	Control	Irradiated Cultures	
		5 mW/cm²	20 mW/cm²
After Microwave Irradiation			
1 hr	10 (++)*	30 (++)	5 (++)
	60 (+)†	45 (+)	35 (+)
	20 (−)‡	25 (−)	60 (−)
24 hr	20 (++)	40 (++)	40 (++)
	40 (+)	20 (+)	10 (+)
	40 (−)	40 (−)	50 (−)
48 hr	20 (++)	30 (++)	5 (++)
	50 (+)	50 (+)	10 (+)
	30 (−)	20 (−)	85 (−)
Virus Infected After Irradiation			
1 hr	50 (++)	10 (++)	5 (++)
	40 (+)	90 (+)	5 (+)
	10 (−)		90 (−)
24 hr	45 (++)	20 (++)	15 (++)
	30 (+)	80 (+)	20 (+)
	25 (−)		65 (−)
Virus Infected Before Irradiation			
2 hr	60 (++)	30 (++)	
	10 (+)	50 (+)	
	30 (−)	20 (−)	
8 hr	55 (++)	35 (++)	
	25 (+)	60 (+)	
	20 (−)	5 (−)	
16 hr	70 (++)	30 (++)	
	10 (+)	60 (+)	
	20 (−)	10 (−)	

*Diffuse staining, large granules, and/or formazan deposits.

†Small distinct granules, without diffuse staining.

‡Without formazan.


a
b
c
d

5 mW/cm²

WISH cultures irradiated at 5 mW/cm² showed no differences in the percentage of cells stained with nigrosine, as compared to controls, 1, 24, and 48 hr after treatment. In the phase-contrast system, the only deviation from normal was that large granules stained with Janus green B were present in about 30% of the cells (FIGURE 3,b). No diffuse staining with the supravital dyes was found.

The reduction rate of nitro blue tetrazolium increased in cells 24 hr after irradiation at 5 mW/cm² and returned to normal 48 hr after treatment. The percentage of cells with succinic dehydrogenase activity was slightly higher in irradiated cultures than in controls, although the exact figure was very different from that seen in cultures exposed to 20 mW/cm² (TABLE 3).

Virologic Studies

The $TCID_{50}$/ml of control cultures was found to be $10^{5.00}$ (100,000 infectious particles/ml), and this parameter equalled $10^{3.67}$ in cultures infected 24 hr later (TABLE 4); the hemagglutination titer in both cases was 1:16. This was accompanied by a large number of nigrosine-stained cells (TABLE 1), a decline in nitro blue tetrazolium (TABLE 2), and an increased percentage of cells that exhibited succinic dehydrogenase activity, in the form of large granules and formazan deposits (TABLE 3). In addition, multiple syncytia were formed (FIGURE 3,c).

Cultures infected 1 hr after microwave treatment at 20 mW/cm² had a reduced virus multiplication rate ($TCID_{50}$/ml $10^{3.67}$, TABLE 4); the hemagglutination titer was only 1:4. Under these conditions, very few syncytia were visible, and large multinuclear cells were the dominant feature in the culture (FIGURE 3,d).

Cultures infected 24 hr after irradiation at 20 mW/cm² possessed a slightly higher virus titer than did control cultures (TABLE 4). The decline in virus multi-

TABLE 4

TISSUE CULTURE INFECTIOUS DOSE OF PARAINFLUENZA 3 VIRUS FOR WISH CELLS IRRADIATED WITH MICROWAVES ($TCID_{50}$/ML)

	Control	Irradiated Cultures	
		5 mW/cm²	20 mW/cm²
Virus Infected After Irradiation			
1 hr	$10^{5.00}$	$10^{5.77}$	$10^{3.67}$
24 hr	$10^{3.67}$	$10^{3.67}$	$10^{4.50}$
Virus Infected Before Irradiation			
2 hr	$10^{4.53}$	$10^{5.33}$	
8 hr	$10^{4.53}$	$10^{5.50}$	
16 hr	$10^{4.53}$	$10^{5.50}$	

FIGURE 3. Morphology and cytochemistry of WISH cells exposed to microwave radiation. a, Reduction of nitro blue tetrazolium in WISH cells 48 hr after microwave exposure at 20 mW/cm², × 9000; b, large granules stained with Janus green B in WISH cells 1 hr after microwave exposure at 5 mW/cm², phase-contrast system, × 900; c, syncytia in control cultures infected with virus parainfluenza 3, phase-contrast system, × 360; d, multinuclear large cells in cultures infected with virus parainfluenza 3 1 hr after microwave exposure at 20 mW/cm², phase-contrast system, × 360.

plication rate in cultures irradiated at 20 mW/cm² was accompanied by a decrease in the percentage of succinic dehydrogenase-active cells (TABLE 3). Irradiation of cultures at 5 mW/cm² stimulated the multiplication of parainfluenza 3 virus at all conditions investigated, regardless of whether exposure occurred before or after virus infection (TABLE 4). The $TCID_{50}$ was higher in irradiated cultures than in controls. This was accompanied by markedly higher reduction of nitro blue tetrazolium in treated cells (TABLE 2), particularly those infected before irradiation.

