# THE CYTOTOXIC ACTION OF IMMUNE GAMMA GLOBULIN AND COMPLEMENT ON KREBS ASCITES TUMOR CELLS*

## I. Ultrastructural Studies

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Plates 56 to 64

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The cytotoxic properties of anti-tumor cell antisera, *in vitro* and *in vivo*, have been described by various investigators and recently reviewed (1). Kalfayan and Kidd (2), Miller and Hsu (3), Flax (4), Goldstein (5), and Easty and Ambrose (6), in particular, have studied these effects in detail by means of phase and light microscopy. The morphologic studies of antisera-treated tumor cells have emphasized cytoplasmic swelling and vesiculation and rapid loss of cytoplasmic basophilia. It has also been shown that antibody causes *in vitro* agglutination of Krebs ascites tumor cells and that cells so treated will not reproduce the tumor when injected into the peritoneal cavity of mice (7).

The present study utilizes phase and electron microscopy to examine in detail the structural changes occurring in Krebs ascites tumor cells treated *in vitro* with antibody and complement. The ultrastructural studies provide an interpretation of cell injury and death in terms of subcellular structures of known function.

### *Materials and Methods*

*Tumor Cells.*—Krebs-2 ascites tumor cells were withdrawn from the peritoneal cavity of adult male Swiss mice inoculated 4 to 5 days previously with 0.2 ml. of tumor-bearing ascitic fluid. The removed cells were suspended in 40 ml. of Eagle's balanced salt solution (8) containing 1 mg./ml. of glucose (BSS), and centrifuged in the cold at 400 R.P.M. for 5 minutes (International refrigerated centrifuge). The supernatant fluid was decanted and the cells resuspended in 3.0 ml. of BSS to yield a concentration of approximately $2 \times 10^7$ cells/ml. A 0.3 ml. aliquot of this suspension was added to each control and experimental flask. Cell counts and all phase observations were performed with a phase hemocytometer counting chamber (American Optical Company, Buffalo).

*Antibody and Complement.*—Washed whole tumor cells from 0.5 ml. of ascitic fluid were injected into rabbits twice weekly for at least 4 weeks. Rabbit gamma globulin was prepared from the serum of the immunized animals by the addition with stirring of an equal volume of

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30 per cent $Na_2SO_4$, at room temperature. The precipitate was centrifuged and washed once with 15 per cent $Na_2SO_4$. It was then taken up in water and dialyzed twice against phosphate buffered saline (pH 7.4) and finally against BSS, to achieve the same salt composition as the incubation medium.

Mouse gamma globulin was prepared from the serum of normal adult male Swiss mice by the above procedure.

Human serum albumin (Cutter Laboratories, Berkley, Calif.) was dialyzed in the same manner as the gamma globulin preparations.

As a source of complement, serum pooled from at least two normal rabbits was divided into small volumes and kept frozen until used. Inactivated complement was prepared by heating the rabbit serum at 56°C. for 30 minutes.

## *Test Systems*

*1. Control Cells.*—

(*a*) 0.3 ml. cell suspension + 0.3 ml. human serum albumin (40 mg./ml.) + 0.3 ml. BSS.

(*b*) 0.3 ml. cell suspension + 0.3 ml. mouse gamma globulin (40 mg./ml.) + 0.3 ml. BSS.

(*c*) 0.3 ml. cell suspension + 0.3 ml. mouse gamma globulin (40 mg./ml.) + 0.3 ml. fresh rabbit serum (complement).

*2. Cells + Immune Gamma Globulin.*—

(*a*) 0.3 ml. cell suspension + 0.3 ml. rabbit immune gamma globulin (40 mg./ml.) + 0.3 ml. BSS.

(*b*) 0.3 ml. cell suspension + 0.3 ml. rabbit immune gamma globulin (40 mg./ml.) + 0.3 ml. inactivated complement.

*3. Cells + Immune Gamma Globulin + Complement.*—

(*a*) 0.3 ml. cell suspension + 0.3 ml. rabbit immune gamma globulin (40 mg./ml.) + 0.3 ml. complement.

Complement addition was always performed last and incubation times for these flasks were measured from the time of this addition. The incubations were performed in 5 ml. volumetric flasks in a rotary shaker water bath maintained at 37°C., after gassing the flasks with a mixture of 95 per cent $O_2$ and 5 per cent $CO_2$ for 3 minutes.

Fixation for electron microscopy was effected by adding the contents of each flask to 11.0 ml. of cold (0°C.), veronal buffered (pH 7.2–7.4), 1 per cent $OsO_4$ in 12.0 ml. glass centrifuge tubes and stirring gently. The cells were fixed at 0°C. for 7 minutes and centrifuged at low speed (International clinical centrifuge). The $OsO_4$ was decanted and the cell pellet suspended for 5 minute intervals in 50, 70, 95, and 100 percent ethanol. The cells were then resuspended for 5 minute intervals in several changes of methacrylate (8:2, *n*-butyl:methyl), placed in gelatin capsules and the methacrylate polymerized at 50°C. Thin sections were cut on a Porter-Blum microtome, placed on copper grids, and examined in an RCA EMU-2E electron microscope.

## RESULTS

*1. Controls.*—Controls *a*, *b*, and *c* gave identical results.

*Phase microscopy:* (Fig. 1.) All control cells displayed a general uniformity of size, sharp cytoplasmic borders, moderate cytoplasmic granulation, and indistinct nuclear outlines. No debris was present in the suspending medium.

*Electron microscopy:* (Fig. 2.) The cells displayed good preservation of nuclear and

cytoplasmic organization. They possessed the same components described in high resolution studies of many other cell types and conformed qualitatively to previously published descriptions of the similar Ehrlich ascites tumor cell (9). Of special note was the small degree of microvilliform (pseudopod) projection of the plasma membrane in control cells. Cells incubated with complement (system 1 *c*) appeared similar to the other control cells (1 *a* and *b*).

*2. Cells + Immune Gamma Globulin and Cells + Immune Gamma Globulin + Inactivated Complement* gave identical results.

*Phase microscopy:* After 15 minutes incubation the most prominent change was that of agglutination (Fig. 3). The degree of agglutination increased as incubation was prolonged to 60 minutes. Thread-like debris was observed in the suspending medium at 15 minutes and grew more prominent with prolonged incubation (Fig. 4). Cell lysis was not observed throughout this period and thus could not be invoked as a source of the debris. The unagglutinated cells appeared similar to the controls.

*Electron microscopy:* Cells were studied after 15 and 60 minutes' incubation. The changes observed were qualitatively similar but more advanced after the longer incubation period. The presence of inactivated complement in the test system (2 *b*) had no apparent effect upon the frequency or nature of the structural changes described below.

