ELECTRON MICROSCOPE STUDIES OF BLOOD CELLS

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In an earlier report we have described briefly the technics utilized in the application of the electron microscope to the study of the blood cells and the preliminary results obtained by these technics. They consisted of the study of lymphocytes, macrophages and neutrophilic leukocytes of experimental inflammatory exudates of man as well as the neutrophiles, neutrophilic metamyelocytes, lymphocytes and their precursors, red corpuscles and the later stages of normoblasts obtained from the hematopoietic organs of man.

Several excellent, electron micrograph studies of other tissue cells have already been reported, even though the study of these relatively large, thick and more delicate tissue cells with the electron microscope has imposed somewhat greater problems than has the study of bacteria and viruses. Scott and Packer reported on the localization of minerals, particularly calcium and magnesium, in striated and smooth muscle, and Scott and Anderson studied connective tissue. Schmitt, Hall, and Jakus have contributed much to our understanding of protoplasmic fibrils in their studies of collagen fibers, protozoan trichocysts, muscle fibers, sperm, flagella, cilia and fibrin. Richards, Anderson and Hance depicted electron micrographs of striated muscle. Clark, Barnes and Baylor and later Buchholz have reported on chromosome structure. Claude and Fullam studied isolated mitochondria and sections of guinea pig liver with the electron microscope. Porter, Claude and Fullam devised a method of electron microscopy of tissue cultures of fibroblasts and nerve endings.

A small number of papers have dealt with electron microscope studies of the blood cells or their derivatives. To the best of our knowledge, save for our preliminary report mentioned above, they have concerned themselves with studies of the blood platelets or the mature red blood corpuscles. Wolpers and Ruska reported on the structure of the blood platelets and their relation to fibrin. The platelet granulomere was found to consist of from 60 to 120 granules; rarely as few as 20 granules were seen. The hyalomeric protoplasm exhibited a fine framework-like structure (their figure 9). In the course of the clotting process their electron micrographs depict: the platelets swelling, aggregation of granules, vacuole and process formation by the hyalomere, loss of all save a remnant of hyalomere, but finally, persistence of the granules as a place of deposition or attachment for the fibrin micelles. In a later paper Ruska and Wolpers detected regularly spaced, dark cross bands in fibrin fibrils. Wolpers separately investigated the structure of the red corpuscular membranes. Electron micrographs of red blood corpuscles and hemolysed erythrocytes are depicted by him. The membranes of the latter were studied after lipid extraction and were composed of an intricate meshwork of long and slender protein fibrils. Barnes, Burton and Scott portrayed in their Fig. 5 a polystyrene-silica replica of red blood corpuscles. Jones recently

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was able to make methacrylate surface replicas obtaining micrographs of blood cells of a quality similar to those of polystyrene.

**Materials and Methods**

We have employed several technics. First, lymphocytes, macrophages and neutrophilic leukocytes of the inflammatory exudate of man were obtained by applying formvar-covered glass cover slips over small lesions produced in the corium of the forearm of human volunteers. To produce these small lesions, the forearm was first cleansed with alcohol, then by means of a sterile scalpel or sterile razor blade, the epithelium was slowly scraped away over a circumscribed area 2 to 3 mm. in diameter, until the papillary layer of the corium was exposed. A few minute bleeding points indicate the proper depth. Care must be taken to avoid lacerations or obvious hemorrhage into the bed of the tiny lesion. In keeping with Kolouch's\(^2\) investigation of lymphocytic functions in rabbits with the light microscope, a small drop of egg white was next placed on the lesion, to increase the lymphocytic content of the cellular exudate.

A formvar-covered glass cover slip was next placed over the lesion, film side down. The cover slip was then covered with a square of cardboard and the entire preparation was covered with surgical adhesive tape, approximately 2 by 4 inches in size. Additional pressure over the lesion can be obtained by placing a flat-bottomed cork over the adhesive surmounting the cover-slip area; the cork is then held in place by a long narrow adhesive band.

