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DIAGNOSTIC ULTRASOUND: TIME-LAPSE AND TRANSMISSION ELECTRON MICROSCOPIC STUDIES OF CELLS INSONATED *IN VITRO*

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Summary.—A fibroblast cell line (3T3) and normal rat peritoneal fluid cells were exposed *in vitro* to pulsed ultrasound from a diagnostic instrument (Smith-Kline "Ekoline 20"). We report here on ultrastructural changes in both cell types and on altered motility patterns in 3T3 fibroblasts. Abnormal motility was detectable 10 generations after exposure. X-irradiation and ultraviolet light elicited similar effects on cell motion. It is suggested that the cellular effects of diagnostic levels of ultrasound be further examined both *in vitro* and *in vivo*.

THE APPLICATION of ultrasound technology to medical diagnosis, especially in obstetrics, has increased markedly. It is estimated that by the mid-1980s all infants in the U.S.A. will have been exposed *in utero* to diagnostic ultrasound (Neill, 1977). Ultrasound is generally regarded as safe to the developing foetus. Two basic modalities of exposure are used: (1) pulsed, for imaging of the foetus, and (2) continuous wave, for the monitoring of the foetal pulse. The bioeffects of pulsed and continuous wave ultrasound may differ substantially since the pulsed mode results in a low average power output with microsecond bursts of extremely high instantaneous peak intensity, whereas the continuous mode has an average output which is generally greater but lacking in bursts of high intensity.

Recent reports have described the bioeffects of both continuous wave and pulsed ultrasound on the same organism. The data show that continuous wave insonation for 5–20 min (Cachon & Cachon, 1981) induced cytological effects that could be reproduced with only 10 sec of pulsed ultrasound having an average power output of 2·5 mW/cm^2 (Cachon *et al.*, 1981).

Investigations of cells *in vitro* in liquid suspension may be analogous to the *in vivo* situation where the cells are carried in suspension in a fluid such as blood or various transudates; however, the situation in solid tissue might not be comparable. Foetal tissues generally contain a looser network of ground substance that is more hydrated than in the adult, resulting in a soft gel having properties intermediate between a liquid medium and solid adult tissues. Biological effects observed with cells suspended in fluid media *in vitro* may be relevant to the situation in certain tissues in intact animals or humans.

Our laboratory has examined the effects of pulsed ultrasound *in vitro*. We have demonstrated that low level (15 mW/cm^2) pulsed ultrasound generated by a commercial diagnostic instrument (Ekoline-20, Smith–Kline): (a) elicits unscheduled DNA synthesis (Liebeskind *et al.*, 1979*a*), morphological transformation (Liebeskind *et al.*, 1979*a*) as determined by light microscopy, and alterations of cell surface architecture as seen by scanning electron microscopy (Liebeskind *et al.*, 1981); (b) alters immunoreactivity of DNA to anti-nucleoside antibody (Liebeskind *et al.*, 1979*a*); (c) increases the frequency of sister chromatid exchanges (Liebeskind

et al., 1979*b*); and (d) disturbs the phagokinetic track patterns of cultured cells (unpublished). Some of these changes persist for many generations after insonation. Other investigators (Morris *et al.*, 1978; Wegner *et al.*, 1980) have failed to obtain increases in sister chromatid exchanges with lymphocytes or cell lines after treatment with ultrasound. They studied continuous wave ultrasound in-

