Cytolysis Induced by Human Lymphotoxin

Cinemicrographic and Electron Microscopic Observations

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By phase-contrast cinemicroscopy, highly purified human lymphotoxin induced two forms of cytolysis—one characterized by slow (1 to 2 hours) swelling (*ballooning*-type) and the other by sudden (3 to 5 minutes) shrinkage of the cell body and violent agitation of residual debris (*popcorn*-type). Specifically sensitized lymphocytes likewise caused both forms of lysis, morphologically indistinguishable from lymphotoxin-induced cytolysis. Lymphotoxin also inhibited cell division; mitoses diminished and virtually ceased before the onset of cytolysis. Many of the ultrastructural features preceding early lysis were similar to those associated with failure of osmoregulatory mechanisms—*ie*, condensation of mitochondria, dilation of rough-surfaced endoplasmic reticulum, separation of polysomes into individual units and expansion of the cell sap. After exposure to lymphotoxin for 12 hours or more, most remaining cells exhibited these same ultrastructural changes but lacked striking mitochondrial lesions (Am J Pathol 69:103–118, 1972).

LYMPHOCYTES are an integral part of the mononuclear inflammatory infiltrate characteristic of cell-mediated immune reactions. The ability of lymphocytes to destroy other cells *in vitro* (target cell destruction) and to mediate tissue injury *in vivo* (allograft rejection, graft *vs* host reactions) suggests that these cells may cause at least a portion of cell-mediated immune injury. If this premise is correct, it is probable that the effector of injury is a cytotoxin(s) released from stimulated lymphocytes.

Rich and Lewis [1] noted that cytolysis followed the addition of tuberculoprotein to splenic explant or buffy coat cultures prepared from tuberculous guinea pigs. Ruddle and Waksman [2] extended and refined this observation, later attributing cell death to a toxic mediator liberated from immune lymphocytes in response to specific antigen.[3] Granger and Kolb [4,5] demonstrated that nonimmune lymphocytes, if stimulated by mitogenic agents such as phytohemagglutinin (PHA), also produced a soluble cytotoxin, which they later named *lymphotoxin*. From

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studies performed with crude lymphocyte culture supernates,[6] it was concluded that lymphotoxin effects cytolysis by "acting on the cell membranes" of susceptible tissue culture cells, causing "disintegration" of the plasmalemma.

The primary objective of this *in vitro* study was to document the sequence of changes which occur in mouse fibroblasts after exposing them to lymphotoxin. The use of highly purified test material minimized the possibility that the results were due to lymphocyte products other than lymphotoxin. Our findings demonstrate that, at the light microscopic level, lymphotoxin causes two morphologically distinct types of cytolysis, and that many of the ultrastructural lesions preceding cytolysis are those associated with impaired osmoregulation.

Materials and Methods

The preparation and purification of human lymphotoxin have been described.[7] Briefly, adenoidal cells consisting of 95% or more lymphocytes were cultured at 37 C in RPMI 1640 tissue culture medium (Associated Biomedic Systems, Buffalo, NY) containing 10% fetal bovine serum (FBS), antibiotics and 5 to 7 μg/ml phytohemagglutinin (PHA-P, Difco Laboratories, Detroit, Mich). After 24 hours, the cells were collected by centrifugation and resuspended in serum-free RPMI containing a reduced amount of PHA (usually 3 μg/ml). After an additional 4 to 6 days' incubation at 37 C, cell-free supernates were harvested by centrifugation. Control material was prepared similarly, except that PHA was added at the time of harvest, after all cells had been removed.

Supernates from lymphocyte cultures were concentrated and sequentially fractionated by column chromatography (Sephadex G-150 followed by DEAE-cellulose) and preparatory slab acrylamide gel electrophoresis. The final product was purified greater than 2000-fold. Identically fractionated control material lacked cytotoxicity. Briefly, a cytotoxic unit (cu) was defined as sufficient lymphotoxin activity to reduce the number of cells in experimental tubes by 1000, compared with control cultures. The details of cytotoxic unit calculation have been described previously.[7]

Electron Microscopy

Stock cultures of logarithmically growing mouse fibroblasts (L-cell strain 929) were treated with 0.25% trypsin in Hanks' balanced salt solution for 5 minutes at room temperature. The freed cells were washed once with RPMI and resuspended in RPMI + 5% FBS at a concentration of 1.4×10^5/ml. Disposable sterile Petri dishes (60 × 15 mm), containing pieces of 300-gauge Melinex O polyester sheeting[8] (Transilwrap West Corp, San Francisco), each received 4 ml of the L-cell suspension and were subsequently incubated for 24 hours at 37 C in an atmosphere of 95% air and 5% CO_2. The supernatant medium was then replaced with RPMI + 5% FBS, containing either purified lymphotoxin (1200 cu/ml) or an equivalent amount of control material; incubation was continued under the same conditions. This larger dose of lymphotoxin insured that there were enough affected cells to permit representative sampling. At subsequent intervals of 6, 12, 24 and 48 hours, cells were fixed with distilled, ice-cold 1.5% glutaraldehyde containing 2% (w/v) sucrose. This was accomplished by adding an