In an hour or two the cells of the inflammatory exudate migrate to the under surface of the formvar film of the cover-slip, spreading themselves out in a thin, single-celled layer. When at definite timed intervals, it is desired to sample the cellular exudate, the film-covered cover-slip is removed from the lesion and the lesion is immediately recovered by a second film-covered glass cover-slip. The cells which migrated to the undersurface of the film-covered cover-slip, while still on the cover-slip, are immediately quick-frozen and dehydrated in vacuo according to Wyckoff's\(^2\) modification of the Altmann-Gersh\(^2\) technic. It should be noted that earlier, Scott and Packer had prepared striated muscle for electron microscopy by plunging the muscle into chilled isopentane before dehydration.

The final specimen mounting is performed as follows: Place the specimen screen over a suitable cell-containing area which has been selected for observation in the electron microscope. The suitability of such areas first may be adjusted roughly by examination under the optical microscope. Next a strip of Scotch tape is affixed over the specimen screen as well as over the entire slide. The Scotch tape is then removed and the film adheres to it. Inasmuch as the specimen screen is interposed between a small portion of the film and the Scotch tape, removal of the specimen screen at this stage will carry along an equal area of the film. The intact cells are thus actually mounted and observed between the specimen screen and the film. This is a direct mounting technic, then, and these specimens are not replicas.

Another satisfactory method of sampling the fresh exudative cells of man was obtained by imbedding a formvar-covered 200 mesh specimen screen, film side down in the lesion instead of surmounting the lesion with the formvar-covered
glass cover-slip. The screen is covered as before, the sterile glass cover-slip is also included, this time serving merely as a bland protective surface about the screen. For a few hours the diapedesis and extravasations of red corpuscles into the lesion, as well as the cellular debris, will obscure the screens for electron micrographic purposes. But if the film-covered screens in the lesion are replaced by means of fine sterile forceps at intervals of several hours, the exudate will gradually thin out and satisfactory preparations can be obtained. These formvar-covered specimen screens were prepared in the usual manner as outlined in current texts of electron microscopy.

A third technic employed for the study of the blood cells of the lymph nodes, spleen and bone marrow of man consisted of obtaining imprint preparations of portions of such organs upon formvar-covered glass slides. Both fresh surgical pathology specimens and specimens obtained at autopsy were utilized. The freshly cut surface of a small portion of the organ is very gently brought in contact with a formvar-covered glass slide, without any smearing or pressure. The preparations so obtained were also frozen and dehydrated in vacuo. The films were then transferred to screens by the modified stripping technic described above in which the cells were again retained intact. Rarely in clinical work when more elaborate apparatus was not available, the imprint preparations were merely air-dried.

Downey and his associates in this country have been persistent advocates of the imprint technic as an adjunct to the detailed cytologic examination of the blood cells with the optical microscope. Sweitzer and Winer have recently published an illustrated description of this technic for optical examination. Once experience has been gained in optical studies by means of the imprint technic, its application to electronic studies is not difficult.

RESULTS

Fig. 1 is an electron micrograph of a mature red blood corpuscle (X 5400). It is shown merely for purposes of size orientation. A micron scale is also shown. Such a corpuscle shows no stromal details, but Wolpers' work on the membranes of hemolysed corpuscles has shown that the surface of the membrane is composed of a fibrous network following the surface curvature. Fig. 2 (X 5400) is an electron micrograph of an orthochromatic normoblast from a bone marrow imprint. The distinct, fine chromatin particles, are partially obscured by denser, coarse chromatin clumps as the red cell undergoes its natural pyknosis. The central cell in the next electron micrograph, fig. 3, corresponds to the polychromatophilic stage of normoblastic development; this cell was magnified to X 5400. Note the larger areas of finely dispersed chromatin in this nucleus, indicating less nuclear maturity. The cytoplasm of both these cells (fig. 2 and 3) is scant and not remarkable at this magnification.