The principal change was a dramatic and complex projection and folding of a restricted portion of the surface membrane of the cell (Figs. 5 to 7). This labyrinthine folding resulted in the formation of cleft-like channels extending into the cell interior whose continuity with the surface membrane was not always apparent in a given plane of section (Fig. 8). In some of the less well developed examples of this surface alteration, the membrane assumed spike-like projections. Where cells were apposed, there was a suggestion of interdigitation of the projections (Fig. 9). The change, by virtue of its complex branching, folding, and invagination, differed from the simple villiform projections noted in control cells.

Electron-dense debris was present outside of the cells and similar material appeared to be adhering to the cell membrane, producing a hazing of the cell outline. This material was found along the smooth portions of the cell membrane as well as in the interstices of the folded zones (Figs. 5 to 8).

Only the cell membrane exhibited structural alteration in this test system. Intracytoplasmic components, such as mitochondria, endoplasmic reticulum, and lipide droplets appeared unaltered.

Approximately 170 cells were examined in thin section and about one out of every five cells exhibited the surface alteration. Of 200 control cells studied only one cell displayed a comparable change. This occurred in control system 1 *c* containing mouse gamma globulin and fresh rabbit serum. It is possible that natural antibodies present in the latter were responsible for the sporadic appearance of the surface phenomenon.

*3. Cells + Immune Gamma Globulin + Complement.*

*Phase microscopy:* After less than 2 minutes' incubation virtually all the cells displayed a remarkable cytoplasmic swelling. The cytoplasmic ballooning was at first

focal, producing gross pouching at two to seven visible points on the circumference (Fig. 10), but by 20 minutes' incubation the entire circumference became uniformly involved.[1] The final result was a greatly expanded cell possessing an unbroken cell membrane, with a uniformly clear cytoplasmic space, a distinct nucleus residing at the margin of the cell membrane with granules clustered close to the nucleus (Figs. 11, 12). Particulate debris, similar to that observed in cells treated with immune globulin alone, was present in the medium by 20 minutes' incubation. With incubations of 60 minutes or more, and if the shaking was vigorous, some cells underwent lysis, leaving agglutinated masses of naked nuclei and large amounts of debris in the medium.

The above phenomena were completely prevented by the presence of 0.01 M disodium ethylenediaminetetraacetate (Versene) and restored by the addition of 0.02 M $Ca^{++}$ and 0.02 M $Mg^{++}$.

*Electron microscopy:* Cells were examined after 15 to 20 minute incubations. Almost every cell examined displayed a moderate to marked degree of vacuolar change (Fig. 13). Normal cytoplasmic organization was disrupted. Large areas of cytoplasmic matrix were cleared of all particulate structures including the small electron-dense particles that form the background density in normal cells (Fig. 14). The expanded cytoplasmic zone contained large membrane-bounded spaces of varying size. These large vesicles tended to cluster near the nucleus; the "cleared" zones of the cytoplasm were more often peripheral, adjacent to the cell membrane.

Three classes of membrane-bounded vesicles were noted (Fig. 15). One type with a smooth outer membrane possessed a system of double membranes internal to the bounding membrane. These internal structures, though lacking the normal arrangement, resembled cristae mitochondriales and thus, these altered, swollen cytoplasmic bodies were judged to represent damaged mitochondria. Another type of vesicle was distinguished by circumferentially arranged electron-dense granules and no internal structure (Fig. 16). The third type lacked the peripheral electron-dense granules and the internal membranes as well. The latter two classes of vesicles, lacking internal membranes, are thought to represent respectively swollen rough and smooth surfaced endoplasmic reticulum (10). The space between the two layers of the nuclear double membrane was also focally expanded (Figs. 13, 17). As this space is known to communicate with the cavities of the endoplasmic reticulum (11), this observation supports the interpretation that this subcellular system was involved in the degenerative process.

The nuclei appeared generally intact and the nucleoplasm free of distinctive changes in aggregation or dispersal. However, the nuclear pores (11) were occasionally seen to be markedly widened, and it appeared as if nuclear material was passing into the cytoplasmic zone (Fig. 17). This phenomenon was not seen in the majority of cells examined after a 20 minute incubation, however, and may have represented the sporadic appearance of a more advanced form of injury.

Nearly all the cells possessed an intact cell membrane. The surface membrane folding, as described with cells treated with immune gamma globulin alone, was still discernible. In the presence of complement, however, the projections appeared consider-

[1] The progress of this change was best followed in separate experiments in which the components were all mixed at 0°C. and a sample observed as it warmed to room temperature in the hemocytometer chamber.

ably simplified, widened, and blunted owing to swelling of the subjacent cytoplasmic matrix (Fig. 18). Osmiophilic material was seen as in test systems 2 *a* and *b*, in the medium and along the cell membrane.

Incubations of cells with antibody and with antibody plus complement (test systems 2 and 3) were performed at 0°C. None of the above described changes noted by phase and electron microscopy occurred at this temperature.

## DISCUSSION

The demonstrated structural alteration of the cell membrane resulting from *in vitro* exposure to antibody alone, appeared quite different from surface structures described in control cells or in the biologically and morphologically similar Ehrlich ascites tumor cell (9). In both untreated cell types small canals or vesicles, even of the simplest form, are not often observed next to the cell membrane nor are invaginations noted between the surface pseudopods. It is suggested that the surface alteration in the antibody-treated cells is the result of antigen-antibody complexing in the surface membrane.

The focal nature of the change suggests the possibility that the membrane is functionally heterogeneous. An alternative and favored possibility is that the cell membrane is homogeneous and the alteration is random and perhaps reversible. The observed phenomenon suggests an exaggerated form of pinocytosis, and the two processes may be functionally related.

Agglutination of the antibody-treated cells occurred together with the membrane change. The irregular projections of the surface membrane may have increased the likelihood of apposed cells sticking together. The agglutination and the membrane change both required metabolic reactions for their development, since neither occurred when the incubation was performed at 0°C. for 90 minutes.[2]

The osmiophilic material noted adjacent to the membrane and in the medium in antibody-treated systems may have been related to the surface alteration. It is possible that the change might be accompanied by the loss of some structural elements from the membrane.

The electron micrographs show that the presence of antibody and complement induced a marked swelling of the whole cell, and of all the membrane-bounded compartments within the cell. These effects represent changes in fluid distribution which might be the result of damage to the mechanisms of permeability control in the membranes. In addition, the clearing of the cytoplasm indicates loss from the cell of small cytoplasmic granules. Since the cell membrane remained unbroken, the loss of such granules may have depended upon a prior solubilization.