appropriate amount of 15% stock glutaraldehyde (containing 20% sucrose) directly to the medium in tissue cultures. Fixation was continued at 4 C for at least 12 hours (up to 72 hours) before cells were postfixed for 30 minutes with 1% osmium tetroxide. After dehydration in a graded series of ethanol and in propylene dioxide (10 minutes each step), the Melinex O coverslips with attached cells were inverted on embedding capsules filled with Araldite. After polymerization, the coverslips detached easily from the plastic, leaving the fibroblasts embedded in the face of the block. Ultrathin sections were cut, stained with lead citrate and uranyl acetate and examined in a Siemens 1A electron microscope equipped with a 35-μ objective aperture.

Phase-Contrast Cinemicrographic Studies

Rose chambers were inoculated with a cell suspension prepared by the procedures described above. After incubation for 24 hours at 37 C, when the fibroblasts were firmly attached to the glass, the supernatant medium was withdrawn and the chamber was refilled with RPMI + 5% FBS containing either purified lymphotoxin (400 cu/ml) or an equivalent amount of control material. Cells were photographed with an inverted phase-contrast microscope, using a 16-mm motion picture camera and Plus-X (Kodak) black-and-white reversal film. A frame interval of 30 seconds was employed in low-magnification (22 ×) studies, while a 5-second interval was used at higher (90 ×) magnification. We analyzed films with a variable-speed projector and, frame-by-frame, with a 16-mm film-editing machine, which additionally magnified the image seven times. The cells pictured in the initial frame of each film were identified by number and, together with any progeny, were followed thereafter.

Results

Control Cultures

It was observed by phase-contrast microscopy, 24 hours after seeding, at the time control material was added, that the individual cells were uniformly distributed and formed a complete monolayer within the subsequent 25 to 30 hours. Thereafter the frequency of mitosis diminished but did not stop entirely.

Mitosis was heralded by a sudden cessation of cell movement and the retraction of cytoplasmic processes. At the magnification employed, a dividing cell became increasingly refractile as it rounded into a small sphere; a cleavage furrow appeared; and the cell separated into two smaller, refractile spheres. The two daughter cells then flattened on the glass, losing their refractility, meanwhile pulling apart. Cytokinesis was frequently incomplete and, in these instances, a cytoplasmic bridge could be detected joining the two daughter cells for up to 2 or more hours.

During interphase, cells in all control cultures varied only slightly in their morphologic appearance and behavior. They were polygonal, flattened on the glass, and they frequently extended cytoplasmic processes with wavy or ruffled leading margins. Cellular movement often

followed the extension of such a process. Activity diminished, however, as the cultures became more populous. The cytoplasm of L-cells was finely granular, except for the presence of round to rod-like mitochondria and occasional pinocytic vacuoles. Nuclear rotation was occasionally seen.

Ultrastructurally (Figure 1), the chromatin of normal L-cells was dispersed, except for occasional aggregates and a thin layer along the nuclear membrane. Nucleoli were prominent and often multiple. Mitochondria were large, oval-to-elongated and myriad polyribosomes were a conspicuous cytoplasmic feature. Microtubules, microfilaments, various types of membrane-bound inclusions and narrow profiles of rough-surfaced endoplasmic reticulum (RER) were also frequently observed. In older cells, digestive vacuoles and lipid droplets were common. Golgi complexes were an infrequent finding; rarely, a structure morphologically akin to a virus particle was detected budding from a membranous structure. The plasma membrane characteristically formed numerous microvillous projections.

Lymphotoxin Treated Cells

Initially, by phase-contrast cinemicroscopy, 400 cytotoxic units of lymphotoxin per milliliter had no observable effect. Fibroblasts continued their movement and division without apparent change. By 12 hours, however, diminished movement and fewer mitoses were evident, and occasional cells had begun to lyse. Thereafter, the L-cells became increasingly granular and, by 18 to 24 hours, cytolysis had become a widespread phenomenon. Two morphologically distinct types of cytolysis were observed.

Popcorn-type Cytolysis. This name was given to the most common form of cytolysis because the residual cellular mass often resembled a kernel of popped corn (Figure 2). This lytic event, sudden in onset, progressed to completion within 3 to 5 minutes and was characterized by sudden shrinkage and violent *bubbling* of the cell body. Attenuated cytoplasmic processes formed during shrinkage (Figure 2B and C) quickly withdrew and rounded into small refractile spheres which clustered around the central granular mass of cellular debris (Figure 2D). Nuclear architecture and detail were lost. Remaining debris detached from the glass and either stuck to an adjacent cell or floated freely in the medium.

Ballooning-type Cytolysis. This form of cytolysis lacked the dramatic immediacy and violent bubbling characteristic of the popcorn-type (Figure 3). Quite simply, it consisted of slow swelling and

rounding-up of the cell (Figure 3B and C), until a large cytoplasmic bleb abruptly appeared (Figure 3D). The process frequently required 60 or more minutes (occasionally longer than 2 hours) and always involved both the nucleus and the cytoplasm. Residual debris detached from the glass as an amorphous granular mass.

