LYSOSOMAL AND ULTRASTRUCTURAL CHANGES IN HUMAN "TOXIC" NEUTROPHILS DURING BACTERIAL INFECTION*

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Changes in human peripheral blood neutrophils are frequently seen during serious bacterial infections and certain other inflammatory states (1–3); they are usually identified by staining peripheral blood smears with one of the Romanowsky stains. Best known is the "shift to the left," defined by the presence of band forms of neutrophils, metamyelocytes, and sometimes myelocytes. The cytoplasmic changes in individual neutrophils have been emphasized less frequently but are amply described in most hematology texts. They include: (*a*) light blue amorphous inclusions—the Döhle bodies, first described in 1911 (4); (*b*) "toxic" granules, which are more prominent than granules of normal neutrophils; and (*c*) cytoplasmic vacuoles (5). Cells showing such changes are sometimes referred to as "toxic" neutrophils, and their presence and persistence in bacterial infections is associated with a poor prognosis (6). The nature of "toxic" granules, Döhle bodies and vacuoles, and the pathophysiologic events from which they result are still poorly understood.

Application of the lysosome concept (7) to the cellular biology of the neutrophil has clarified many aspects of its physiology and pathophysiology. The granules of the neutrophil are primary lysosomes (8); they are involved in the killing and digestion of bacteria following phagocytosis (9), in tissue damage during inflammation (10), in the production or release of endogenous pyrogen (11), in experimental hypersensitivity states (12), in experimental endotoxic shock (13), and in certain human diseases characterized by recurrent bacterial

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infection, such as chronic granulomatous disease (14) and the Chediak-Higashi-Steinbrick anomaly (15).

The studies on the ultrastructure and lysosome activity of human toxic neutrophils presented in this paper were designed to help clarify the nature of the toxic granules, Döhle bodies, and vacuoles.

Materials and Methods

Neutrophils from 50 patients were studied by light and phase microscopy. Neutrophils from 12 patients with severe bacterial infections were used, in addition, for electron microscopy and biochemical studies; 10 of the 12 had an associated bacteremia, and four subsequently died of their infection. The only criterion for selection of all patients was the occurrence of toxic granulation in at least 90% of peripheral blood neutrophils, but a "shift to the left" was also present in all. Döhle bodies were identified by light microscopy in 5–25% of the neutrophils of each of the 12 selected patients. Control neutrophils were obtained from healthy adult male volunteers.

Light Microscopy.—Most blood smears were prepared on cover slips flooded with Wright stain for exactly 3 min and then buffered with tap water (pH 5.0–6.0) for exactly 4 min. Some preparations were stained with the May–Grünwald-Giemsa stain. Wright-stained cover slip preparations of sternal bone marrow were also studied in three patients.

Wet Mounts.—Unstained wet mounts were prepared by dispersing a drop of heparinized fresh blood between a cover slip and glass slide and sealing it with petroleum jelly. Repeated observations by phase microscopy were carried out at both 20° and 37°C, determining in 100 neutrophils the proportion of cells in which one or more vacuoles could be identified after 1, 2, and 3 hr of incubation at each temperature.

Supravital wet mounts were prepared in a similar fashion on a thin film of neutral red (Harleco Chemical Specialties, Philadelphia, Pa.) (0.025% in absolute ethanol) which had been evenly dispersed, drained, and allowed to dry on the glass slide. These slides were kept in total darkness until they were examined.