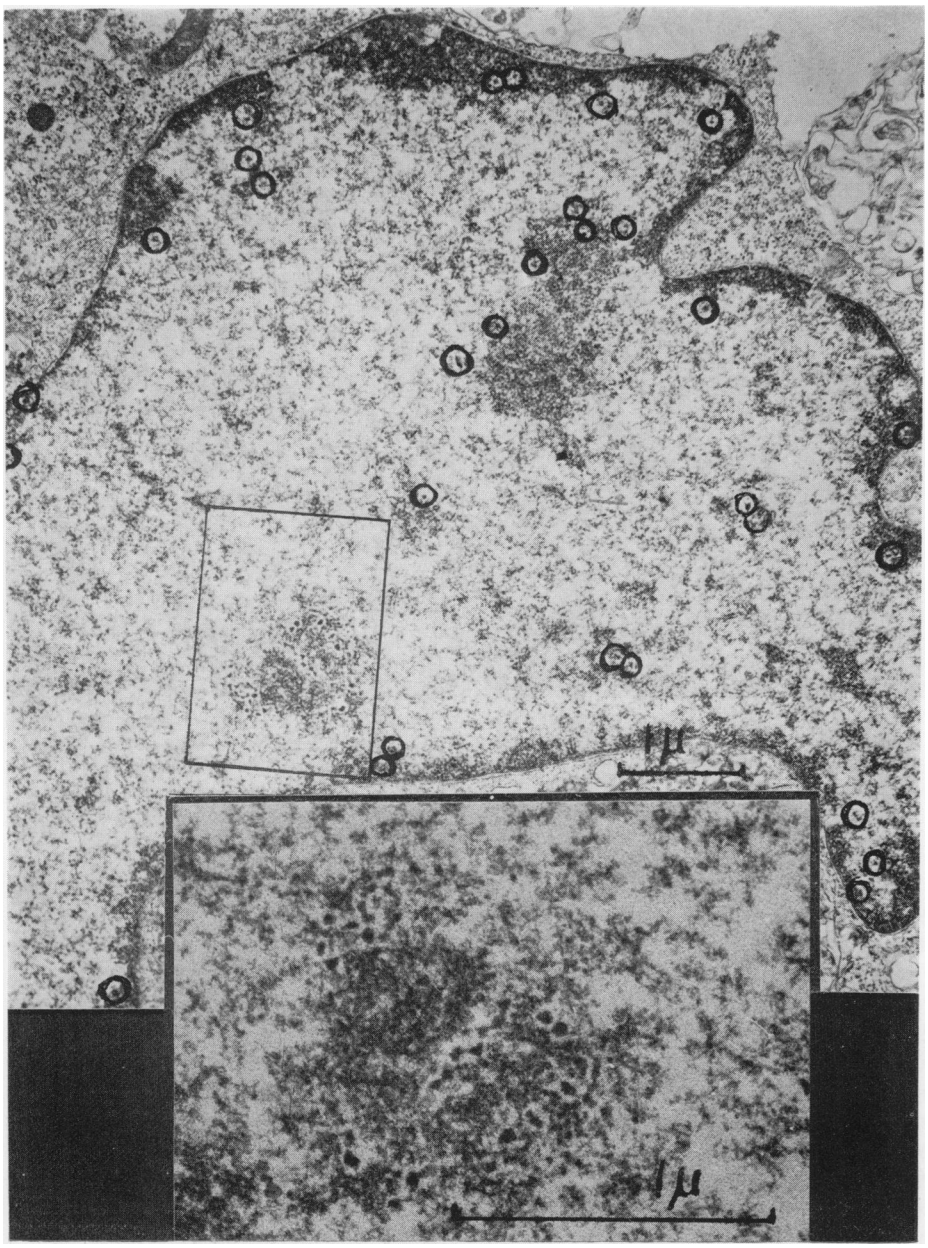

FIG. 1.—Electron micrograph. Nucleus of 3T3 cell, 30 min post-insonation. Note numerous perichromatin granules (*e.g.* circles), some clearly clustered (inset). Controls did not display as many.

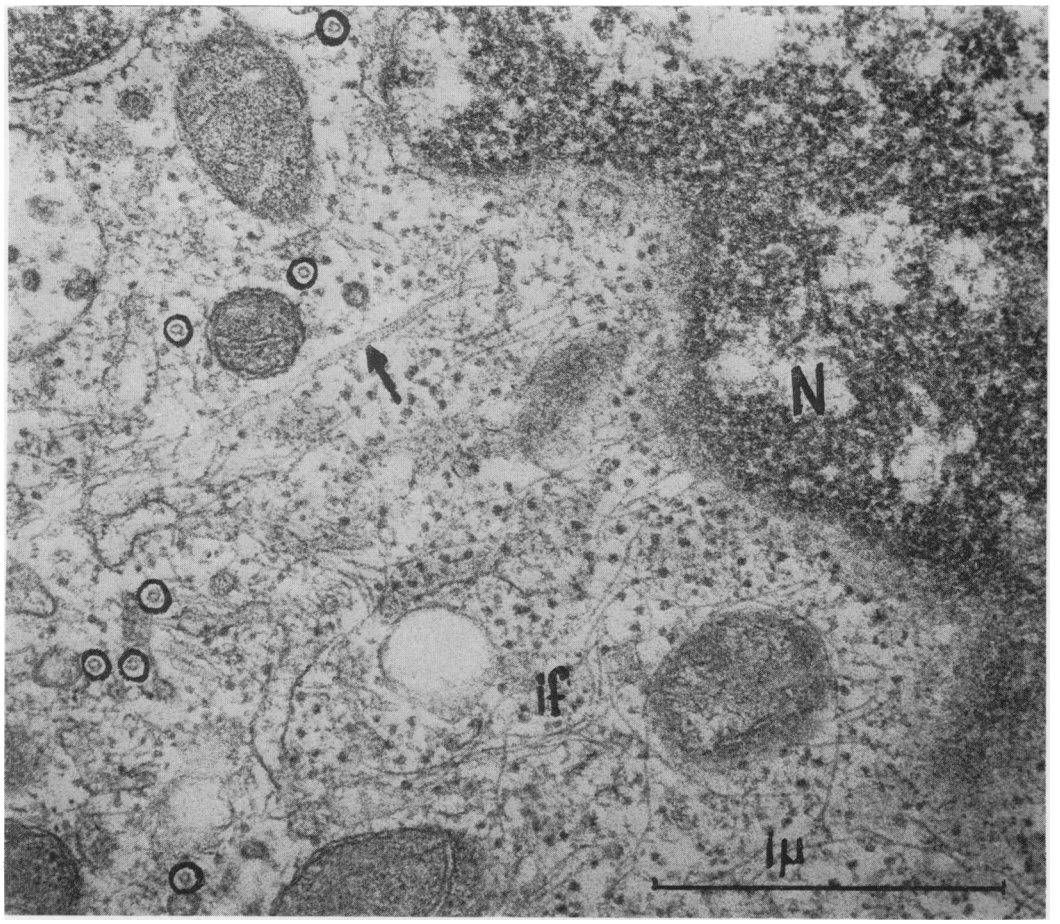

FIG. 2.—Electron micrograph of paranuclear area of 3T3 cell, 30 min post-insonation. Microtubules in both longitudinal (arrow) and cross sections (circles) are seen as well as many intermediate filaments (i.f.). The latter are randomly oriented in the ground cytoplasm, but may originate at the nuclear (N) envelope.

stead of pulsed ultrasound, and their protocols differed from ours in other important features.