Electron microscopic examination of L-cells exposed to lymphotoxin (1200 cytotoxic units/ml) for 6 hours revealed extensive ultrastructural lesions in some; many, however, appeared unaffected. The least severe alterations detected (Figure 4) were dilation of RER, separation of polyribosomes into individual units and condensation of mitochondria. Chromatin was clumped along the nuclear membrane. At this stage, microvillous processes still protruded from the cell surface. More extensively affected cells (Figure 5) exhibited marked dilation of RER and other membrane-bound structures. The matrix of some mitochondria appeared homogeneous and had become intensely black, with transverse lines of lesser osmiophilia. There was extensive clumping of chromatin, principally along the nuclear membrane, and the angularity of cells and their microvillous processes had been lost.

At 12 hours, fewer cells were present within the cultures. Those which remained on the cover slips exhibited many of the changes (including dilated RER, disintegration of polysomes and expansion of the cell sap) described at 6 hours; however, the majority of the cells now lacked striking mitochondrial changes (Figure 6). Structures resembling virus particles were seen with increasing frequency as degenerative changes in L-cells became more severe. Small discontinuities in the plasma membrane could occasionally be detected.

After 24 hours, very few cells remained attached to the cover slips. Those present (Figure 7) consisted of little more than degenerating nuclei surrounded by cytoplasmic debris. Mitochondria, when present, were swollen, usually spherical and exhibited dilated intracristal spaces. Extensive breaks were found in the plasma membranes of these cells.

By 48 hours, no cells adhered to coverslips in lymphotoxin-treated cultures.

In addition to causing cytolysis, lymphotoxin (400 cytotoxic units/ml) inhibited mitosis (Table 1). In control cultures photographed for 28 hours or more, every cell initially present divided at some time during the course of the film, with the rare exception of those which developed into giant cells. In striking contrast, after exposure to lymphotoxin, many cells present in the initial microscopic field failed to divide,

Table 1—Effect of Purified Human LT (400 cytotoxic units/ml) on L-cell Strain 929 Target Cells (Representative Films)

	Control		Lymphotoxin	
No. of cells initially in field	51		57	
	No.	%	No.	%
Divide	50	98	18	31.6
Lyse	0	0	22	38.6
Neither divide nor lyse	1*	2	17	29.8
No. of daughter cells observed	100		36	
	No.	%	No.	%
Divide	33	33	0	0
Lyse	3	3	7	19.4
Neither divide nor lyse	59	59	28	77.8
Lost to view	5	5	1	2.8
Total number of mitroses	83		18	

* Developed into giant cell

Film durations were 28 hours for control cells and 32 hours for cells exposed to lymphotoxin. Control medium consisted of identically purified supernate from unstimulated lymphocyte cultures, diluted similarly (1:150) to the lymphotoxin-containing medium. **Daughter cells** are the progeny of **cells initially in the field**.

These data show that, in addition to causing cytolysis, lymphotoxin also inhibits the proliferation of L-cell 929 fibroblasts. Lysis which occurred in control cultures was observed late in the incubation period. Nonspecific cytolysis is a well-documented phenomenon in aged L-cell cultures.[9]

and after 18 to 24 hours, when cytolysis had begun in earnest, mitoses were extremely rare.

Discussion

By phase-contrast cinemicroscopy, there were two morphologically distinct forms of lymphotoxin-induced cytolysis, one characterized by cell swelling (which we termed ballooning-type) and the other by sudden shrinkage of the cell body followed by violent agitation of the residuum (popcorn-type). These were indistinguishable from the two kinds of cytolysis previously observed when L-cell 929 fibroblasts were exposed to specifically sensitized lymphocytes.[10]

The cardinal feature of ballooning-type cytolysis—early cell swelling—suggests that lymphotoxin may impair target cell osmoregulatory mechanisms. Many of the ultrastructural changes we found are consistent with this hypothesis in that they closely resembled the alterations observed by Trump and his colleagues.[11,12] In cells which had lost the capacity to regulate their volume, these investigators described swelling as the principal light microscopic feature, while the earliest

ultrastructural changes reported were dilation of the endoplasmic reticulum and nuclear envelope. Subcellular alterations of moderate severity included contraction and condensation of the inner mitochondrial compartment, with relative expansion of intracristal spaces and enlargement of the cell sap. Advanced stages of injury were heralded by severe swelling of mitochondria, loss of polysomes, karyolysis and interruptions in the plasma membrane. In spite of the marked similarities between our observations and those of Trump *et al*, we stress that the finding of morphologic alterations compatible with osmoregulatory failure does not indicate a primary site of action for lymphotoxin. Numerous agents, acting at different levels within the cell, are capable of interfering directly or indirectly with the maintenance of cell volume.[12]

Since cell swelling was not associated with popcorn-type lysis, we expected to find a second group of ultrastructural changes which would correlate with this form of cytolysis. We were unable to do so, in spite of examining hundreds of lymphotoxin-treated cells by electron microscopy. Among a number of plausible explanations for this apparent discrepancy, two seem most likely. First, the mechanism of injury was the same in both types of cytolysis and caused similar ultrastructural changes in all affected cells. However, because of differences in our target cell population (these cells had not been cloned for many years), the morphologic expression of injury varied at the light microscopic level. Alternately, we were simply unable to detect the ultrastructural changes associated with popcorn-type lysis because of inherent sampling difficulties. By light microscopy, this event was unheralded and occurred rapidly (3 to 5 minutes). Individual cells were affected at different times after exposure, and the residuum usually quickly detached from the glass. To clarify these points, further studies are planned, employing cloned and synchronized cells.