Fig. 4 is an electron micrograph of a small macrophage or histiocyte, obtained from the inflammatory exudate of the arm, magnified to X 5400. This cell measures 9 by 15 microns. It was obtained 16.5 hours after inflammation had been produced. The horseshoe shaped nucleus, the nuclear-cytoplasmic interface, the cytoplasm and the cytoplasmic surface are plainly seen. A portion of the same cell magnified
to $\times 18,000$ in fig. 5 shows an interlacing network which goes to form a type of cellular skeleton. The greater portion of fig. 5 depicts the delicacy of the nuclear framework. Only the lower central portion of the micrograph contains a small area of the cytoplasmic bay, but it is sufficient to afford ample study of its contrasting structure. The nuclear interstices, are, on the average, smaller than those within the cytoplasmic protoplasm, although some of the smaller cytoplasmic spaces measure only $15 \times 30$ millimicrons and some of the larger nuclear spaces measure $120 \times 200$ millimicrons. The nuclear strands or fibrils appear to be thicker than the corresponding structures in the cytoplasmic protoplasm.
Fig. 6 (×21,000) is an electron micrograph of this cell depicting the major portion of the nuclear indentation and cytoplasmic bay areas. The U-shaped portion of nuclear membrane can be observed as an anchoring membrane for both cytoplasmic and nuclear structural bands. Fig. 7 (×94,000), is again a micrograph of a portion of this same cell. The upper portion of this figure is cytoplasm, the lower portion nuclear protoplasm. The nuclear membrane runs obliquely across
the lower third of the figure. The individual fibrillar cross-pieces, which can be made out easily in either the nuclear or cytoplasmic protoplasm, actually measure
only 140 to 200 or so Angstrom units in breadth and about 1000 Angstrom units in length.

Fig. 8 is a micrograph of a second macrophage from the 16.5 hour stage of inflammation in man. To the best of our knowledge this is the first electron micrograph depicting phagocytosis. Celluar debris has been ingested by a small mononuclear. This phagocyte only measures 7 x 10 microns. A delicate rim of cytoplasm is depicted bridging over the particle. The vacuolar lining wall is formed by loops of cytoplasmic fibrillae which usually arch out towards the vacuolar lumen and return into the cytoplasm after forming a scallop-like lining for the vacuole. Occasionally free ends of these fibrillae appear to project for short distances into the vacuole.

Fig. 9 (X 5400) is a micrograph of a third macrophage from the eleventh hour of inflammation in another lesion of the forearm. Fig. 10 (X 18,000) is a high power view of a portion of the nucleus and cytoplasm of the same cell. The structural features of the horseshoe shaped nucleus, cytoplasm and nuclear membrane can also be observed in this cell. In all of these macrophages at the site of the cytoplasmic surface, the fibrillar structures of the protoplasm seem to project quite freely, without forming a distinct membrane. This could be due to slight shrinkage of the cytoplasm with the initial impact of the electrons; if not, the well known sticky character of the surface of such cells may be in some way related to this finding.

Fig. 11 (X 11,000) is a micrograph of the peripheral portion of the cell body of a fourth macrophage taken from the same lesion as the previous cell at the eleventh hour of inflammation. Several linear arrangements of feather-like, clear areas radiate throughout the cytoplasm. This finding warrants some discussion of ice crystal artefacts, although, for reasons which will appear below, we do not feel that such spaces represent ice crystal artefacts. Simpson analyzed the various factors influencing ice crystal artefacts produced in protoplasm by the Altmann technic. Although his work was performed with the light microscope, he found that such artefacts were small enough to disrupt nuclear detail and produce fine reticulation of the cytoplasm in sections of guinea pig liver cells at some distance from the surface of the tissue block. The operation of such factors in the production of the extremely small interstitial nuclear and cytoplasmic spaces of our figs. 4 to 11 cannot, of course, be definitely excluded at this time. Examination of Simpson's fig. 3, plate I, reveals, however, that his ice crystal artefacts are many hundreds of times larger than the protoplasmic spaces of our macrophages. Kistler's concept must also be considered, namely that the water of biological tissues is in the form of small isolated droplets and may undercool without freezing. It is probable that the water content of the macrophages is higher than that of the blood cells themselves. Certainly it is true that according to Simpson's standards, our preparations approach the optimum as to size of the piece of tissue frozen and ratio of large surface area to small tissue volume. It is unlikely that an insulating gaseous phase of any consequence enveloped the cells of our preparations under the conditions of the technic employed. Wolpers and Ruska demonstrated a type of protoplasmic framework similar to that under discussion, in the hyalomere of blood platelets. Their electron micrographs are of platelets not submitted to
freezing of any type. Wolpers'22 electron micrograph of an unfrozen red corpuscular membrane likewise shows a type of delicate fibrillar structural framework, dem-