We report here on a 3T3 mouse fibroblast cell line and on normal rat peritoneal fluid cells insonated with pulsed ultrasound.

The arrangement for the ultrasound exposure has been previously described (Liebeskind *et al.*, 1979*a*, *b*, 1981). Briefly, a sterile polypropylene test tube filled to the brim with the cell suspension was covered with a parafilm strip so as to exclude all air bubbles and placed in a 37°C water bath. The transducer was coupled to the Parafilm strip (American Can Co.) with a layer of Aquasonic gel and the cells were insonated for 30 min. Dosimetry measurements were made with a force balance (Liebeskind *et al.*, 1979*a*). Total acoustic power output was determined to be 17 mW/cm^2. Temporal average/spatial average intensity across the cross-section of the test tube was measured to be 15 mW/cm^2. Pulse length was 3 μs, repetition rate was 200 Hz and SPTP intensity 35·4 W/cm^2. No standing waves were detectable by Schlieren

techniques and, at most, a local two-fold increase due to focused reflections could have occurred (Liebeskind *et al.*, 1979*a*).

Balb/c 3T3, clone I-13 cells were obtained from Dr Andrew Sivak (Arthur D. Little, Cambridge, MA). Stock cultures were grown in Minimal Essential Medium (Eagle) supplemented with penicillin, streptomycin, and 10% foetal bovine