At the ultrastructural level, severe mitochondrial vacuolation has been described as the earliest discernible lesion caused in L-cell strain 929 fibroblasts by specifically sensitized lymphocytes.[13] Mitochondrial vacuolation was also observed in our target cells, but this change was detected in both control and experimental material, and the incidence increased greatly when fixative was placed directly onto the cells rather than into the tissue culture medium. We therefore believe that this mitochondrial alteration was an artifact of fixation rather than a lesion induced by lymphotoxin.

The inhibition of mitosis we observed probably reflects cellular injury caused by lymphotoxin. This is supported by our finding that the frequency of mitosis gradually diminished and virtually ceased just

before the onset of cytolysis. Proliferation inhibitory factor (PIF), an agent released from PHA-stimulated human lymphocytes, also interferes with cellular division.[14] The relationship of our highly purified lymphotoxin to this agent is unknown; however, PIF was described as having no effect on L-cell 929, the strain of fibroblast employed in our investigation.

Thus, highly purified human lymphotoxin causes both cytolysis and inhibition of mitosis, and many of the subcellular alterations it produces may reflect failure of osmoregulatory mechanisms. We are currently extending these morphologic studies with biochemical investigations designed to elucidate specifically how lymphotoxin mediates such changes.

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[*Illustrations follow*]

