

PHASE CONTRAST AND INTERFEROMETRIC MICROSCOPY OF THE L.E. CELL PHENOMENON*

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The L.E. cell phenomenon (1), considered pathognomonic of systemic lupus erythematosus, occurs in two phases. The primary change results in a peculiar nuclear alteration in lymphocytic or polymorphonuclear leukocytes with the production of swollen, structureless masses which are the L.E. bodies. Subsequently these bodies may be ingested by viable blood phagocytes, commonly polymorphonuclear leukocytes, to form completed L.E. cells. This sequence of events has been demonstrated in supravital preparations by Rohn and Bond (2) and by Moyer and Fisher (3). The technique of supravital staining is of limited value, however, in demonstrating morphologic changes occurring within the nucleus, which is presumed to be the site of primary alteration. The active agent initiating this chain of events has been found in the gamma globulin fraction of the serum of patients with systemic lupus erythematosus (4). Other cofactors may also be required (5, 6).

Recent cytochemical studies by Godman and Deitch (7) have demonstrated that the primary intranuclear event in the formation of L.E. bodies is a profound alteration in the nature and quantity of the protein components of the deoxynucleoprotein complex. The most specific changes appear to occur during the production of the L.E. body, while those changes occurring after phagocytosis are probably less pertinent to the basic L.E. phenomenon.

Certain questions relating these cytochemical studies to the morphological and physical characteristics of the L.E. phenomenon must be answered in order to understand this process, the events of which are considered pertinent to the pathogenesis of systemic lupus erythematosus (8, 9). It would be desirable to assess the relative contribution of nuclear and cytoplasmic material to the L.E. body and subsequently to the L.E. cell. Information regarding changes in physical properties and particularly of anhydrous mass is required in order to interpret the previously documented cytochemical changes (7, 10), which are dependent upon the dye binding capacities of as yet unidentified proteins. Specifically, it must be established whether the increased dye binding capacity of the L.E. body for the dye naphthol yellow S (flavianic acid) compared with

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that of the leukocyte nucleus represents a net gain in protein content and hence of mass, or an increased availability of dye binding sites already present in the parent nucleus. The increase of tyrosine residues in the L.E. body demonstrable with the cytochemical Millon reaction have strongly suggested an actual increase in protein (7). If by techniques independent of cytochemical reactions for protein groups there can be demonstrated a significant increase in total dry mass during the transformation from nucleus to L.E. body than the hypothesis that increased protein staining reflects actual increased protein mass could be established.

A study was therefore undertaken to observe, in the unstained leukocytes, by means of phase contrast microscopy, the earliest detectable changes in cellular and nuclear structure under the influence of L.E. serum. Secondly, interference microscopy has been employed to determine the comparative dry masses of the parent leukocyte nucleus and the L.E. body as a measure of incorporation or loss of material, presumed to be protein or nucleoprotein, in the course of L.E. body formation.

Materials and Methods

L.E. Preparations.—L.E. bodies and completed L.E. cells (*i.e.* phagocytosed L.E. bodies) were produced by means of two techniques. That of Davis and Eisenstein (11) employs a dried smear of normal donor buffy coat as substrate for the action of L.E. serum. The technique of Lee (12) utilizes a solution of atabrine to provide the critical damage to the leukocyte substrate. This method employs a suspension of leukocytes at concentrations of from 1 to 3×10^6 cells per c. mm., a stock solution of atabrine, 20 mg. per 10 ml. of 0.1 M phosphate buffer at pH 7.0, and L.E. serum. A mixture of 0.5 ml. of L.E. serum, 0.1 ml. of cell suspension, and 0.1 ml. of atabrine solution is incubated at 37°C. for 2 hours, then smeared and stained or examined in wet mount. In both these techniques, as in all the methods described in the literature (13), the basic requirement is a damaged, presumably non-viable leukocyte the nucleus of which is the site of the changes described. Viable polymorphonuclear leukocytes must be added in either technique if phagocytosis is to occur. In the technique of Lee, however, enough leukocytes remain undamaged by the atabrine to provide adequate phagocytes. For confirmation of the L.E. phenomenon by the usual criteria, all preparations examined in this study were subsequently fixed and stained with Wright's stain. Potent sera were obtained from patients with systemic lupus erythematosus, the principal features of whose sickness have been given in (7). For control preparations pooled normal serum was substituted for L.E. serum in either of the techniques employed without altering the method in any other way.