Figs. 8 to 10

onstrating that the fibrillar structure of protoplasm need not necessarily be a product of ice crystal artefact.
Chambers and Hale,\(^3\) on the other hand, employed internal freezing to throw light on structural elements within the cell instead of looking upon it as a possible source of ice crystal artefacts. They demonstrated that the advance of ice columns...
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Fig. 13 to 15
within the protoplasm of frog muscle cells occurred by congealing of the water present in advance of the crystal. Hence it is reasonable to suppose that the steady and uniform advance of the longitudinal ice columns during the internal
freezing of a muscle fibre is along uninterrupted fluid interstices between more solid constituents." To them, the random spreading of fine feathery crystals within the protoplasm of amoebae indicated a lack of a complicated framework structure. Because of temperature differences between the interior of the cell and its environ-
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In their report, they assumed the existence of "still finer capillary spaces within the cell." The finding of free fibrillae at the cytoplasmic surface of our macrophages is open to the criticism of their view that to attain internal freezing, the plasma membrane breaks down.

Fig. 12 (X5400) is a micrograph of one neutrophilic leukocyte and a portion of two others. Neutrophil granules, seen only indistinctly heretofore with the optical microscope, when seen at X18,000 (fig. 13) are round, oval, or rod-shaped structures. At X47,000 (fig. 14) and X72,000 (fig. 15) the neutrophil granules are clearly demarcated. They measure from 70 by 85 millimicrons up to as much as 465 by 650 millimicrons. Figs. 13 and 15 are from the same frozen and dehydrated preparation. Fig. 14 was taken from a preparation which was merely air-dried.

Fig. 16 is an electron micrograph (X5800) of a neutrophilic metamyelocyte or juvenile from the bone marrow. Its deeply indented nucleus and granular cytoplasm are characteristic features. Fig. 17 is a micrograph (X5400) of a neutrophilic myelocyte with its round nucleus and the less abundant granular content of its cytoplasm. Fig. 18 (X5400) is a portion of another neutrophilic myelocyte. Fig. 19 (X5800) depicts a portion of the nucleus (to the left center) and cell body of an eosinophilic leukocyte. The eosinophil granules are nearly spheres and are quite clear and can be best made out as they overlie the nucleus. Eosinophil granules range from 780 by 830 up to 1050 by 1150 millimicrons in size. Fig. 20 (X5800) represents one of the granulocyte precursors at the promyelocyte or possibly the leukoblast stage. The granules in the cytoplasm are less well-defined, and it is impossible to state whether they represent specific or azurophil granulation. The cytoplasm is more abundant than the preceding stages. The nuclear membrane is exceedingly thin, the chromatin pattern is much finer than the older developmental forms and three or four nucleoli are in evidence. All the developing granulocytes were obtained from bone marrow preparations.

Fig. 21 (X5400) is a micrograph of a group of three normal, small and medium-sized lymphocytes from a lymph node. This preparation was not frozen or dehydrated, it was merely air-dried. All other preparations with the exception of Fig. 14 were quick-frozen and dehydrated. The coarse chromatin pattern and scant cytoplasm are well shown. Fig. 22 (X5400) is a micrograph of an extremely immature lymphocyte. The fine chromatin pattern and distinct nucleoli are in sharp contrast to the cells in fig. 21. The immature lymphocyte of fig. 22 was taken from the spleen of a patient with lymphatic leukemia. Fig. 23 (X5400) depicts a leukemic lymphocyte in amitosis, from a lymph node.