The observed structural damage of cytoplasmic matrix, mitochondria, and endoplasmic reticulum would provide a reasonable functional basis for the death of the cells.

[2] The inagglutinability of the tumor cells at 0°C. applies only to a rabbit antibody system. Chicken antibody, for example, agglutinates the cells strongly at 0°C.

SUMMARY

Electron microscopic studies of Krebs ascites tumor cells treated *in vitro* with rabbit immune gamma globulin revealed a distinctive alteration in the structure of the cellular surface membrane. The subsequent addition of complement to this system severely damaged the cytoplasmic matrix, mitochondria, and endoplasmic reticulum. This sequence of events provides a structural basis for the cytotoxic action of antibody and complement.

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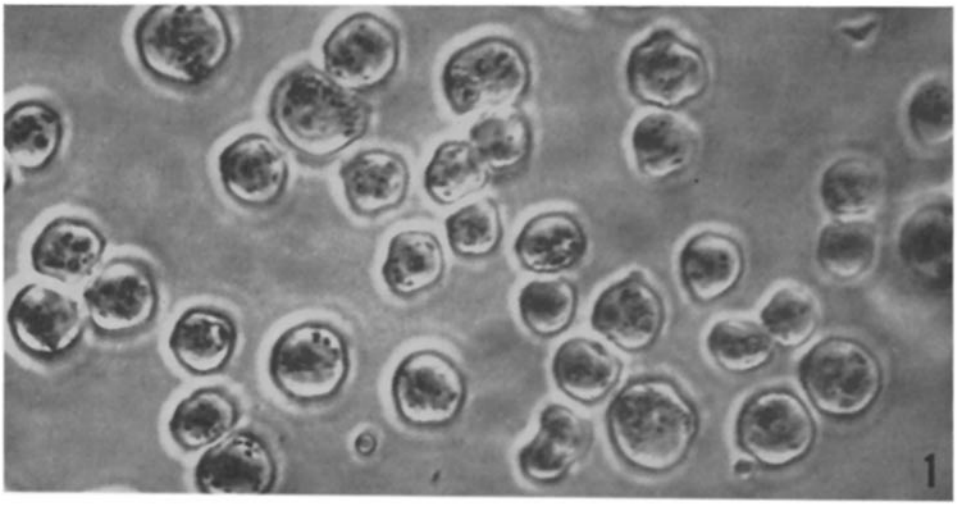
EXPLANATION OF PLATES

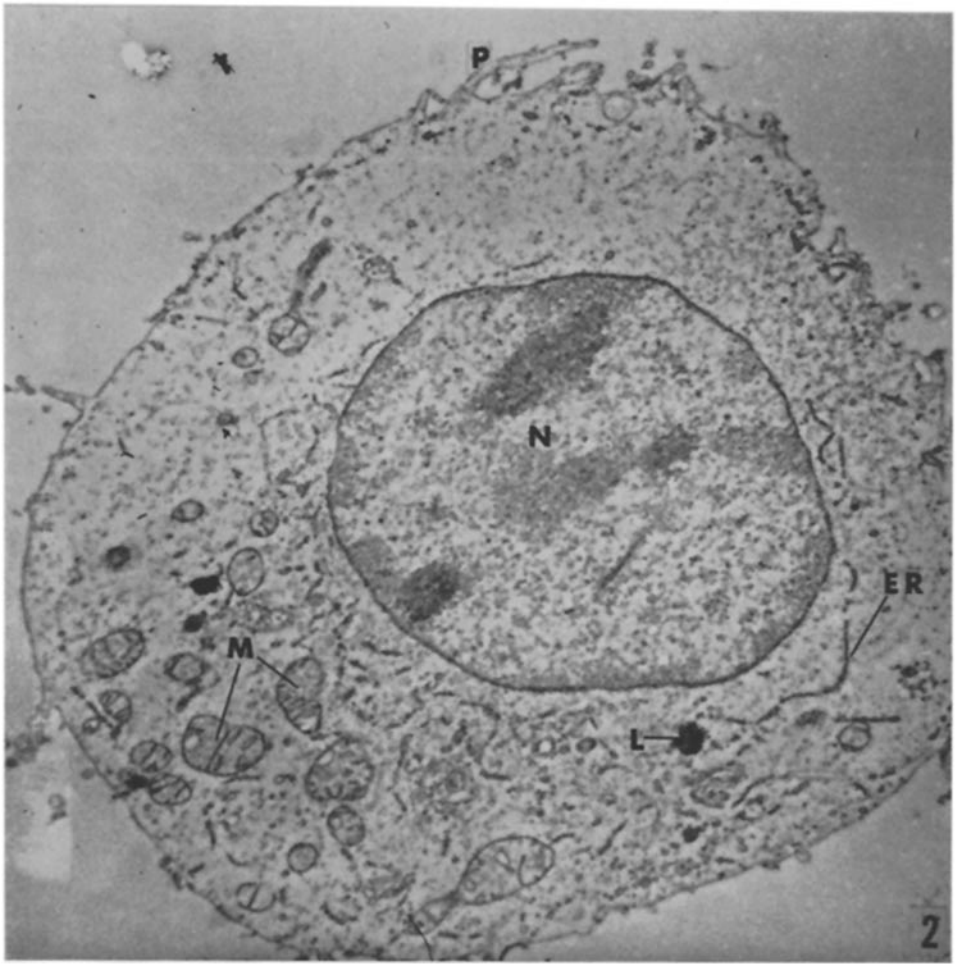
PLATE 56

*Control Cells*

FIG. 1. Cells display general uniformity of size, sharp cytoplasmic borders, moderate cytoplasmic granulation and indistinct nuclear outlines. Phase, × 640.

FIG. 2. Mitochondria (*M*) tend to display variability in shape and irregular arrangement of the cristae. The endoplasmic reticulum (*ER*) is not highly developed and small free osmiophilic granules provide the background density of the cytoplasmic matrix. A "pseudopod" projection of the cell surface (*P*) is shown. (*L*) denotes a lipide droplet. These droplets were frequently larger and more numerous than illustrated in this section. (*N*) indicates the nucleus. Electron micrograph, × 10,500.