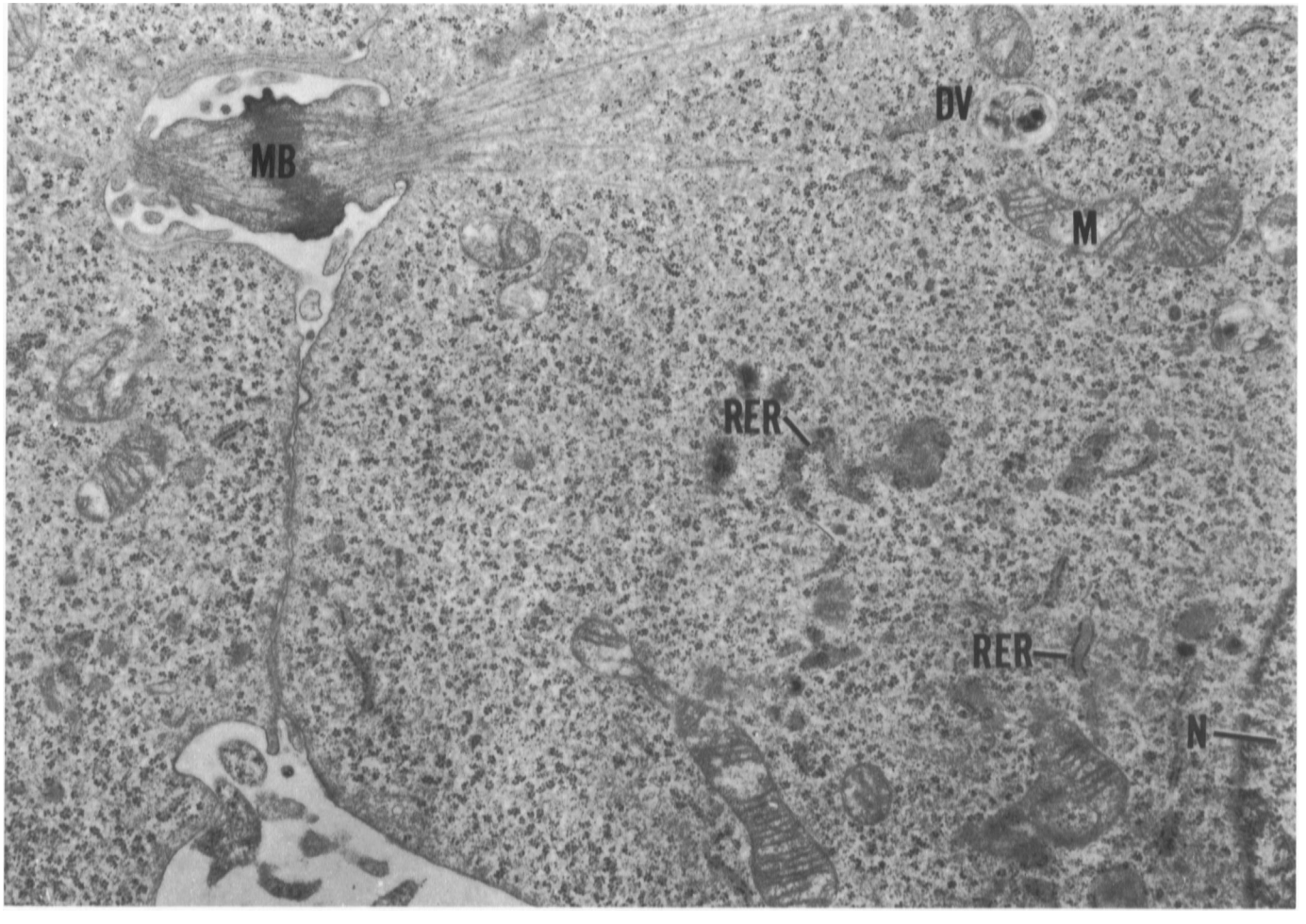

Fig 1—Normal fibroblasts of L-cell strain 929. Numerous polyribosomes are a characteristic cytoplasmic feature of these cells. Incomplete cytokinesis is a common, though transient, finding. The two cells depicted have recently divided, but remain joined by a tangentially sectioned cytoplasmic bridge. A midbody (*MB*) and attached spindle fibers are seen in the bridge. *M* = mitochondrion, *RER* = rough-surfaced endoplasmic reticulum, *DV* = digestive vacuole, *N* = nucleus (× 21,000).