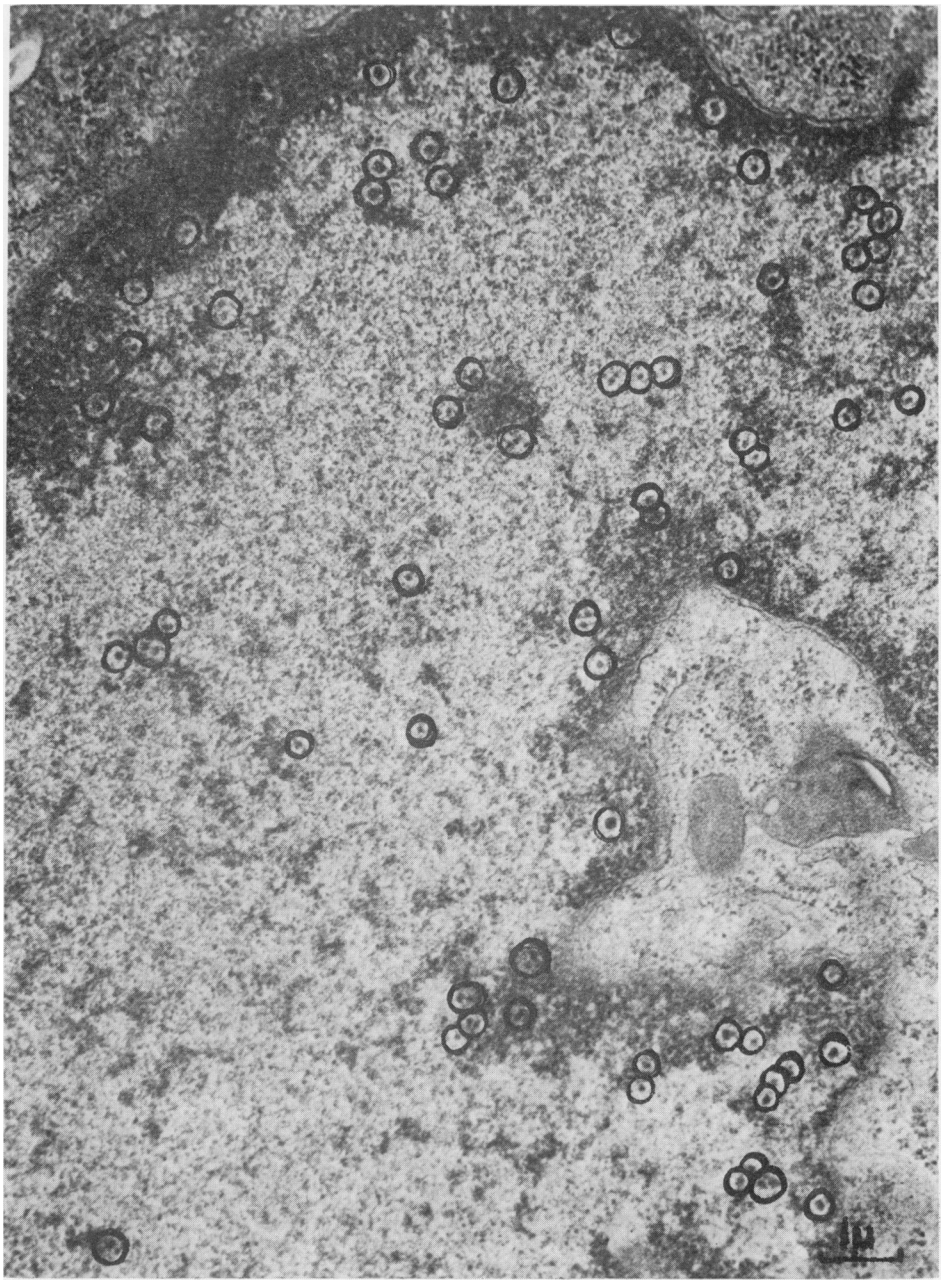

FIG. 3.—Electron micrograph of rat peritoneal fluid macrophage nucleus 30 min post-insonation. Numerous perichromatin granules are seen (*e.g.* circles). Otherwise, this cell appeared normal.

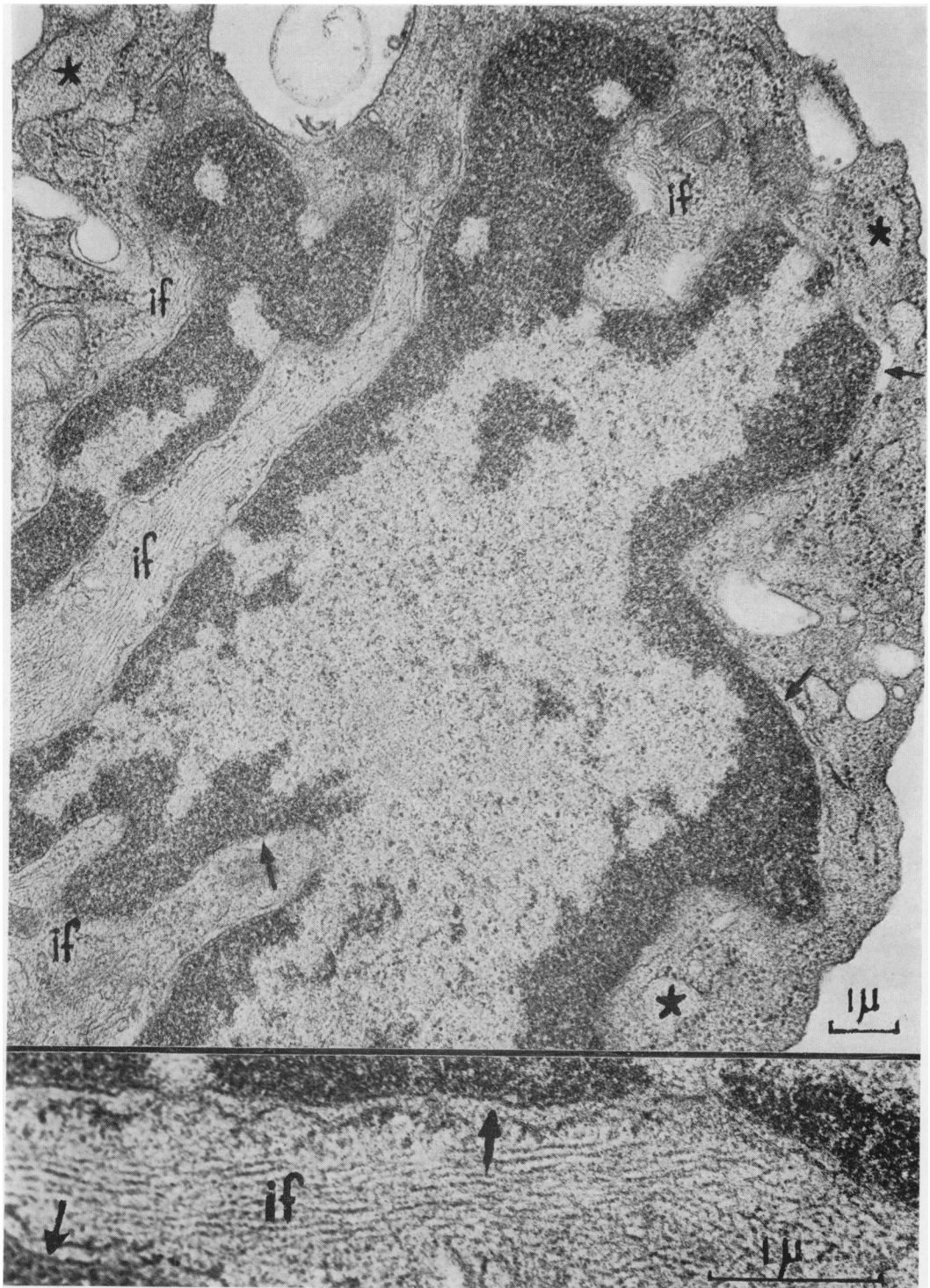

FIG. 4.—Top: electron micrograph of rat peritoneal fluid macrophage 30′ post-insonation. Several bundles of intermediate filaments (i.f.) are seen closely associated with the nuclear envelope. Cisternae of rough endoplasmic reticulum (*e.g.* *) are conspicuously engorged. At places, the outer nuclear membrane has lifted somewhat (arrows). Bottom: enlarged portion of an i.f. bundle in above cell; note electron-dense material between nuclear membrane leaflets (arrows).