Phase Contrast Microscopy.—Leukocytes and L.E. bodies formed by both techniques were examined with a Zeiss opton 100 X apochromat medium dark contrast phase objective of N.A. 1.30 on a Zeiss "lumipan" stand. Photographs were made on 35 mm. Adox KB-14 film. Most observation on the early stages of the L.E. phenomenon were made with the Davis technique (11) utilizing a stage incubator and a wet mount preparation made by sealing a coverslip at the edges with silicone grease to a microscope slide.

Interference Microscopy.—Preparations of control cells and L.E. bodies made according to the atabrine method of Lee were used for determination of dry mass by interferometry. The control cells were handled in the same manner as the L.E. preparations save for the substitution of pooled normal serum for L.E. serum. Lymphocytes from the blood of patients with

chronic lymphocytic leukemia have been demonstrated to serve as excellent material for L.E. preparations (3) and were employed because of the relative ease of measurement of these geometrically regular bodies.

The principles and practice of interference microscopy have been discussed extensively by Barer (14). The interference microscope, like the phase contrast microscope, employs differences in refractive index to permit visualization of materials which are almost or completely transparent to visible light. Basically, both these techniques make transparent objects visible by means of the constructive or destructive interference produced on recombination of two beams of light, one passed through the object and the second bypassing the object. The interference microscope enables direct measurement of the optical retardation (phase shift) produced by a transparent object. Determination of dry mass by interference microscopy was made according to the method of Davies *et al.* (15). The anhydrous mass of small objects suspended in a medium of refractive index of water can be derived from the formula $m = \frac{\phi A}{100\alpha}$ in which ϕ is the optical retardation of the object in wave lengths, A is the area in cm.^2 , and α is a constant taken as 0.0018 for the type of biological material being studied. The variations in this constant for different proteins and DNA from the value 0.0018 are so small as to introduce negligible error into the calculation of total dry mass (14-16). When objects are suspended in a fluid of refractive index greater than that of water a correction must be applied if the results are to be expressed in absolute units. However, the correction is small and is not necessary when making a comparative study of similar and comparable objects (16). The result obtained without the correction is termed the "reduced" mass. Measurements were made upon the nuclei of whole lymphocytes suspended in the normal serum-atabrine mixture and upon L.E. bodies suspended in a similar L.E. serum-atabrine mixture after 2 hours of incubation. Both control and L.E. preparations were mounted wet under one rectangular coverslip on the same slide, the two areas being separated by a silicone grease seal. This technique permitted interferometric measurements on both L.E. and control material without appreciable delay.

The interference microscope employed was a Cooke-Dyson model with a 95 \times objective of N.A. 1.3 and a 10 \times ocular. The 546 $m\mu$ green line isolated by a Wratten 58 filter from an AH4 mercury vapor lamp was used to furnish Köhler illumination. Individual nuclei or L.E. bodies were examined and their optical retardations determined visually by the technique of successively placing the background and the object at minimum intensity in a uniform field fringe system. Under the conditions of this experiment, making 10 successive measurements on a single lymphocyte, this method gave reproducible results with a standard error of 2.6 per cent in the measurement of optical retardation. Nuclear diameters were determined by means of a calibrated filar micrometer ocular and the area computed as πr^2 . Similar determinations were made on L.E. bodies.

RESULTS AND COMMENTS

Phase Contrast Microscopy.—Stages in the development of the L.E. body observed from the moment of addition of L.E. serum to the dry buffy coat under the coverslip are shown in Figs. 1 to 9. The morphology of the normal leukocyte seen with the phase contrast microscope has been reviewed by Ackerman and Bellios (17). The substrate leukocytes in the Davis dry buffy coat L.E. preparation are not viable and hence do not display the amoeboid movement of the living polymorphonuclear leukocyte. The nucleus of the polymorphonuclear leukocyte can be seen as a multilobed structure with an internal differentiation into areas of greater and lesser phase darkness. Fig. 1 demonstrates such a cell at the instant of addition of L.E. serum and before

detectable change has occurred. Fig. 16 demonstrates a viable, actively phagocytic, polymorphonuclear leukocyte. Both illustrate the characteristic peripheral and central clumping of nuclear phase-dark material. Cytoplasmic granules are readily apparent in the cells of Fig. 1.