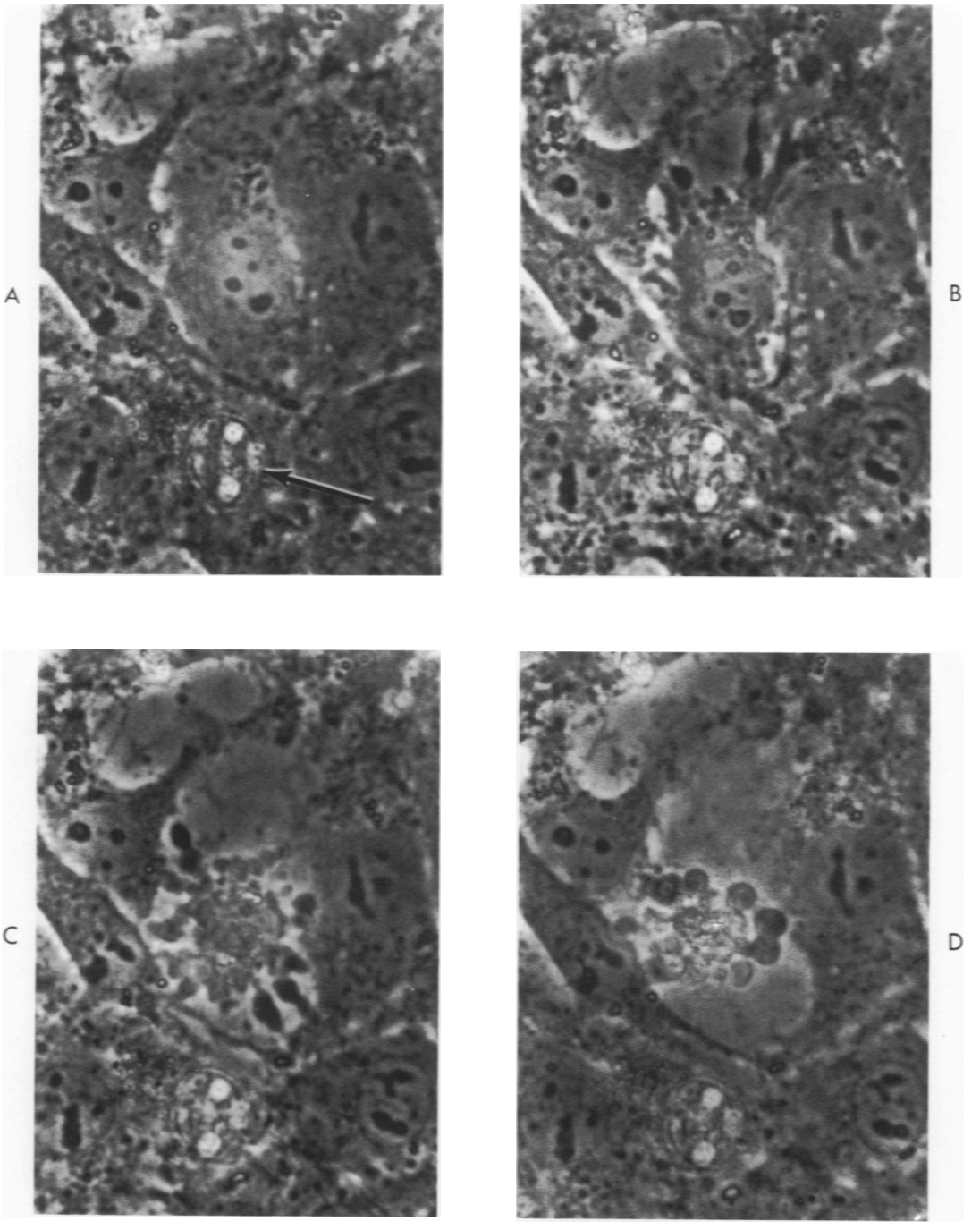

Fig 2—*Popcorn*-type lysis of L-cell strain 929 fibroblast exposed to 400 cytotoxic units of purified human lymphotoxin/ml. The events depicted here occur within 3 to 5 minutes (phase-contrast, × 1150). **Fig 2A**—Fibroblast (*center*) 30 seconds prior to initiation of the lytic process. There are no specifically discernible degenerative changes which suggest impending cytolysis. Previously lysed cell (*arrow*). **Fig 2B**—The initial event is sudden retraction of the cell body, leaving attenuated cytoplasmic processes still attached to the glass. Early distortion of the nucleus is evident. **Fig 2C**—The cell body has fully shrunk and there is severe distortion of the nucleus. The peripheral cytoplasmic processes are retracting and becoming globular. **Fig 2D**—The fully retracted cytoplasmic processes have become refractile spheres peripherally arranged around the main mass of granular debris. Violent *bubbling* of the entire debris mass and detachment from the glass follow.