serum. Peritoneal exudate cells from normal male rats (Sprague–Dawley strain, Charles River, COBS) were obtained by lavage of the abdominal cavity after decapitating the animals under ether anaesthesia. Lavage fluid consisted of calcium- and magnesium-free Dulbecco's phosphate buffered saline, pH 7·4. Cells from 6 rats were centrifuged individually, resuspended in cell culture medium and pooled. Aliquots were insonated for 30 min, while control aliquots were incubated without insonation. Five or 30 min later both insonated and control cells were fixed for electron microscopy.

Various ultrastructural changes have been observed in insonated 3T3 cells and in peritoneal cells insonated *in vitro*. With 3T3 cells, there was an increased frequency of perichromatin granules (PCG) at 30 min post-insonation. These were often clustered, sometimes in appreciable numbers (Fig. 1). Cytoplasmic changes included increased frequency of intermediate (10 nm) filaments and of microtubules (MT). Both showed a tendency to associate with the nuclear envelope, though not exclusively. The MT tended to associate in parallel perinuclear bundles. The intermediate filaments appeared randomly oriented and formed a loose, thin, criss-crossed network (Fig. 2).

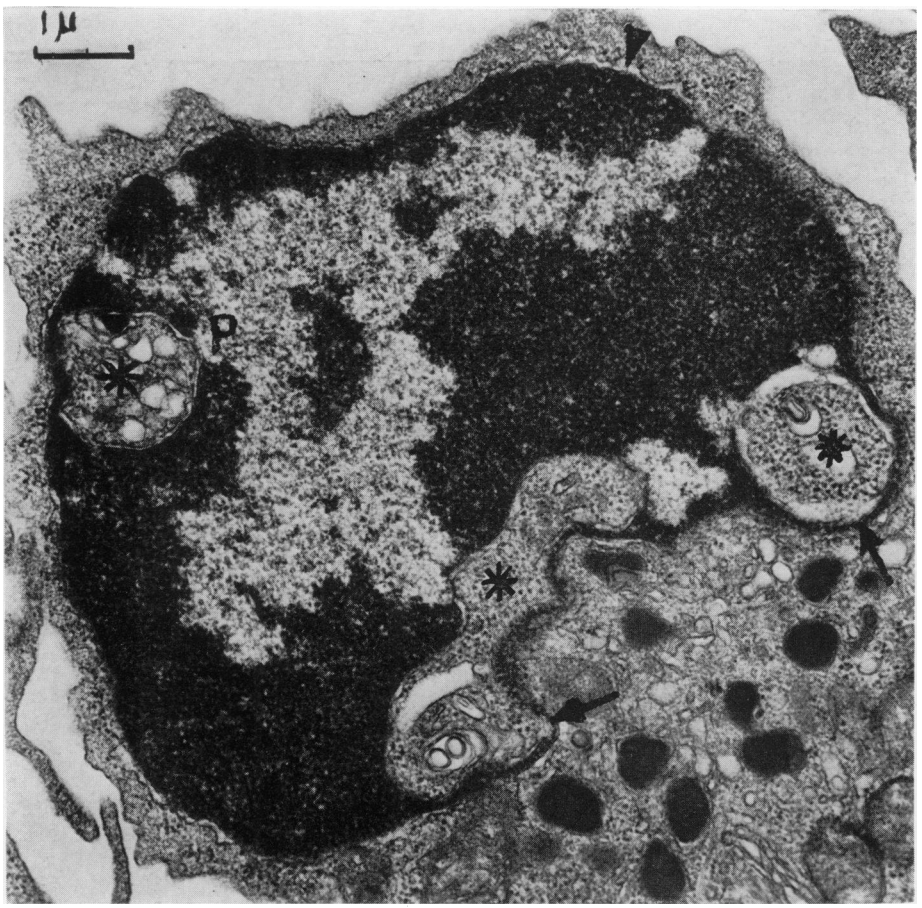

FIG. 5.—Electron micrograph of peritoneal cell 5 min post-insonation. Three pockets of cytoplasm (*) are invaginated into the nuclear domain. Such inpocketings retain nuclear pores (P); characteristically they are delimited on one side by a thin leaflet of nucleoplasm (arrows). The outer nuclear membrane has clearly lifted in several areas (*e.g.* arrow head).

Some of these changes were also observed in rat peritoneal fluid cells. There was an increased number and clustering of PCG, (Figs 3 and 6), especially 30′ post-insonation (Fig. 3), as well as more frequent bundles of intermediate filaments (i.f.) (Fig. 4). In addition, several other changes were noted in insonated cells. For example, bulk invagination of cytoplasm into the nuclear domain (Fig. 5) was noted. Lifting of the outer nuclear membrane was seen (Figs 4 and 5). Both of these could be observed most prominently 5 min after insonation. At 30 min after insonation, bundles of i.f. were seen (Fig. 4) and engorged cisternae of rough endoplasmic reticulum (RER) were often seen (Fig. 4). Centriole morphology was not overtly affected.