In Figs. 1 to 4 the sequential changes during the formation of the L.E. body can be followed in the same cells. The earliest detectable change in the polymorphonuclear leukocyte nucleus, occurring within 5 to 15 seconds of the addition of L.E. serum, is a sudden rather uniform homogenization of the normal nuclear pattern. The altered nucleus appears as a multilobed structure of uniform density of somewhat greater phase-darkness than the original nucleus and without visible internal differentiation (Fig. 2). Fig. 5 and 6 likewise demonstrate this early nuclear change. There is no loss of the individuality of the lobes of the nucleus during this or any subsequent phase of the L.E. phenomenon; each lobe usually becomes one L.E. body. The loss of internal nuclear pattern is followed by rapid and progressive swelling of the altered nuclear lobes. Frequently a break occurs in the cell membrane and a swollen nuclear lobe may be partially or totally extruded from the cell to form a characteristic L.E. body (Fig. 3, 4, 7, and 8). A similar field, fixed and Wright's stained, is seen in Fig. 21 and may be compared with the control cells demonstrated in Fig. 20. The changes noted with phase contrast can also be seen here and consist of a total loss of the normal chromatin pattern, swelling of the nuclear lobes, frequently with disruption of the filaments between them, and at least partial extrusion of the resulting bodies from the cytoplasmic remnant. The entire process is usually completed within 5 to 10 minutes of incubation.

Lymphocytes in the buffy coat substrate are similarly affected by the L.E. serum but the process is slower in its inception, usually requiring several minutes of exposure to the serum before the onset of the nuclear changes. The degree of homogenization of the L.E. body so formed is frequently somewhat less than that of the polymorphonuclear L.E. body. Figs. 10 to 15 demonstrate the changes in a lymphocyte substrate under the influence of L.E. serum. Fig. 10 shows a control group of lymphocytes exposed to normal serum and incubated for 20 minutes. In the upper left is a normal lymphocyte suspended in its own serum and examined promptly on isolation. Both show the normal clumping of phase-dark material at the nuclear membrane and centrally. Figs. 11 to 13 show the early changes following exposure to L.E. serum. The progressive loss of the normal distinction between phase-dark and light areas in the nucleus is apparent, and is similar to that seen in the polymorphonuclear leukocyte. Fig. 14 contains an L.E. body derived from a lymphocyte nucleus which has progressed to the stage of maximal swelling and almost complete loss of visible cytoplasm. A few shreds of cytoplasmic material adhere at its lower left margin. Two unaltered lymphocytes flank this L.E. body. Fig. 15 is a Wright's stained smear of a similar lymphocyte-derived L.E. preparation showing an L.E. body and an unaltered lymphocyte.

The cell cytoplasm and the cytoplasmic granules appear to be mechanically displaced by the expanding L.E. body and on rupture of the cell may either be dispersed in the suspending medium or remain as a cytoplasmic "ghost" of the original cell. Frequently a very small number of cytoplasmic granules adhere to the periphery of the fully formed L.E. body and may be ingested with it by a viable phagocyte. Gross incorporation of cytoplasmic material is not seen to occur.

L.E. bodies formed from single lymphocyte nuclei may attain a size considerably greater than those derived from single lobes of polymorphonuclear leukocytes. The relative size of nucleus and L.E. body, in the absence of any accurate and feasible method of measuring actual thickness in the wet mount preparation, may be taken as proportional to the area of the object and hence to the square of its radius. The ratio of the mean area of lymphocyte-derived L.E. bodies to that of lymphocyte nuclei is 3.1. The ratio of the area of lymphocyte-derived L.E. bodies to that of L.E. bodies derived from polymorphonuclear lobes is approximately 3.0. These ratios approach those we would expect, assuming first that the average number of polymorphonuclear lobes is three (18), second that the degree of swelling is proportional to the amount of nuclear material present initially in the unaltered leukocytes, and finally that the amounts of alterable nucleoprotein in the lymphocyte and the polymorphonuclear nucleus are approximately equal. Fusion of two or more L.E. bodies has not been seen to occur although several may lie in close proximity.