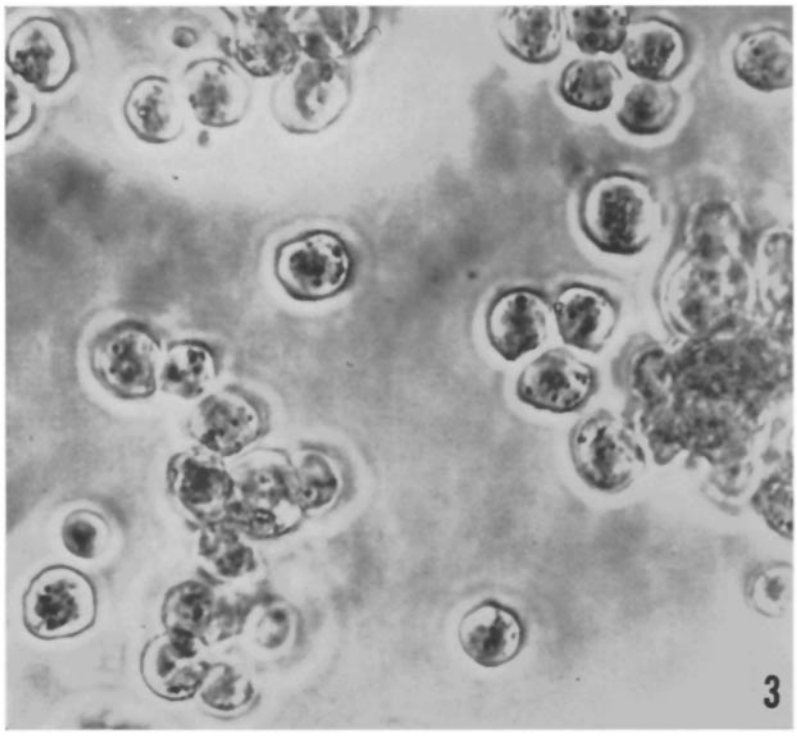

(Goldberg and Green: Antibody-complement cytotoxicity)

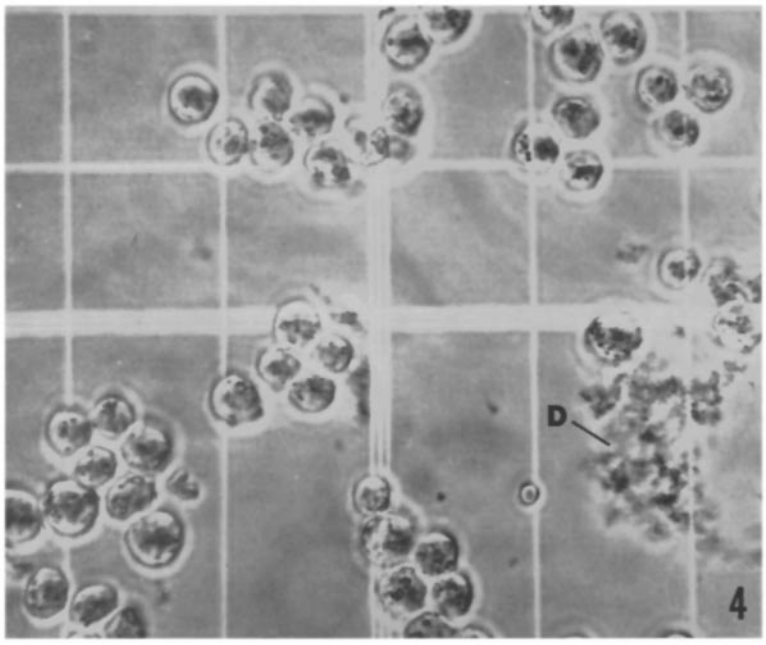
PLATE 57

*Cells + Immune Gamma Globulin*

FIG. 3. 15 minute incubation. Except for agglutination, cells appear similar to controls. Phase, × 640.

FIG. 4. One hour incubation. Debris (*D*) is now prominent in the medium. Phase, × 460.



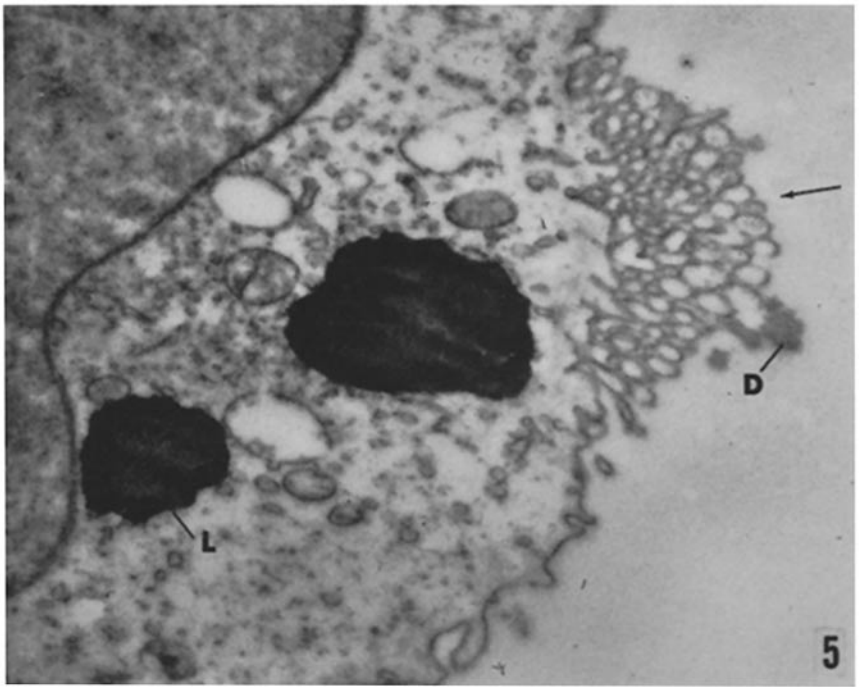



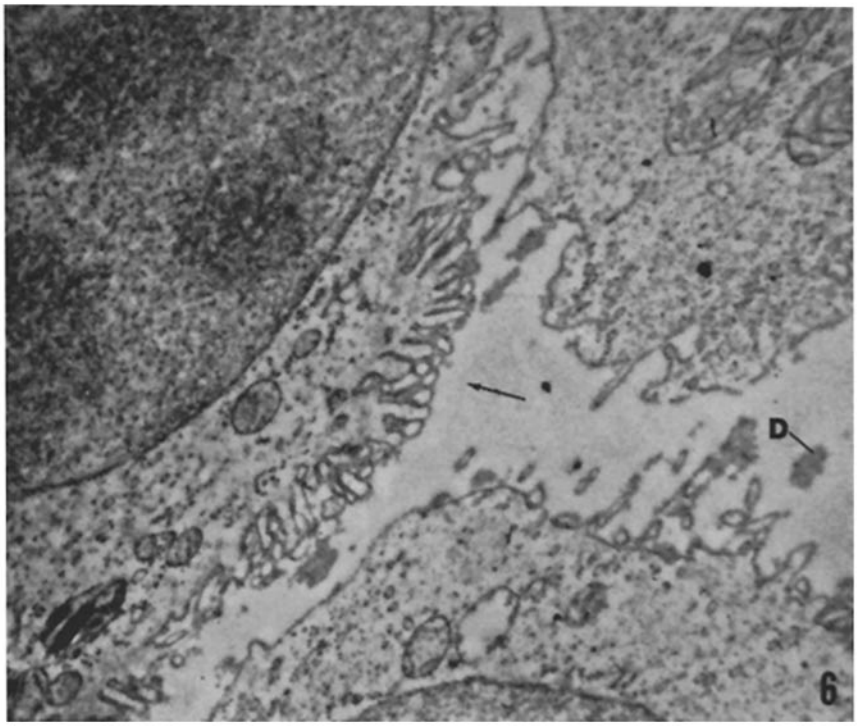

(Goldberg and Green: Antibody-complement cytotoxicity)