DISCUSSION

Newer concepts concerning the finer structure of protoplasm are just beginning to find their application to the study of the white blood cells. De Bruyn36 has recently interpreted the amoeboid movement of leukocytes in the light of Frey-Wyssling's37 structural schemata for protoplasm. The modern concepts37-39 of protoplasmic structure comprise molecular configuration of polypeptide chains with their associated intermolecular forces. Between such molecular protein structures are water, salts, mobile protein, and lipid, as well as submicroscopic
particulates which serve in the mediation of special chemical processes. The structural units themselves include at least fibrous and nucleoproteins as well as lipids. The intermolecular forces of structural proteins result in part in their being joined at certain points by chemical bonds. De Bruyn's envisions contraction of leukocytic protoplasm as being due to increased binding of such molecular structures at more and more points by chemical bonds, thus narrowing "the meshes of this three-dimensional reticulum," or in keeping with Astbury's findings, as due to actual folding of the polypeptide chains. Solation, then, would involve the loosening of many, but not all, bonds with a resultant loosening of the structural mesh-work, whereas cytoplasmic gelation would mean the locking of more side chains with the structural proteins approaching the formation of a three-dimensional reticulum.

Wolpers' micrograph (his fig. 7) of the structure of the nonmotile red blood corpuscle depicts a tightly knit framework suggesting that this may be composed of a mesh-work of long and narrow protein fibrils. The fibrillar structures of platelet hyalomere are not so closely bound as an examination of Wolpers and Ruska's micrographs of such structures reveals. We would expect less definite orientation of the structural proteins of the more motile leukocytes and definite orientation in the depolarized derivatives. Menkin has commented on the fact that macrophages are much more resistant to deformation than polymorphonuclear leukocytes and cites as evidence the fact that rabbit macrophages resist stresses at water-oil interfaces which severely damage polymorphonuclear leukocytes.

The macrophage stage of the various hematogenous and histogenous cellular sources of macrophages is likewise apparently marked by a tendency to sluggish pseudopodial activity. Our micrographs (figs. 4 to 11) of macrophages reveal a complex fibrillar mesh-work with definite structural orientation of both cytoplasm and nucleus. These findings are at least in keeping with modern functional and structural concepts of protoplasm and are suggestive that some of the structural units of protoplasm can be oriented at times to form a framework of linear molecules when the situation requires such reorientation.

**Summary**

Several technics were employed for the application of the electron microscope to the study of the blood cells. First; lymphocytes, macrophages, and neutrophilic leukocytes of acute inflammatory lesions in man were prepared by imbedding formvar-covered screens in the corium of the forearm. Exudative cells which migrated to the undersurfaces of the specimen screens were quick-frozen and dehydrated in vacuo, according to a modified Altmann-Gersh technic. In some cases formvar-covered glass cover slips were substituted for the screens in the lesions, in which case an extra step was needed to transfer the preparations to specimen screens.

In a further technic, blood cells of lymph nodes, spleen and bone marrow of man were imprinted upon glass slides covered with formvar. These preparations were also frozen and dehydrated in vacuo. The films were then transferred to screens by a direct method in which the cells were retained intact.

Neutrophil granules, seen only as irregular, indistinct granules with the optical
microscope, are actually round, oval, or rod-shaped structures measuring from 70
by 85 millimicrons up to as much as 465 by 60 millimicrons.

Immature blood cell precursors were found to possess fine chromatin patterns
with distinct chromatin-parachromatin distinction and well-demarcated nucleoli.

At the higher magnifications made possible by electron microscopy, a new level
of cytostructural organization appeared in the mononuclear phagocytes or macro-
phages. Individual fibrillar structural units can be made out in both the nuclear
and cytoplasmic protoplasm which can be measured in Angstrom units. The
nuclear protoplasmic interstices so formed are smaller than the corresponding cyto-
plasmic areas. The nuclear membrane is actually an anchoring structure for both
nuclear and cytoplasmic fibrillar cross-pieces. Phagocytosis is depicted and the
wall of the phagocytic vacuole is described. Finally, the observed cyto-structural
features of the blood cells are discussed in the light of newer concepts of the finer
structure of protoplasm.

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