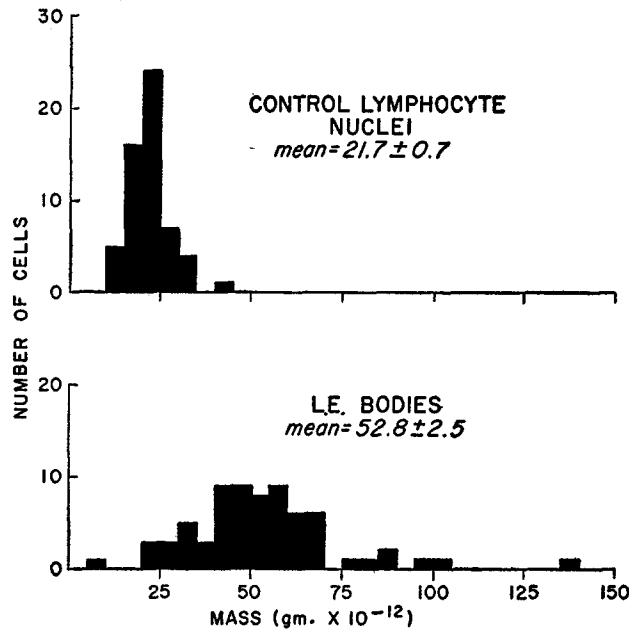
Phagocytosis of these bodies producing complete L.E. cells occurs if incubation with viable polymorphonuclear leukocytes is continued, the optimal yield being at about 20 minutes of incubation. Figs. 16 to 19 demonstrate the process of phagocytosis of an L.E. body to form the L.E. cell. Figs. 16 and 17 show partial ingestion of a large lymphocyte-derived L.E. body, the first being a photomicrograph of a phase contrast preparation and the second a similar Wright's stained field. Figs. 18 and 19 show completed phagocytosis of one and two L.E. bodies, respectively.

When the Lee atabrine method is used to prepare L.E. bodies and cells, changes similar to those just described occur, but there is a delay of at least 90 minutes before their onset; the delay is presumably due to the time needed for atabrine to appropriately damage the most susceptible cells and allow the L.E. factor to act. Once initiated in any one cell, the changes proceed as quickly as they do in the Davis preparation, and extrusion of altered nuclear lobes may be noted within 30 seconds after the first onset of visible nuclear damage (homogenization etc.).

Leukocytes treated in the Davis method, using normal instead of L.E. serum, showed variable changes. A frequent finding was a rapid loss of nuclear phase-darkness in some leukocytes, leaving an apparent negative image of the nucleus within the cytoplasmic "ghost," without, however, undergoing nuclear homogenization, and without the extrusion of bodies resembling the L.E. body (Fig. 9). Comparison of Fig. 9 with Fig. 6 demonstrates clearly the difference in nuclear appearance in L.E. and control preparations. Occasionally small blebs containing material which appeared light under phase contrast were formed on the cell surface. These were, however, quite distinct in appearance from L.E. bodies. In the Lee technique the control preparations usually underwent no cellular alterations save for the appearance with time of increased numbers of round, non-motile cells. Wright's stained smears of the control preparations showed morphologically intact cells and various degrees of cellular autolysis and disintegration (Fig. 20). Occasional bodies somewhat resembling the L.E. body in size and tinctorial properties were seen on the control slides, but these were never found phagocytosed as L.E. cells, and while presumably representing some stage of cellular degeneration, were lacking in their ability to attract phagocytes and undergo the remainder of the L.E. phenomenon. In addition, whereas these bodies were generally quite rare in control preparations, the L.E. body was invariably found in profusion in positive L.E. preparations, in addition to various

stages of phagocytosis and fully formed L.E. cells. The frequency of all such abnormal and degenerating cells in control preparations appeared to be inversely correlated with the freshness of the substrate material.

Interference Microscopy.—The frequency distributions of the reduced dry masses of control lymphocyte nuclei incubated in normal serum and of L.E. bodies derived from lymphocytes as determined by interference microscopy are presented in Text-



TEXT-FIG. 1. The frequency distribution of "reduced" dry mass values for control lymphocyte nuclei and of L.E. bodies prepared according to the Lee atabrine technique (12). No correction is made for suspension in a medium of a higher refractive index than water.

fig. 1. A total of fifty-seven control and sixty-eight L.E. body measurements were made on three independently controlled series. The mean reduced dry mass for control lymphocyte nuclei is $21.7 \pm 0.7 \times 10^{-12}$ gm., while that for L.E. bodies is $52.8 \pm 2.5 \times 10^{-12}$ gm., approximately two and one half times the control mass. The difference is of course, extremely significant by the *t* test for difference of means. A smaller series of fifteen control lymphocyte nuclei were measured interferometrically without prior incubation. The mean reduced mass of these nuclei was not significantly different from the control cells incubated over 90 minutes in the Lee mixture. It is thus apparent that the observed difference in mass between L.E. bodies and lymphocyte nuclei is not due to a loss of mass from the control nuclei during the incubation period, but rather to an increase in mass of the L.E. body.