PLATE 58

*Cells + Immune Gamma Globulin*

FIGS. 5 and 6. Arrows point to the surface membrane change. Invaginations extending into cytoplasmic zone are seen to be in continuity with the cell surface. Debris (*D*) is present along the cell membrane and in the medium. (*L*) indicates lipide droplet. Electron micrographs. Fig. 5, × 15,300. Fig. 6, × 14,000.



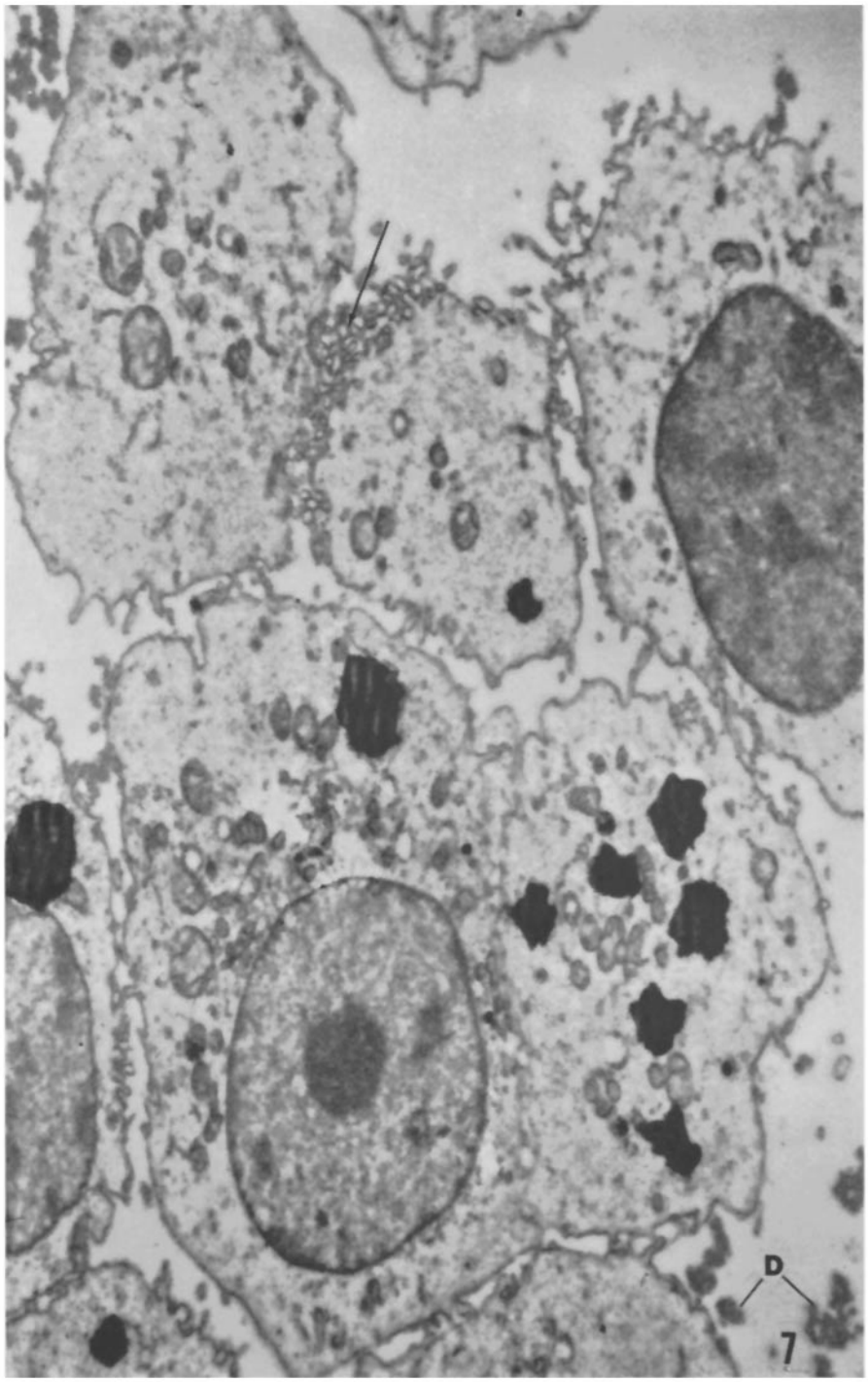




(Goldberg and Green: Antibody-complement cytotoxicity)

PLATE 59

*Cells + Immune Gamma Globulin*

FIG. 7. Apposed cells in antibody treated system. Surface membrane changes (arrow) may aid in agglutination. Electron micrograph, × 8000.



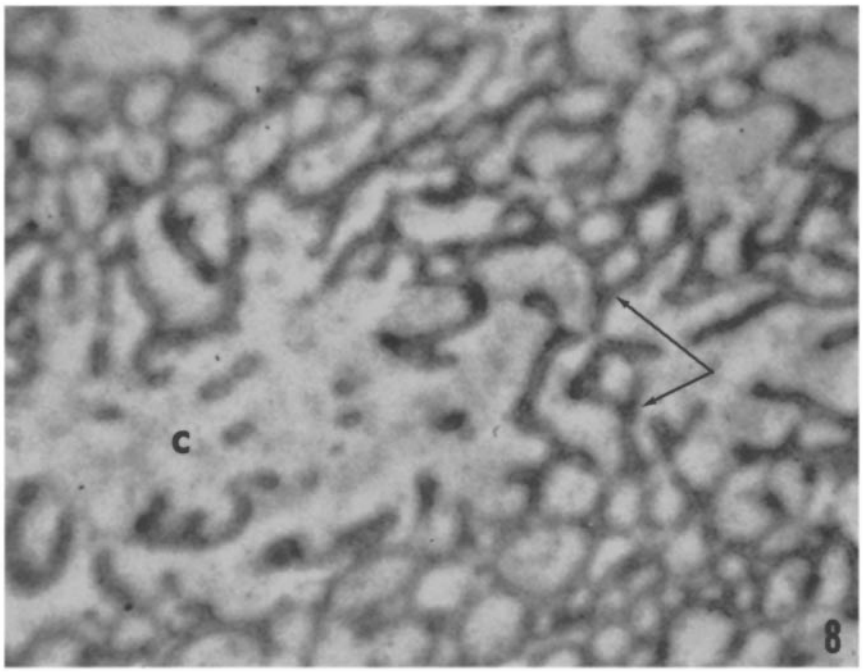

(Goldberg and Green: Antibody-complement cytotoxicity)