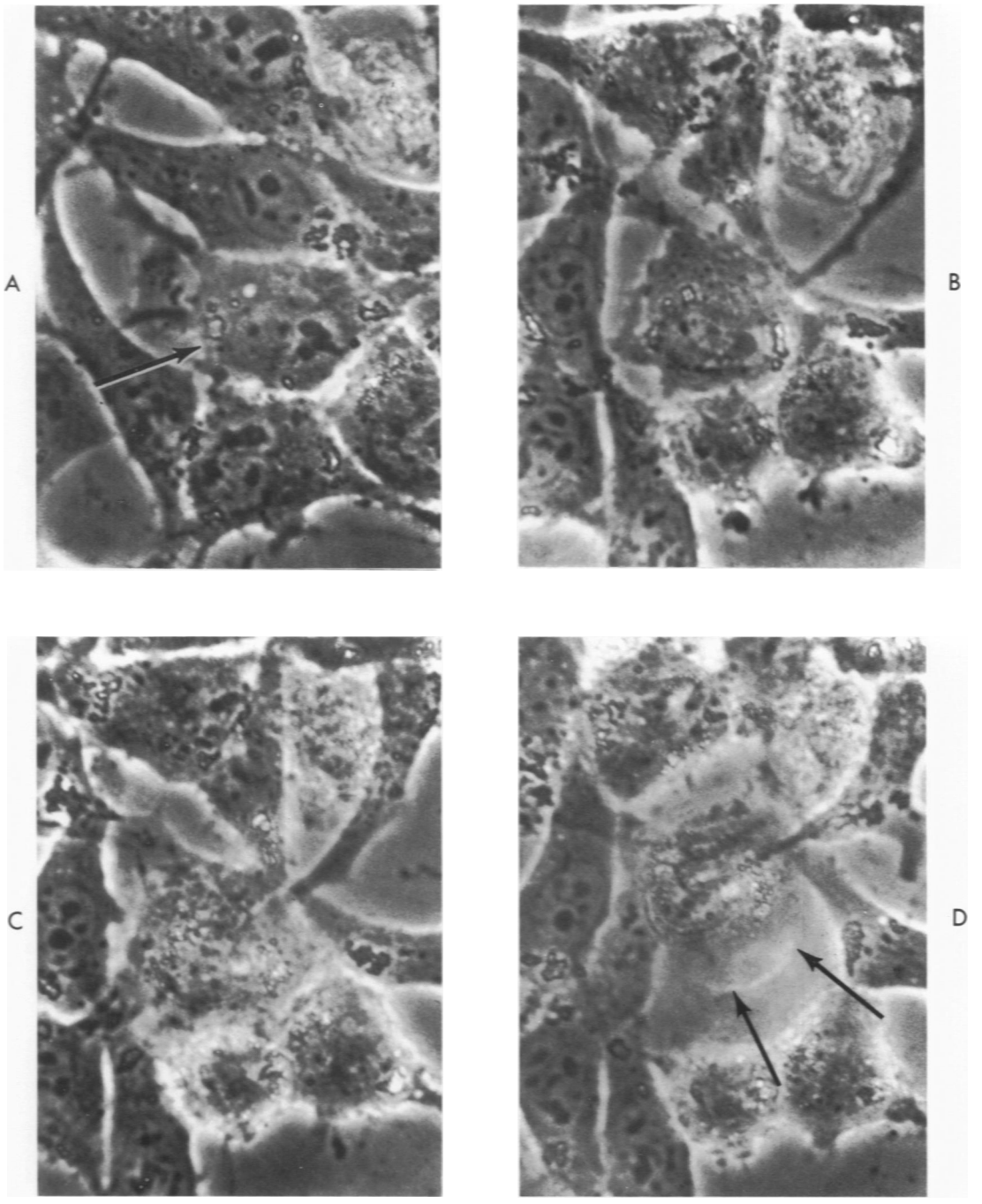

Fig 3—*Ballooning*-type lysis of L-cell strain 929 fibroblast exposed to 400 cytotoxic units of purified human lymphotoxin/ml. In contrast to popcorn-type cytolysis, the events depicted here often require more than 60 minutes and sometimes as long as 2 hours (phase-contrast, × 1150). **Fig 3A**—Fibroblast (*arrow*) approximately 30 minutes before earliest detection of changes associated with ballooning cytolysis. The two cells below the subject fibroblast are similarly degenerating. **Fig 3B**—The earliest detectable changes are slight swelling and rounding-up of the affected cell. **Fig 3C**—Fifty minutes after earliest detectable changes, the affected cell is much more spherical than normal and its cytoplasm has become quite granular. Nuclear architecture has been lost. **Fig 3D**—Termination of the lytic process (100 minutes from earliest detectable changes) is heralded by the sudden appearance of a cytoplasmic bleb (*arrows*). Thereafter, the residual mass increases in granularity, shrinks, and usually detaches from the glass to float freely in the medium.

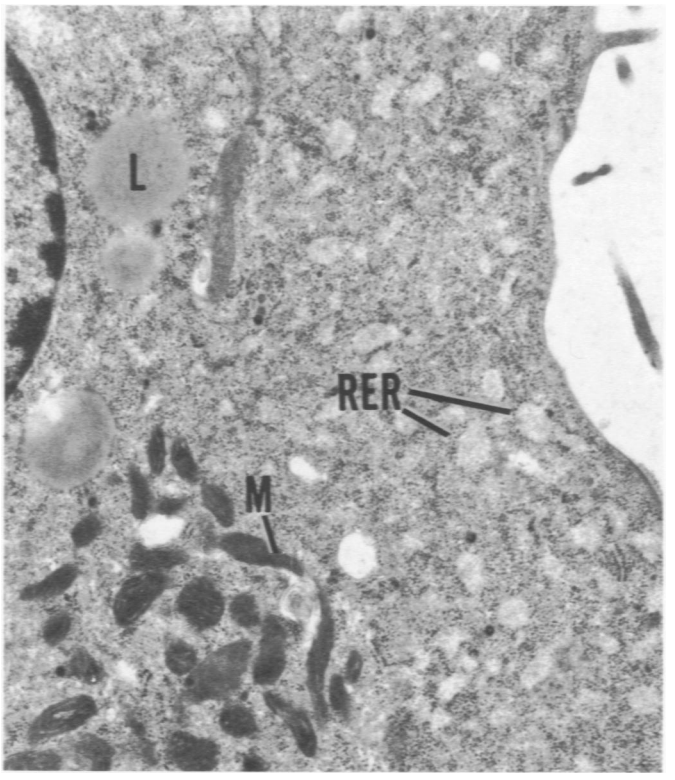

Fig 4—Early stage of lymphotoxin-induced cell injury. Rough-surfaced endoplasmic reticulum (*RER*) is dilated, mitochondria (*M*) appear *condensed* due to increased osmiophilia of their matrix, and polyribosomes have disintegrated into individual units. Microvillous processes, a characteristic feature of normal L-cells, still protrude at the cell surface. There is clumping of chromatin along the nuclear membrane. *L*=lipid droplet. Six-hour exposure to 1200 cytotoxic units lymphotoxin/ml (× 15,000).

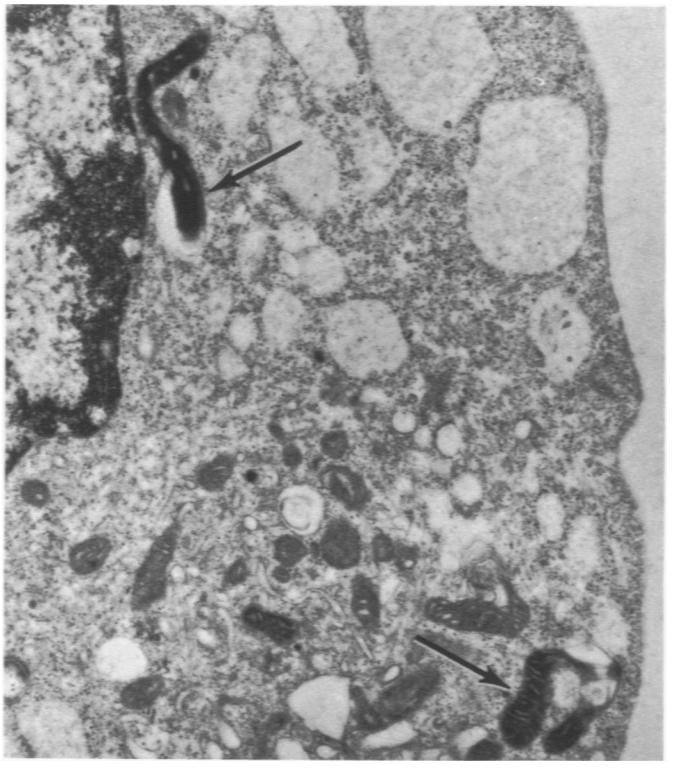

Fig 5—Intermediate stage of lymphotoxin-induced cell injury, also after 6-hour exposure to 1200 cytotoxic units LT/ml. The time-course of injury varied, due to apparent differences in individual cell susceptibility to lymphotoxin. In addition to the type of mitochondrion illustrated in Figure 4, there are some that appear black (*arrow*), with transverse lines of decreased osmiophilia. Dilated endoplasmic reticulum and multiple membrane-bound spaces are also characteristic of this stage of injury. Microvillous processes have been lost as a result of cytoplasmic volume increase (× 28,000).