Time-lapse cinematography of 3T3 cells 5–7 days post-insonation showed that the cells continued to divide and grow normally, but they showed striking differences in cell motility and in surface behaviour. Insonated cells did not spread as well on the substrate. They showed less contact inhibition and displayed remarkable bubbling over their surface, reminiscent of that seen at the poles of untreated cells during cytokinesis. This behaviour was seen throughout the cell cycle when examined at least 10 generations later in culture. Attention to individual cells revealed that there were brief periods

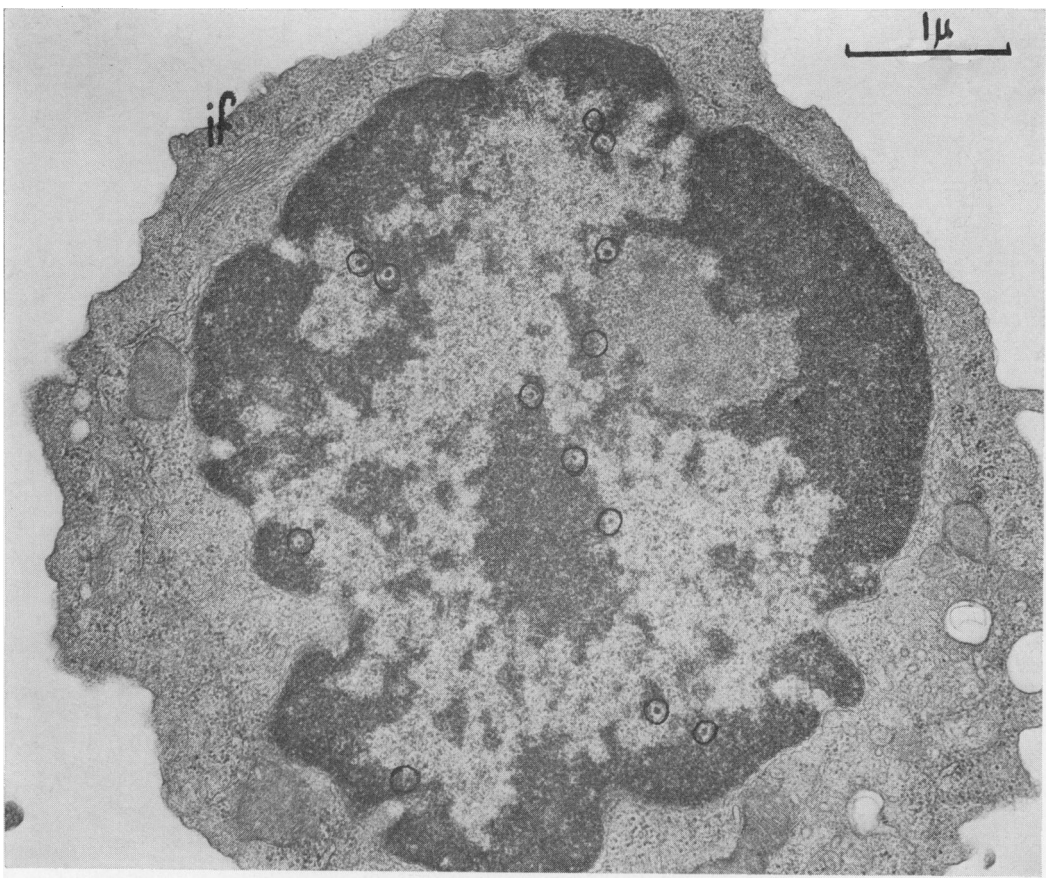

FIG. 6.—Electron micrograph of rat peritoneal fluid lymphocyte 5 min post-insonation. Many perichromatin granules are already present (*e.g.* circles). A small bundle of intermediate filaments (i.f.) is also seen.

during which they assumed a more normal morphology and behaviour and then reverted to the abnormal pattern. In photographic sequences lasting over a period of 18 h (2 frames/min) all recorded insonated cells displayed abnormal motility. The cell cycle time of 3T3 cells in our laboratory is 16 h. In such long sequences, almost all the cells can be observed to divide, with both daughter cells then exhibiting disturbed behavioural patterns. In preliminary studies, insonated cells appeared to have a more random pattern of motion, as revealed by phagokinetic tracks (unpublished results).