DISCUSSION

Continuous cell cultures, WISH cells, manifested temporal functional disturbances, observed with morphologic and virologic techniques, after a 30-min irradiation with 3 GHz microwaves at 5 or 20 mW/cm² power densities, as measured on culture flask surfaces. The rise in both nitro blue tetrazolium reduction and myxovirus multiplication rates in cultures exposed to 5 mW/cm² suggests that this radiation dose stimulates cell metabolism, especially since no changes in supravital staining and phase-contrast observations were found.

Conversely, a 30-min irradiation at 20 mW/cm² resulted in increased membrane permeability (staining with nigrosine, diffuse staining with Janus green B and neutral red), decreased nitro blue tetrazolium reduction rate and succinic dehydrogenase activity, and the appearance of widespread cellular vacuolization. These findings were accompanied by inhibition of myxovirus replication. The above phenomena all suggest severe cellular injury, with a possible disturbing effect on the mitochondrial system. Surprisingly enough, 24 and 48 hr after irradiation at 20 mW/cm², partial regeneration of the cultures was observed; the virus replication rate also returned to normal.

Because staining with nigrosine and cytoplasmic vacuolization are believed to be the symptoms of irreversible cellular injury,[5,13] it is possible that only certain cells were injured at 20 mW/cm² and that regeneration is possible by proliferation of the unaffected cells.

That exposure to 5 mW/cm² stimulates oxidative metabolism and virus multiplication could best be observed in cultures infected 1 hr after treatment and in those infected with virus before irradiation. The ability to reduce nitro blue tetrazolium without any external substrate is a function of oxygen consumption intensity and pentose phosphate shunt[10] activity. The high nitro blue tetrazolium reduction rate in cultures infected with myxovirus and exposed to 5 mW/cm² microwave radiation and the resultant stimulation of oxidative metabolism may be the causes of increased virus multiplication.

Inasmuch as WISH cells react differently to 5 or 20 mW/cm² power densities, quantitation of the energy absorbed becomes a problem. Power density measurements from free field conditions, empty flasks placed in the field, and flasks with WISH cell monolayers only yielded part of the answer. At the 20 mW/cm² power density, as measured on the flask surface, values of 7.5 and 6.5 mW/cm² were found on the other side of the empty and cell monolayer flasks, respectively. This difference may partially be due to increased wave reflection and to their absorption in the monolayer; more precise measurement techniques are needed to distinguish the effects of both components. Nevertheless, continuous cell line monolayers *in vitro* would appear to offer an attractive model for studying the effects of microwave radiation on cell metabolism.

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DISCUSSION

DR. K. D. STRAUB: Your work was very beautiful. You still found stimulation after infection. This finding may localize the changes to a structure other than the plasma cell membrane. Could it be an effect on ribosomes?

DR. SZMIGIELSKI: I agree with you. These changes may be due only partially to increased cell membrane permeability. When you observe better penetration of virus particles through the cell membrane, the conditions for replication of this larger

number of viruses may be present. The absorption and penetration of virus particles are not sufficient for replication in the cell. RNA, in these cases, or DNA, in other viruses, is engaged in the process, and metabolism is also required. All of these systems must be functioning to produce good replication of viruses. I agree that it is quite possible that a stimulating effect on nucleic acid metabolism also occurs, and we are examining this effect with autoradiography in cell cultures by means of thymidine and uridine uptake. It is particularly important, because we found a higher number of mitoses in these irradiated cultures.

DR. J. C. LIN: What are the dimensions of your sample holder?

DR. SZMIGIELSKI: It is 14.5 cm high, 4.5 cm wide, and 3 cm deep.

DR. LIN: The flask is less than 1.5 wavelengths high; therefore, you might have some resonance effect. If you had an antenna, currents may be induced on it; therefore, you may have selective absorption induced in the flask. Did you measure the temperature?

DR. SZMIGIELSKI: Yes. We made some measurements of culture media temperatures, and elevations of about 3 or 4°C were seen in the cells. These cells normally live at 37°C. At the beginning of the experiment the culture was at 25°C, and after irradiation, the temperature had increased to 29–30°C, which is still below normal for the cells. It is well known that cell cultures tolerate temperature decreases of as much as 4°C very well, whereas they tolerate temperatures above 39°C very poorly.

DR. LIN: Did you make one measurement at one point or a series of measurements?

DR. SZMIGIELSKI: One point was measured in the culture medium after irradiation by a thermocouple.

DR. LIN: It is possible that different temperatures existed at different points in the medium.

DR. SZMIGIELSKI: Possibly, but convection and conduction should equalize the temperature. However, we will try to investigate this in the future.