DISCUSSION

Early studies of the morphology of the L.E. phenomenon by Rohn and Bond (2) have suggested that the primary visible changes occur in the nuclei of polymorphonuclear leukocytes. They were unable to demonstrate this phenomenon in lymphocytes, although this was subsequently found to occur (3). Others (19, 20) have suggested that the basic process may be a fusion and autolysis of the cytoplasmic and nuclear components of the leukocyte. It has been amply shown in the past (see references 21, 22 *inter alia*) that the prerequisite for the L.E. phenomenon is the presence of injured and non-viable leukocytes, L.E. serum and blood phagocytes. The substrate of injured leukocytes is not species-specific. These broad conditions required to demonstrate the L.E. phenomenon account for the wide variety of L.E. techniques, the differences among them being essentially in the manner of achieving the proper primary injury to the substrate leukocyte. The present phase contrast observations are in over-all agreement with the supravital studies of Rohn and Bond in the demonstration of nuclear participation in the L.E. phenomenon. Both polymorphonuclear leukocytes and lymphocytes were observed to be sensitive to L.E. serum. Our observations suggest that at the light microscope level, morphologically visible components of the leukocytic cytoplasm play little or no part in the L.E. phenomenon and it would appear that the cytoplasm is mostly discarded during formation of the L.E. body. Some cytoplasmic granules may persist around the periphery of the L.E. body and may appear within the L.E. cell after phagocytosis. The incorporation of small quantities of cytoplasmic material at the molecular level cannot be excluded.

The current studies by Godman and Deitch (7, 10), which are at variance with previously held concepts (23-25), suggest that during the development of an L.E. body there is no detectable depolymerization of nuclear DNA. There is, on the other hand, a loss of stainable basic nuclear protein of the histone type, accompanied, however, by an increase in the total amount of cytochemically demonstrable protein. The changes described in this study, *i. e.* the loss of normal chromatin pattern and formation of a homogeneous-dense body swollen to about three times the size of the parent nucleus or nuclear lobe, appear to be the morphological manifestation of this alteration in the nucleoprotein of the leukocyte nucleus. The interferometric studies demonstrating an increased total dry mass as well as size of the L.E. body compared with the parent nucleus, together with the demonstration of increased amounts of protein groups available to cytochemical tests (7), indicate that the observed nuclear swelling is caused by a marked ingress of protein, and not merely by an osmotic shift of water. The increased affinity for protein stains apparently reflects a real increment in protein mass and not an enhanced reactivity of protein already present in the nucleus. The source of this extraneous protein,

whether from cytoplasm, serum, or both, and its chemical nature are as yet undetermined. The minor extent of visible cytoplasmic contribution to the L.E. body suggests that the blood serum may be the major source of this protein. It has recently been reported by investigators using the fluorescent antibody technique (26, 27) that serum globulin is present in the L.E. body, as contrasted with control material. The mechanism whereby the serum of patients with disseminated lupus erythematosus initiates this process involving both a disruption of the normal nucleohistone organization and an influx of exogenous protein is totally unknown. The suggestion that the serum factor in disseminated lupus erythematosus represents a proteolytic enzyme which is active on a nuclear DNase inhibitor has been made by Kurnick *et al.* (28, 29), but the current cytochemical studies of Godman and Deitch do not lend support to the hypothesis of depolymerization of DNA. If the factor is enzymatic, we still remain ignorant about its precise substrate. Inderbitzen (30) has reported the production of L.E.-like cells on treatment of blood leukocytes with a synthetic heparinoid and normal serum. It is doubtful whether these bodies can truly be interpreted as L.E. cells. Anderson and Wilbur (31) and Roberts and Anderson (32) have reported the extrusion from isolated cell nuclei of a nuclear gel which contains DNA and protein following treatment of the nuclei with heparin. The techniques described in this paper, and those used by Godman and Deitch, should be applied to such preparations before any analogies to the L.E. phenomenon may be considered.