With Nomarski's differential interference contrast optics, the morphology of cells in culture is particularly well demonstrated. Control 3T3 cells are shown in Fig. 7, which shows that the well-flattened cells have cytoplasmic extensions terminating in attenuated hyaloplasmic veils; fine attachment fibres are also present. By contrast, insonated cells (Fig. 8) do not spread as well, and they

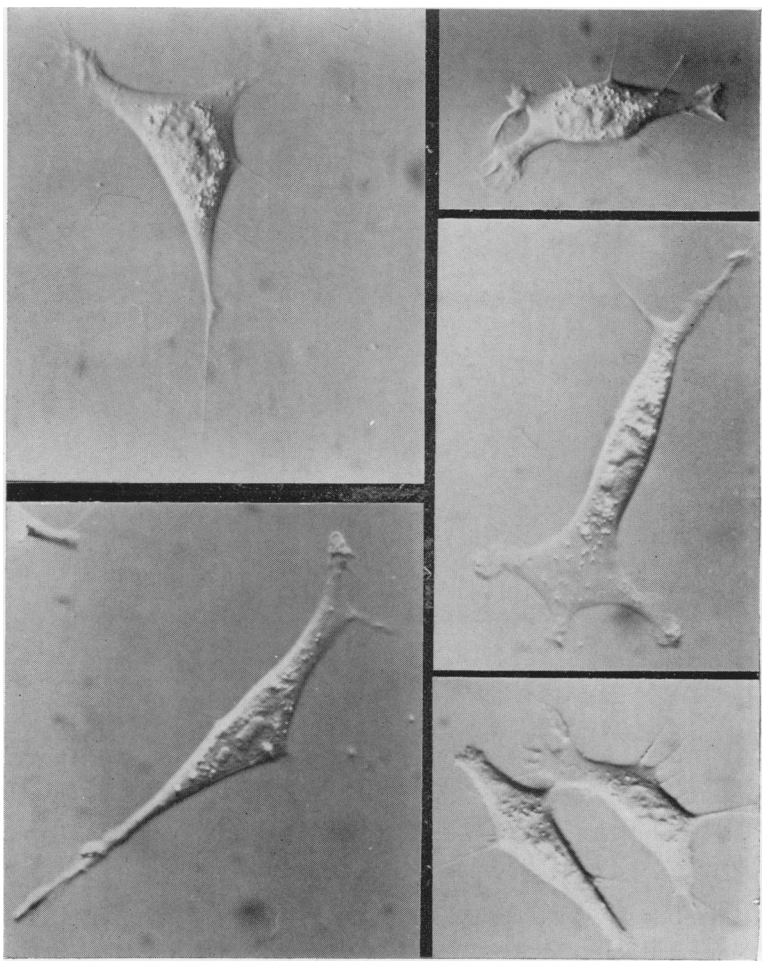

FIG. 7.—Control 3T3 cells in tissue culture photographed with Nomarski's differential interference contrast optics 5 days after plating. Note the cells are well spread and display broad attenuated cytoplasmic extensions (hyaloplasmic veils) at the ends of their cytoplasmic extensions, which are regularly tapered (oil immersion objective).

display grossly abnormal cytoplasmic extensions and fewer attachment fibres. The cytoplasmic extensions are sharply angular or bullous, and attenuated hyaloplasmic veils are rare. Some insonated cells appear normal (Fig. 8, lower right), corresponding to the temporary normal appearance assumed between long periods of abnormal locomotion, as seen in the time-lapse cinematographic sequences described earlier.

We have determined the fraction of time during which insonated cells exhibit normal morphology, by scoring them in live preparations under microscopic observation as either "affected" or "not affected" in coded slides. This method is not entirely impartial, because affected cells are so readily recognized that one is soon aware of the nature of the sample. Nevertheless we noted that approximately 2/3 of cells in the ultrasound