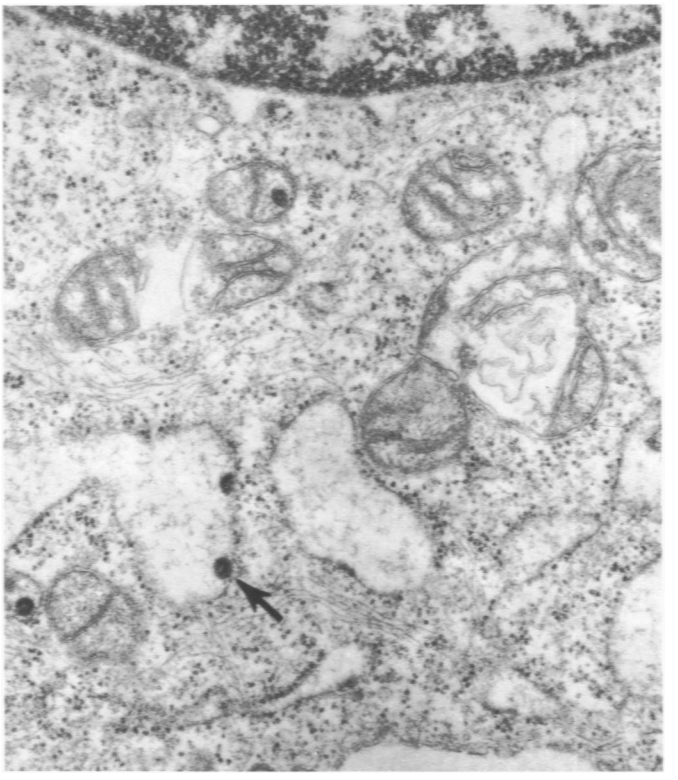

Fig 6—After 12 hours of exposure to lymphotoxin, many of the cells remaining on cover slips exhibit extensive dilation of their endoplasmic reticulum, fragmentation of polysomes and expansion of their cell sap, but lack condensed mitochondria. Structures morphologically consistent with virus particles (*arrow*) are seen with increasing frequency in degenerating cells. *N* = nucleus (× 30,000).

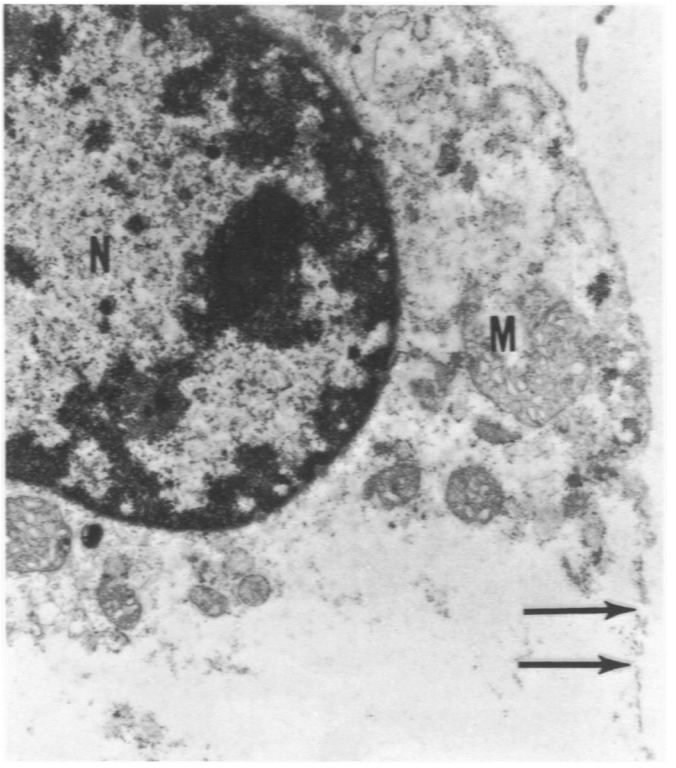

Fig 7—Terminal stage of lymphotoxin-induced cell injury (24-hour exposure to 1200 cytotoxic units lymphotoxin/ml). The plasmalemma of such cells is extensively disrupted (*arrows*) and very few cytoplasmic organelles remain. Mitochondria (*M*), when observed, are swollen and exhibit dilated intracristal spaces. *N* = nucleus (× 13,000).

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