SUMMARY

Alterations in the cellular morphology of polymorphonuclear leukocytes and lymphocytes under the influence of serum from patients having disseminated lupus erythematosus were observed under the phase contrast microscope. These changes appear to involve the cell nucleus without significant visible incorporation of cytoplasm. In the formation of the L.E. body, there is a loss of internal nuclear structure and a subsequent nuclear swelling and extrusion of the nuclear contents from the cell to form the free L.E. body. The possible incorporation of cytoplasmic substance cannot alone account for the large mass of the L.E. body as contrasted with the parent nucleus or nuclear lobe. Measurements of dry mass by means of the interference microscope show a two and one-half-fold increment in dry mass in L.E. bodies compared to parent lymphocyte nuclei. This confirms previous cytochemical studies, and establishes that an influx of protein into the leukocyte nucleus is an integral part of the L.E. phenomenon. That the accumulation of extraneous protein within the L.E. body is simultaneous with or subsequent to a disruption of the normal structure of the leukocyte nucleus is apparent from these studies, but the pathogenesis of this alteration is as yet unknown.

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EXPLANATION OF PLATES

Figs. 1 to 21 represent phases of the L.E. phenomenon made with the technique of Davis and Eisenstein (11). All phase contrast photographs were made at a magnification of 1500; the Wright's stain preparations are reproduced at $\times 1350$ and $\times 950$ as indicated.

PLATE 64

FIG. 1. Polymorphonuclear leukocytes at the instant of addition of L.E. serum showing normal nuclear and cytoplasmic structure. Phase contrast.

FIG. 2. Same field; 5 seconds after addition of L.E. serum. Nuclear homogenization and early nuclear swelling have occurred.

FIG. 3. Same field; 20 seconds after addition of L.E. serum. Further nuclear swelling with extrusion of nuclear lobes from cells at lower left and lower right, forming L.E. bodies.

FIG. 4. Same field; 2 minutes after addition of L.E. serum. Further swelling and extrusion of L.E. bodies. The cell at the lower right hand corner has lost its L.E. body and remains as a cytoplasmic remnant of the original cell.

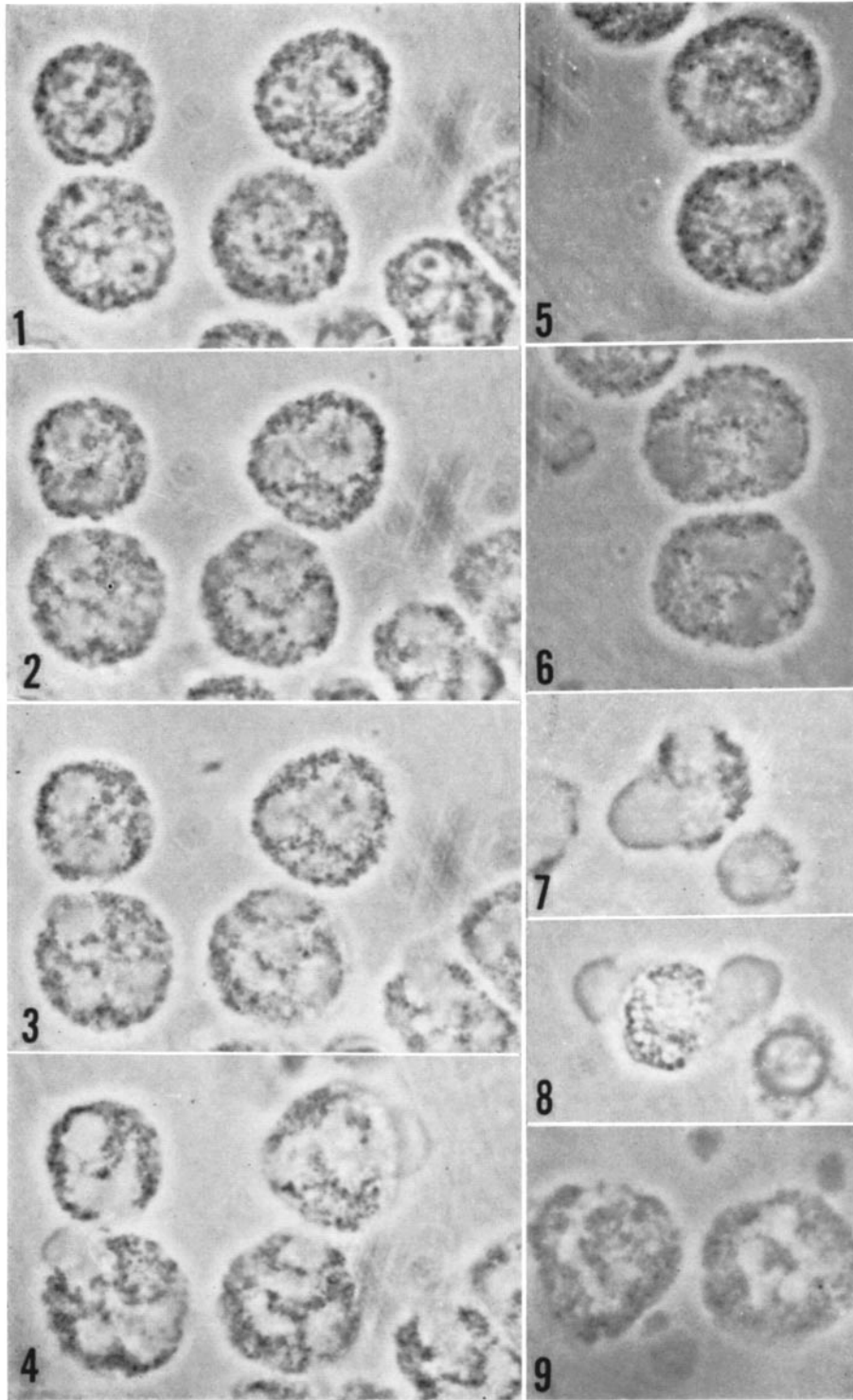
FIG. 5. Two polymorphonuclear leukocytes at the moment of addition of L.E. serum. Phase contrast.