Electron Microscopy.—Venous blood was withdrawn into a heparinized syringe and centrifuged at 100 *g* for 10 min. The plasma was then removed and the buffy coat covered with 3–10 ml of 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4, with 0.01% $CaCl_2$ added. After 15–30 min, the buffy coat solidified sufficiently to be removed as a disc (16); this was cut into 1 mm cubes, fixed further for 1 hr and then washed in 0.1 M cacodylate buffer overnight, at 4°C. The tissues were then postfixed for 1 hr in 1.3% OsO_4 in collidine buffer at pH 7.4 with 5% sucrose and 0.01% $CaCl_2$ added, washed briefly in collidine buffer, dehydrated in graded alcohols, and embedded in Epon 812. The blocks were oriented so that the cut plane of tissue sections would include all layers of the buffy coat disc. By use of either an LKB Ultrotome or a Porter Blum MT_2 Ultra-Microtome, serial thin (0.07–0.1 μ) and thick (0.4–0.7 μ) sections were cut in such a way that a thin section electron micrograph could be compared with a corresponding thick section light micrograph, thus permitting study of the same cells by both light and electron microscopy. The sections were placed on parlodion-coated grids, stained with uranyl acetate and lead citrate, or lead alone, and examined with a Philips EM 200 electron microscope. Thick sections were stained with Azure II–methylene blue, toluidine blue, or Wright stain for light microscopic examination.

Single or duplicate experiments were also performed with other fixation procedures, including (*a*) osmium veronal (17) without prefixation in glutaraldehyde, (*b*) 2.0% paraformaldehyde-2.5% glutaraldehyde (18) followed by osmium collidine, (*c*) 1.5% glutaraldehyde in 0.67 M cacodylate (19), (*d*) 1% glutaraldehyde in phosphate buffer, followed by osmium phosphate, followed by uranyl staining *en bloc* according to method 2 of Breton-Gorius (20),

(*e*) primary fixation in glutaraldehyde-osmium (21) as modified by Hirsch and Fedorko (personal communication),[1] and (*f*) 6.25% glutaraldehyde in 0.1 M phosphate buffer (22). A specimen from a normal control was fixed, processed, and examined in parallel with each patient's specimen.

To demonstrate endogenous peroxidase activity, dextran sedimented leukocytes were fixed in 2.5% glutaraldehyde in cacodylate buffer for 40 min. and washed overnight. They were then resuspended in the complete incubation mixture of Graham and Karnovsky (23) (0.01% hydrogen peroxide, 0.05% diaminobenzidine in 0.05 M Tris buffer at pH 7.6) for 7–10 min, washed three times in distilled water, postosmicated for 1 hr, dehydrated in graded alcohols, and embedded in Epon. Controls for peroxidase activity consisted of cells incubated in H_2O_2-free medium.

Enzyme Studies.—Harvest of cells: 20 ml of heparinized (0.1 mg/ml) venous blood was placed immediately into 10 ml of 6% Dextran (Travenol Lab., Inc., Morton Grove, Ill.) in saline and allowed to sediment for 60 min at 37°C. It was necessary to add 0.1% EDTA to avoid clumping during sedimentation. The white cell fraction was then centrifuged at 100 *g* for 10 min and washed twice with Hanks' balanced salt solution. The number of neutrophils/cubic millimeters was enumerated by phase microscopy in a counting chamber. The red cells were lysed by the method of Fallon et al. (24), the sample again centrifuged at 100 *g* for 10 min and the leukocyte button suspended in 0.34 M sucrose. Some platelets were still present in the final preparation. To minimize clumping, the preparations at each step contained 0.1 μg/ml of heparin.

Using a modification of the method of Cohn and Hirsch (8), leukocytes were lysed in 0.34 M sucrose and fractionated by centrifugation into "nuclear," "lysosome", and "supernate" components. It was necessary to homogenize the cells for 2 min to obtain complete lysis ,as monitored by phase microscopy. The nuclear fraction was obtained after spinning the lysed cells for 10 min at 400 *g*, and the lysosome fraction after centrifugation at 21,000 *g* for 20 min at 4°C. All fractions were frozen and thawed seven times before enzyme analysis. Total enzymatic activities were not altered by the use of 0.1% EDTA during the sedimentation process. All glassware used in working with whole leukocytes was siliconized, sterilized, and depyrogenated by heating at 180°C for 2 hr.

Acid phosphatase activity was determined with *p*-nitrophenylphosphate (Sigma Chemical Company, St. Louis, Mo.) as the substrate, buffered to pH 4.8 with 0.09 M citrate. Reactions were allowed to proceed for 10–30 min, stopped with 0.1 N NaOH, and read at 420 mμ in a Coleman Junior Spectrophotometer. Results were expressed as Sigma units per 10^7 neutrophils.