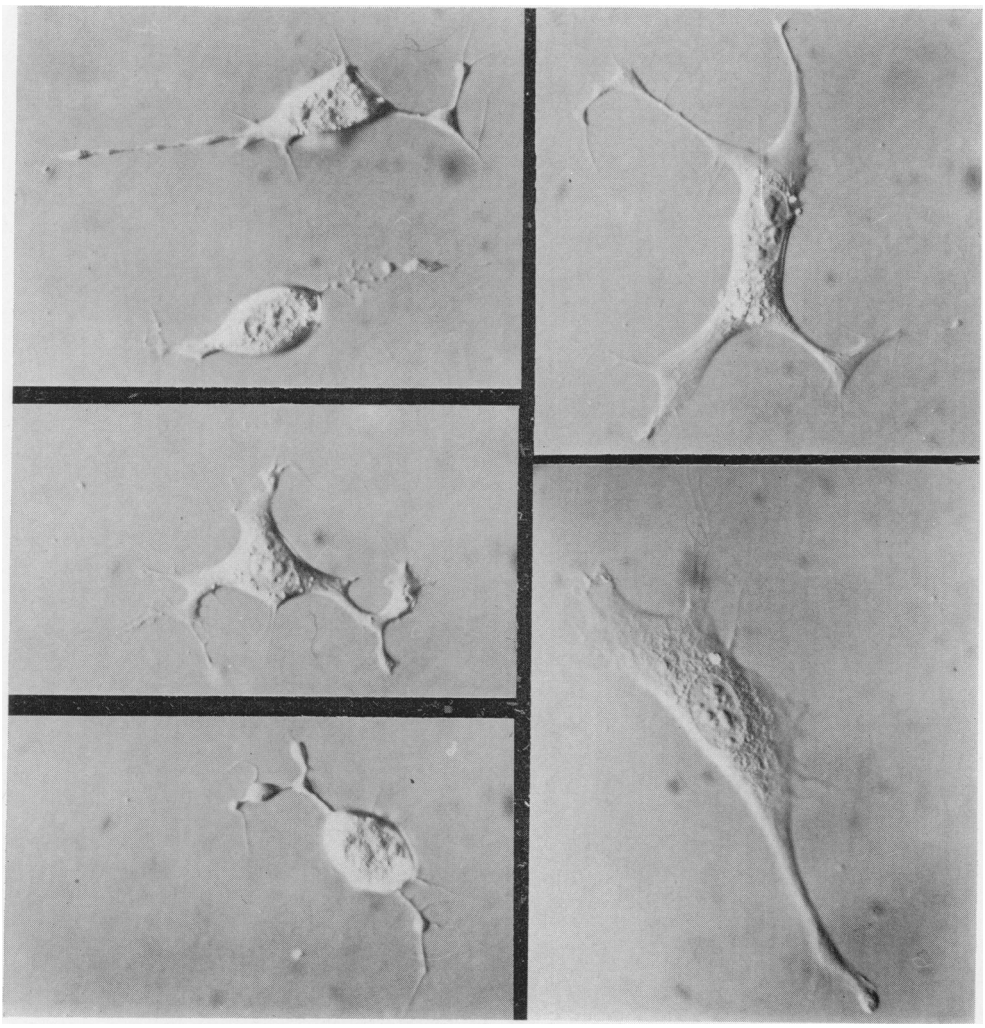

FIG. 8.—Insonated 3T3 cells in tissue culture (aliquot from same material shown in Fig. 7) photographed with Nomarski's optics 5 days post-insonation. Note cell body is thicker, smaller, and more rounded than control cells of Fig. 7. Their cytoplasmic extensions appear grossly abnormal; they are thinner but bullous, and they display sharp angularities. Hyaloplasmic extensions are poorly developed (oil immersion objective).

treatment group demonstrated multipolarity with trabeculation and beading of cell projections. This could be easily differentiated from the appearance of control cells.

We have also examined the influence of U.V. and X-rays to learn if these could elicit the abnormal locomotion seen in insonated cells. Cells were exposed to low doses of X-ray (0·029 Gy to 0·29 Gy) or of ultraviolet irradiation from a germicidal lamp (10 J/m^2 for 1 sec) one week before time-lapse cinemicrography. In the X-irradiated group the response was dose-related; the fraction of affected cells and their lack of spreading increased in proportion to dosage, as did the extent of surface "bubbling". Ultraviolet irradiation induced a similar abnormal motility.

We conclude that low level *pulsed* ultrasound from a clinical instrument induced abnormalities of cellular ultrastructure and motility. The cytoskeleton affects motility, cell shape, and plasmalemmal behaviour as well as translocation of organelles during secretion. The cytoskeleton is intimately related to the centriole and thus may play a key role in the various observed effects. Unwinding of double-stranded DNA (as evidenced by increased immunoreactivity to anti-nucleoside antibodies) and increased PCGs (which are RNA-protein complexes (Daskal *et al.*, 1980; Puvion *et al.*, 1980) apparently related to transcription and/or translation of DNA messages) may reflect molecular damage or repair activity. PCGs are increased in tumours and in transformed cells (Smith, 1979) and are elicited by carcinogens or toxins (Barsotti *et al.*, 1979). Thus, both morphological and immunochemical changes can be demonstrated in the nucleus after pulsed ultrasound. Engorgement of rough endoplasmic cisternae, and plasmalemmal activity, could both be due to altered function of microtubules and microfilaments. Destruction of microtubules by colchicine interferes with various secretory processes and results in engorged RER cisternae in fibroblasts. Our findings that bundles of intermediate filaments are more frequent in insonated cells may reflect disruption of internal cellular redistribution mechanisms by ultrasound and favours the possibility that RER contents accumulate in cisternae through failure of cytoskeletal involvement in the normal transfer of RER intracisternal contents into Golgi saccules.

The persistence of abnormal behaviour and motility in cells exposed to a single dose of diagnostic level ultrasound 10 generations after insonation suggests permanent hereditary effects. The abnormal cellular locomotion may well be responsible for the increased irregular dense microvilli seen in 3T3 cells exposed to ultrasound, as previously reported (Liebeskind *et al.*, 1981). We speculate that this persistence of abnormal behaviour may represent an early physiological step in progression towards transformation. This, in turn, could lead to an enhanced probability of a second event which may lead to eventual transformation. A similar theory has been postulated for ionizing radiation in a different cell system (Kennedy *et al.*, 1980). We believe that this speculation is strengthened by the observation that similar behavioural changes occur with ionizing radiation as well as ultraviolet light, both well-known mutagens.

It is not known whether the *in vitro* effects of ultrasound also occur *in vivo*. We have previously reported that neither the polypropylene test tube nor the parafilm cover contributed to the observed bioeffects (Liebeskind *et al.*, 1979*a*). The persistence of disturbances in cell motility many generations after insonation *in vitro* is especially important. One can speculate that if foetal cells were to be subtly damaged, it might affect patterns of cellular migrations during ontogenesis. If germ cells were so involved, the effects might not become apparent until the next generation. We strongly believe that further studies in both *in vitro* and *in vivo* systems are clearly indicated.

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