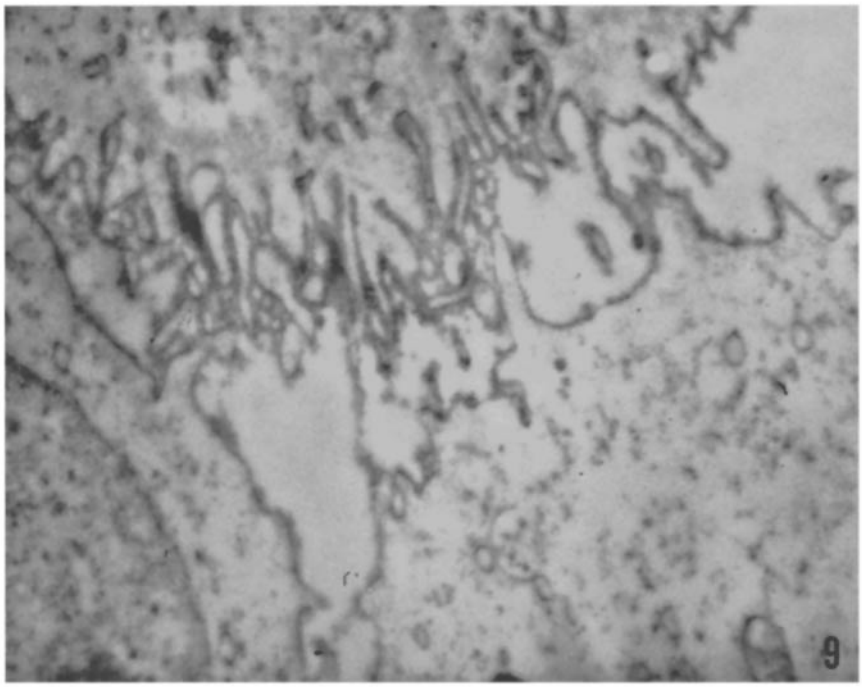
PLATE 60

*Cells + Immune Gamma Globulin*

FIG. 8. Labyrinth of cleft-like channels (arrows) extending deep into cytoplasm as a result of surface alteration. Osmiophilic material is present in the channels. Cytoplasmic matrix (*C*). Electron micrograph, × 36,000.

FIG. 9. Interdigitation of projections from the surfaces of three apposed cells. Electron micrograph, × 20,000.



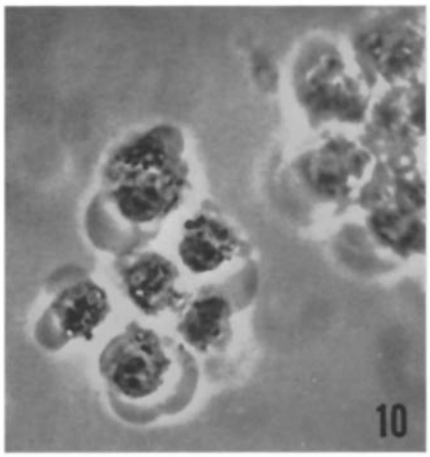

(Goldberg and Green: Antibody-complement cytotoxicity)

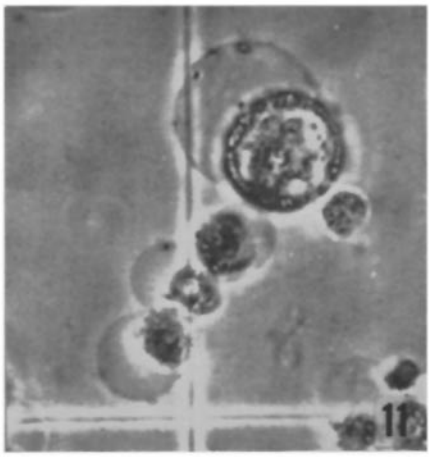
PLATE 61

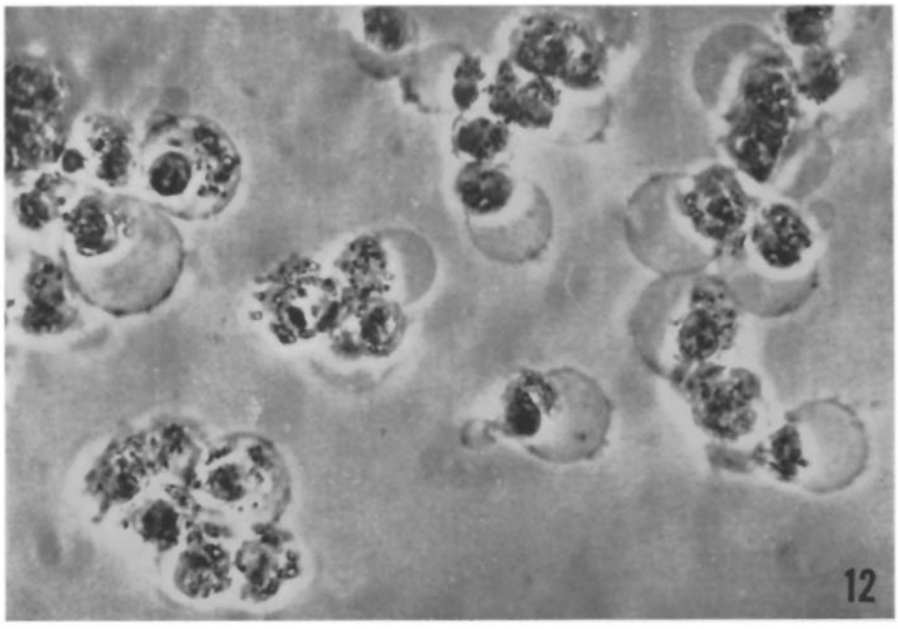
*Cells + Immune Gamma Globulin + Complement*

FIG. 10. 3 minutes' incubation. Focal outpouching at two to three points on the circumference. Phase, × 640.