Alkaline phosphatase activity was determined in the same substrate as that used for acid phosphatase, but buffered to pH 10.5 with 0.1 M glycine. Reactions were terminated with 0.02 N NaOH, read at 420 mμ, and expressed as Sigma units per 10^7 neutrophils.

β-Glucuronidase activity was measured by the procedures of Folette et al. (25) and Fishman et al. (26). Phenolphthalein-β-glucuronide was obtained from Sigma Chemical Co. as 0.01 M solution and reactions carried out at pH 4.5 for 3 hr at 37°C. Results were expressed in units per 10^7 neutrophils, where 1 unit = 1 μg phenolphthalein liberated in 3 hr.

RESULTS

Morphologic Observations

Ultrastructure of Normal Neutrophils.—

Descriptions of the fine structure of normal human neutrophils have been recorded by several investigators (16, 20, 22, 27) and vary according to the

[1] The method has since been published in the *J. Cell Biol.* 1968. **38**:615.

fixation procedure employed. With our routine fixation method the mature neutrophil exhibited the usual segmented, multilobed nucleus, a few mitochondria, a small but occasionally well-developed Golgi complex, an occasional centriole, a rather dense hyaloplasm with scattered free ribosomes, glycogen particles, rare single rows of rough endoplasmic reticulum, variable numbers of empty vesicles, 500–1000 A in diameter, and numerous granules. In sections stained with uranyl acetate and lead, the granules formed a heterogeneous population of organelles that varied in size, shape, electron density, and internal structure. In addition, there was great variation in the relative electron density of various granules in different preparations.

Although several types of granules were seen, two could usually be distinguished (Fig. 3): (*a*) Round to oval, electron-dense granules 0.2–0.7 μ in shortest mid-diameter, henceforth designated "large dense granules." The contents of these granules were sometimes heterogeneous, including stippled and less intensely staining foci, and occasionally the whole granule appeared less dense. In some preparations, a few large granules (especially elliptical ones) were less electron dense and exhibited an internal dark core with a crystalline structure like that described by Breton-Gorius (20) and Watanabe et al. (22) (Fig. 4*a*). However, with our routine fixation the elliptical crystal-containing granules could not be consistently differentiated and were thus counted with the large granules; (*b*) smaller (about 0.15 μ in shortest mid-diameter), either rounded or elongated, sometimes clearly dumbbell-shaped granules, henceforth designated "specific granules." In most preparations these granules could be clearly distinguished from the large granules by their lower electron density (Figs. 3 and 4*b*); in some specimens, however, they were of the same density as larger granules (Fig. 4 *a*) yet most of them could be differentiated by their characteristic size and shape. The relative electron densities of granules varied also according to whether lead, uranyl, or combined uranyl acetate-lead staining was used, and the descriptions given apply to the uranyl acetate-lead staining used in most experiments.

Structure of Toxic Neutrophils.—

General: With the electron microscope, toxic neutrophils (Fig. 5) formed a heterogeneous group of cells that varied from mature neutrophils similar to controls to a few that exhibited little nuclear lobulation and could be identified as metamyelocytes. However, up to one-fourth of the lobulated, toxic neutrophils exhibited lamellae of rough endoplasmic reticulum in one or more foci within the cytoplasm, a feature that could distinguish them from controls. Other differences could also be seen but could not be confirmed by quantitative studies due to difficulties in sampling. These included: a large number of cells with prominent Golgi complex and a greater proportion of large granules that possessed a heterogeneous, stippled, or, occasionally, a moth-eaten internal structure.

Döhle bodies: These were identified in Wright-stained smears (Fig. 6 *c*)

in 5–25% of the toxic neutrophils studied. In the specimens of bone marrow, Döhle bodies were prominent in metamyelocytes and band forms.