FIG. 6. Same field; 15 seconds after addition of L.E. serum. Changes in the nucleus similar to but more pronounced than those in Fig. 2.

FIG. 7. Polymorphonuclear neutrophilic leukocyte 10 minutes after addition of L.E. serum, showing extruded altered nuclear lobe (L.E. body) still attached to the cytoplasmic remnant. Phase contrast.

FIG. 8. Polymorphonuclear eosinophile 10 minutes after addition of L.E. serum showing 2 extruded L.E. bodies, one corresponding to each of the two nuclear lobes of the eosinophilic leukocyte. Phase contrast.

FIG. 9. Polymorphonuclear leukocytes; control preparation after 2 minutes exposure to pooled normal serum. Little internal nuclear structure is apparent as compared to the cells shown in Figs. 1 and 5, but there is no extruded body, no nuclear swelling and no phase-dark nuclear material. Phase contrast.



(Rifkind and Godman: Microscopy of L.E. cell phenomenon)

PLATE 65

FIG. 10. Lymphocytes in a control dried buffy coat (Davis) preparation incubated with normal serum for 20 minutes. Upper left inset is a normal freshly isolated lymphocyte. Phase contrast.

FIGS. 11 to 13. L.E. body formation in lymphocytes of a Davis preparation after 5 seconds', 1 minute, and 3 minutes' incubation with L.E. serum. There is progressive loss of nuclear structure and formation of a homogeneous body with a thin cytoplasmic rim. Phase contrast.

FIG. 14. Large lymphocyte-derived L.E. body after 10 minutes' incubation in L.E. serum; Davis preparation. Fine cytoplasmic tags at lower left of L.E. body. Two unaltered lymphocytes. Phase contrast.

FIG. 15. Similar field as Fig. 14; Wright's stain preparation showing large lymphocyte-derived L.E. body and normal lymphocyte; incomplete nuclear homogenization characteristic of lymphocytic L.E. bodies. $\times 1350$.

FIG. 16. Motile polymorphonuclear leukocyte actively phagocytosing a large lymphocyte-derived L.E. body. Phase contrast.