FIGS. 11 and 12. Fig. 11., 20 minutes' incubation. Fig. 12., 60 minutes' incubation. Shown are later stages of cytoplasmic swelling. Fig. 11, phase, × 650. Fig. 12, phase, × 640.



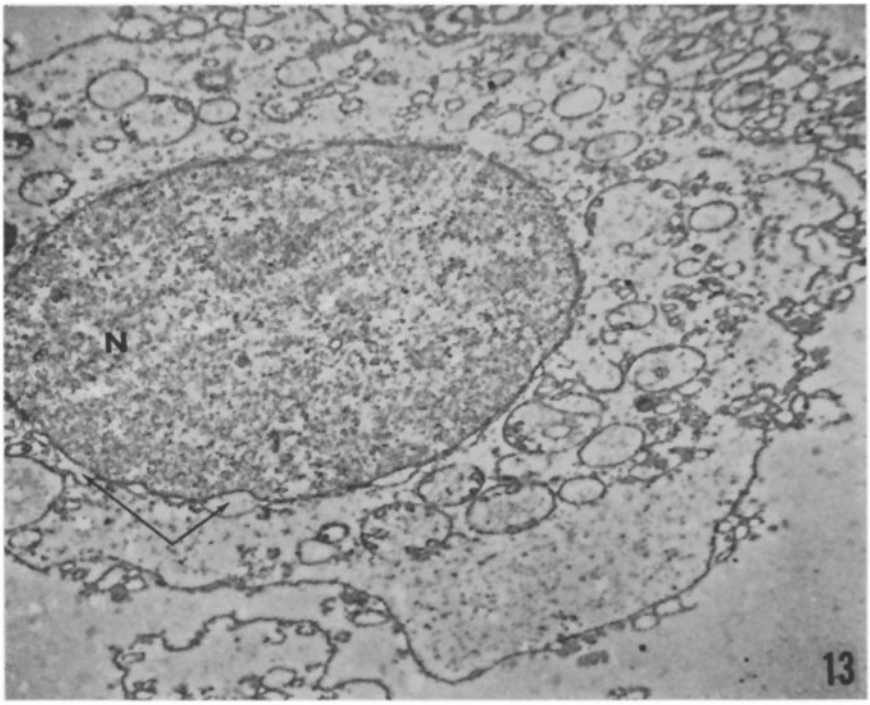

(Goldberg and Green: Antibody-complement cytotoxicity)

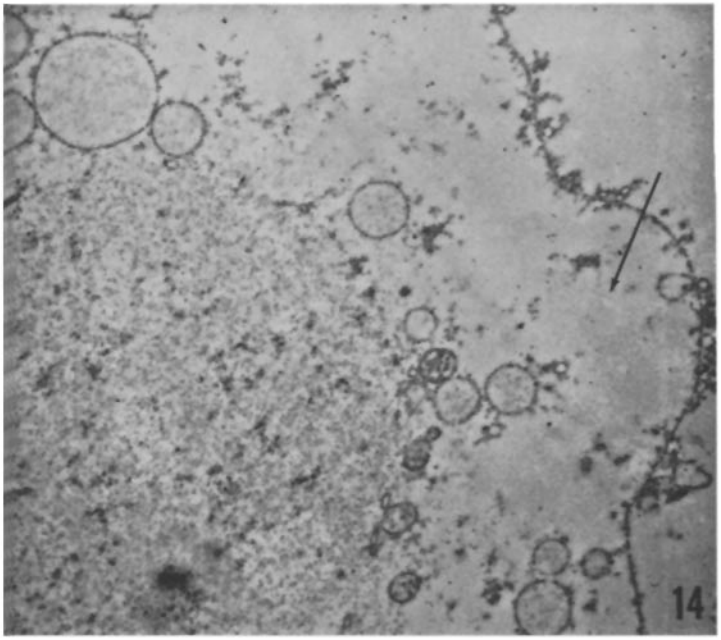
PLATE 62

*Cells + Immune Gamma Globulin + Complement*

FIG. 13. Illustrates swelling of membrane-bounded components of the cytoplasm. Arrows point to expanded perinuclear space. (*N*) indicates the nucleus. Cell membrane is structurally intact. Electron micrograph, × 10,500.

FIG. 14. Arrow indicates "clearing" of peripheral cytoplasmic zone of small osmiophilic granules with consequent loss of back-ground density. Electron micrograph, × 10,000.



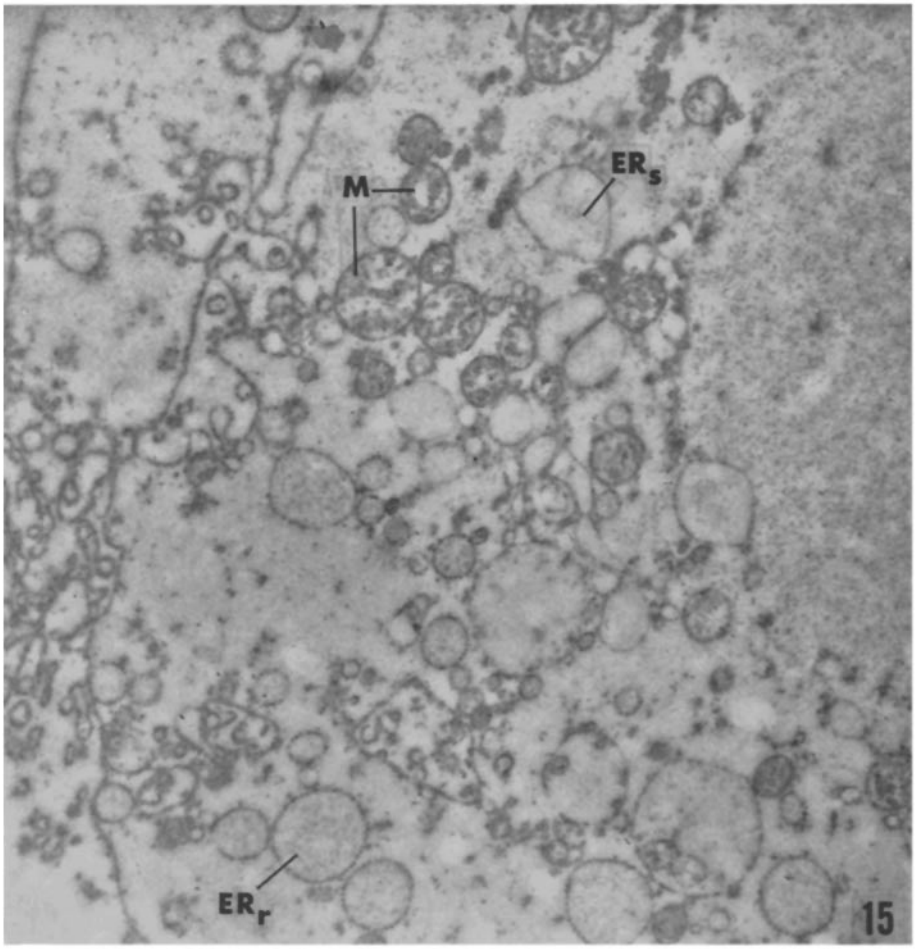

(Goldberg and Green: Antibody-complement cytotoxicity)