By comparing Wright-stained thick epon sections and thin sections observed with the electron microscope (Fig. 7 *a* and *b*) Döhle bodies could be clearly identified in some neutrophils as the aggregates of rough endoplasmic reticulum within the cytoplasm (Fig. 7 *c*). These aggregates were in lamellar form and were best seen in the thick epon sections when three or more rows of rough endoplasmic reticulum were present. Fifty neutrophils each from one patient and one control were randomly selected and the presence of lamellae of rough endoplasmic reticulum was determined; three or more rows were observed in 11 of 50 toxic neutrophils, but none or only rare single rows were observed in controls.

Toxic granules: Light microscopy of peripheral blood and of bone marrow smears from patients and controls suggested a similarity between toxic granules (Fig. 6 *e*) and the azurophilic granules of neutrophil precursors in the bone marrow (Fig. 6 *f*).

In Wright-stained thick epon sections of glutaraldehyde-fixed cells the toxic granules (Fig. 8 *a*) remained azurophilic and similar granules were visible in controls. Since Wright-stained specimens of unfixed blood neutrophils from controls examined in the usual manner contained no azurophilic granules, air-dried control blood smears were fixed with 2.5% glutaraldehyde, dehydrated, and then stained. After this procedure, azurophilic granules were visualized in control preparations and looked like toxic granules. In addition, when Wright-stained blood smears were buffered for 1 hr rather than 4 min, azurophilic granules became visible in control blood smears (Fig. 6 *b*).

When neutrophils from electron micrographs were compared with corresponding cells in adjacent thick sections, azurophilic granules within the control and toxic granules in the toxic neutrophils (Fig. 8 *a* and *b*) could be identified by their relative location as groups of large dense granules. The thickness of the sections relative to the size of the granules precluded definite identification of individual granules, although corresponding *groups* of granules could be distinguished. In the specimen of bone marrow that was studied by electron microscopy, large dense granules were preponderant in neutrophil precursors (Fig. 9), and in the adjacent thick sections such granules were azurophilic. There was a suggestion that the dense granules in immature bone marrow cells were somewhat larger than those in peripheral blood cells; however, a detailed study of normal and toxic marrow was not done and the various stages in the development of azurophilic, specific, or other granules in humans could not be differentiated.

Since no obvious differences were found in the majority of granules from toxic and control neutrophils, other methods of fixation were employed, (see Materials and Methods). Although such procedures resulted in somewhat

different ultrastructural appearance of granules, no consistent differences between those of toxic and control neutrophils were uncovered.

GRANULE COUNTS From scanning some of the electron micrographs the impression was gained that large dense granules were present in larger number or in greater proportion in the toxic neutrophil. Hence although it was realized that many variables preclude exact quantitation of granules, an objective estimate was made of the number of granules in blood smears, thick epon sections, and electron micrographs of 50 toxic and control neutrophils having a mid-transverse diameter of approximately 8 μ, randomly selected from two different preparations. The average number of toxic granules per neutrophil in blood smears of patients was 105; none was identified in controls. In thick epon sections there was an average of 36 azurophilic granules per section in toxic neutrophils and 42 in controls. Total granule counts in electron micrographs average 190 per section in toxic neutrophils and 178 in controls. These differences were not significant. Further division of the granules into large, medium, and small ones also showed no statistically significant differences.

PEROXIDASE REACTIONS In electron micrographs there was no significant difference between the numbers of peroxidase positive granules in control (Fig. 10) and toxic (Fig. 11) neutrophils; the average number was 88 per section in 23 control neutrophils and 98 per section in the 48 toxic cells that were counted. The large dense (azurophilic) granules, including elliptical granules were almost always positive, whereas the specific granules were usually negative. Peroxidase activity was also seen in small round or oval granules which varied in size, perhaps representing azurophilic granules sectioned at different planes. The stacks of rough endoplasmic reticulum in toxic neutrophils were peroxidase negative (Fig. 12).