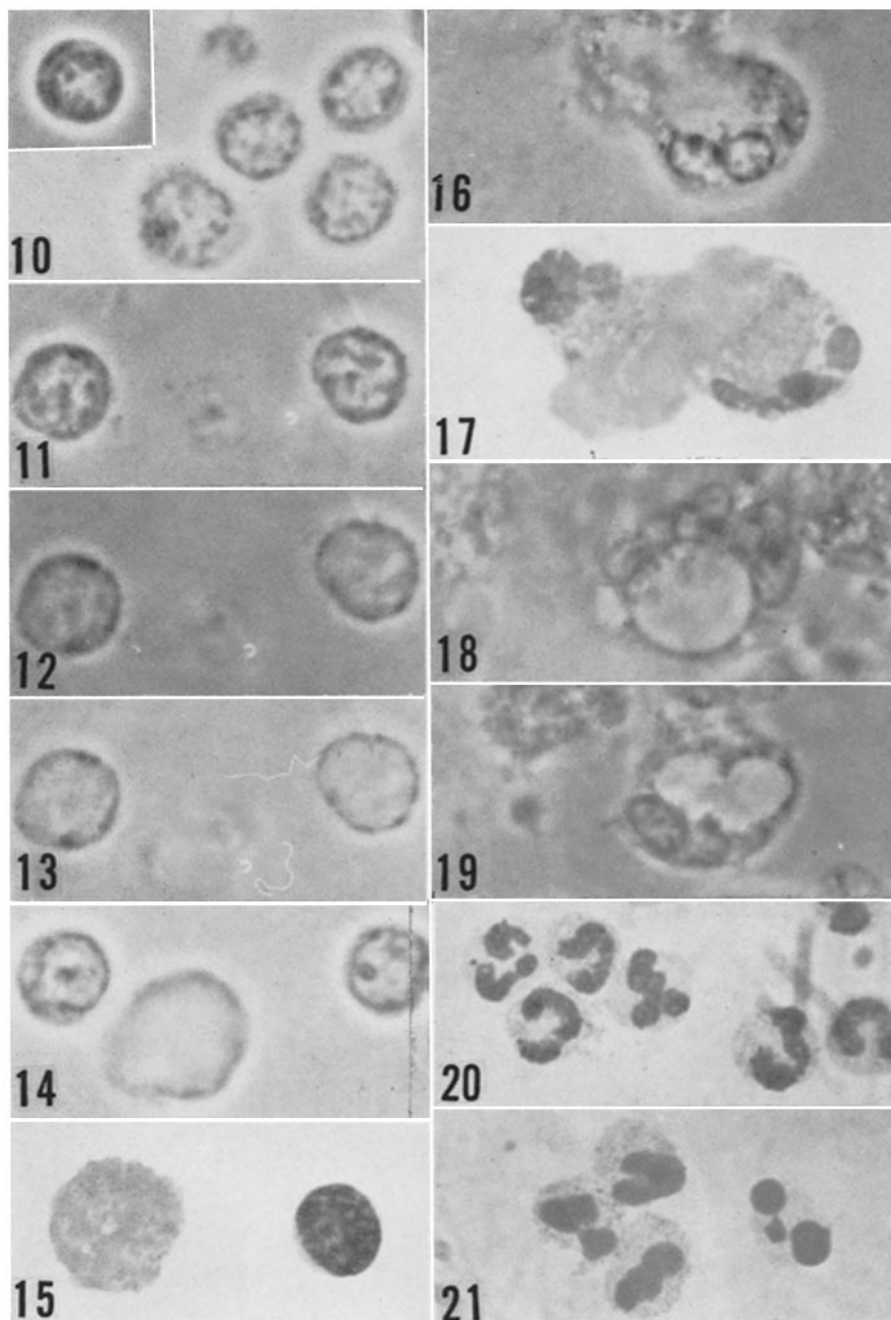
FIG. 17. Similar field as Fig. 16; Wright's stain, showing two leukocytes ingesting a distorted L.E. body. $\times 1350$.

FIG. 18. Completed L.E. cell with a single L.E. body inclusion. Phase contrast.

FIG. 19. Completed L.E. cell with two inclusions. Phase contrast.

FIG. 20. Control Davis preparation; Wright's stained preparation, demonstrating preservation of normal chromatin pattern. $\times 950$.

FIG. 21. L.E. preparation, Davis technique; Wright's stain, showing loss of chromatin pattern, swollen nuclear lobes, and partial extrusion of L.E. bodies. $\times 950$.



(Rifkind and Godman: Microscopy of L.E. cell phenomenon)