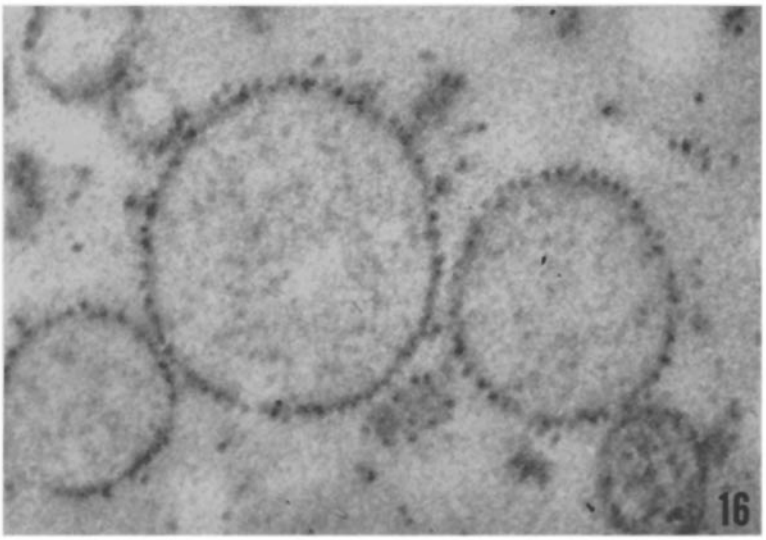
PLATE 63

*Cells + Immune Gamma Globulin + Complement*

FIG. 15. Altered mitochondria ($M$), and swollen smooth ($ER_s$), and rough ($ER_r$) surfaced endoplasmic reticulum are illustrated. Electron micrograph, × 15,000.

FIG. 16. Detail of swollen rough surfaced endoplasmic reticulum characterized by peripheral granules. Electron micrograph, × 50,000.



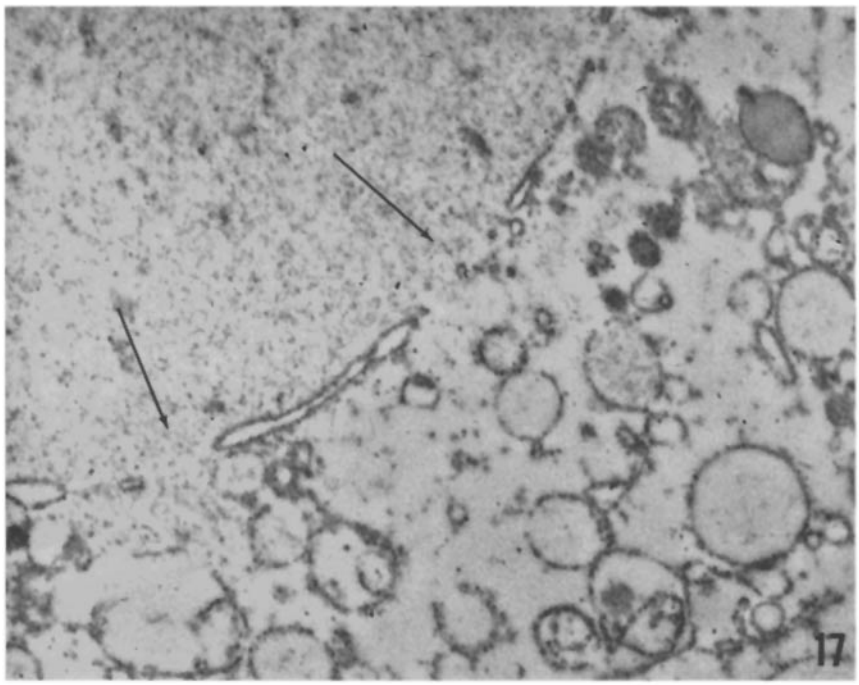

(Goldberg and Green: Antibody-complement cytotoxicity)

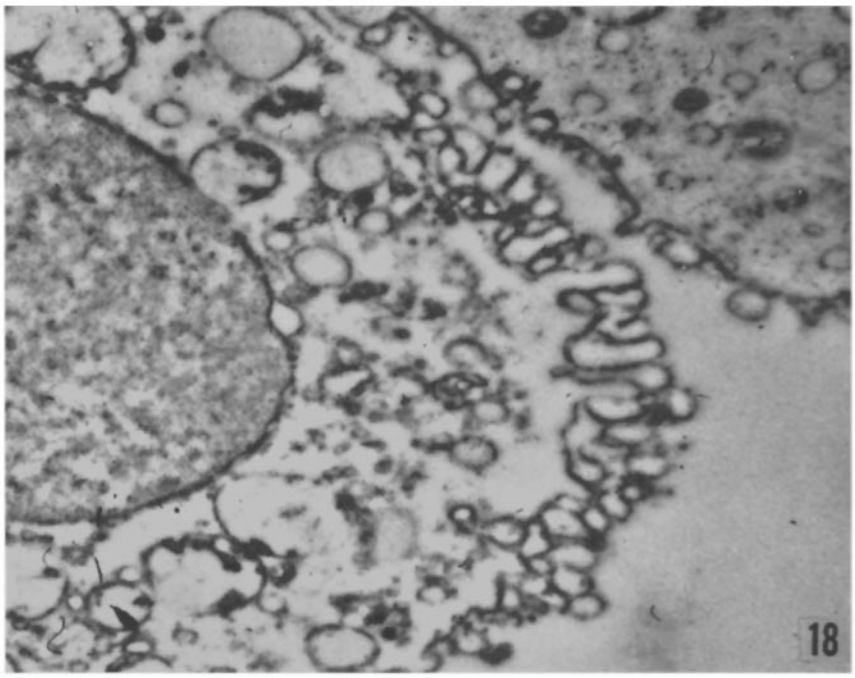
PLATE 64

*Cells + Immune Gamma Globulin + Complement*

FIG. 17. Arrows indicate widening of nuclear pores. Expanded perinuclear space is also shown. Electron micrograph, × 20,000.

FIG. 18. Persistence of folded surface membrane in antibody-complement treated cell. Electron micrograph, × 14,000.




(Goldberg and Green: Antibody-complement cytotoxicity)