Vacuoles: Clear vacuoles in thick epon sections of toxic neutrophils were identified in some of the corresponding electron micrographs as electron-lucent areas of homogeneous matrix bound in a membrane. Occasionally such vacuoles seen in toxic neutrophils contained cytoplasmic constitutents suggesting "autophagy" (38).

When dextran sedimented toxic neutrophils were incubated with or without neutral red (0.01%) or endotoxin (10 μg/ml) for 15 min–1 hr prior to fixation for electron microscopy, there was a definite increase in membrane-bound structures containing remnants of cytoplasmic organelles. In preparations reacted for peroxidase, the enzyme was seen within these structures.

Vacuole Formation In Vitro.—

Unstained wet mounts of toxic neutrophils examined promptly by phase contrast microscopy revealed clear vacuoles within a significant number of cells; such vacuoles were rarely seen in control neutrophils. When reexamined at intervals up to 3 hr at 20°C, toxic neutrophils became increasingly vacuolated and eventually appeared degranulated. Vacuolization occurred more

rapidly when preparations were incubated at 37°C. Control neutrophils vacuolated and degranulated at a much slower rate at both 37° and 20°C. Reaction rates are plotted in Fig. 1.

Reaction with neutral red: Supravital preparations stained with neutral red

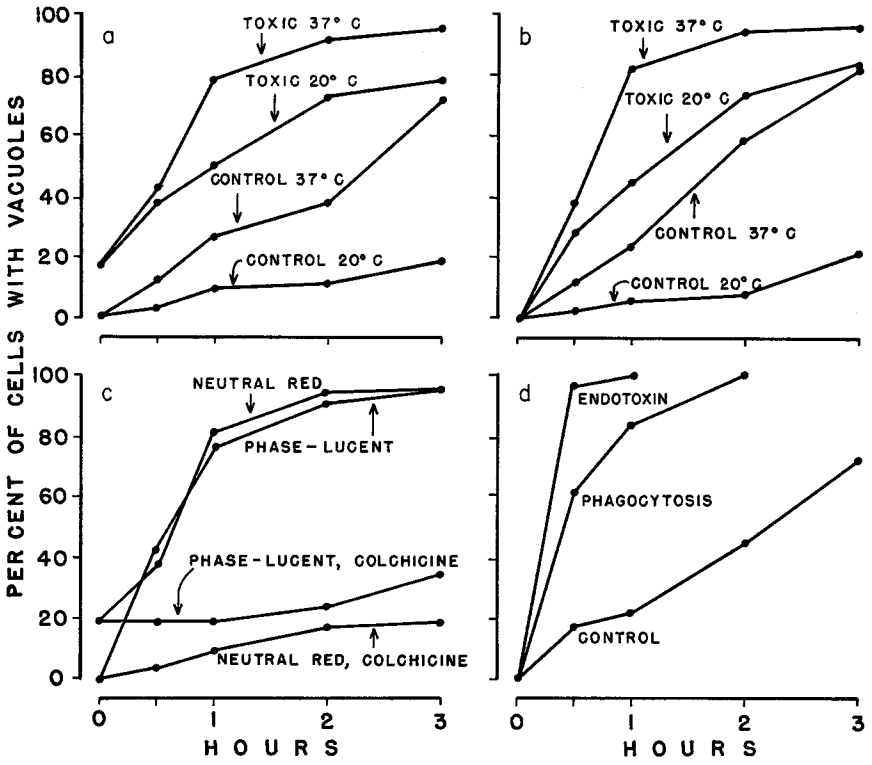

FIG. 1. (*a*) In vitro formation of clear vacuoles in unstained toxic and control neutrophils (*b*) In vitro formation of neutral red vacuoles in supravital preparations of toxic and control neutrophils at 20°C and 37°C. (*c*) Inhibition of clear (phase-lucent) and neutral red vacuole formation in toxic neutrophils incubated at 37°C with colchicine, 10 μg/ml. (*d*) Induction of neutral red vacuoles in control neutrophils by exposure to endotoxin, 1 μg/ml, and by phagocytosis of *Staphylococcus albus* at 37°C.

contained dark red granules within the toxic neutrophils. The granules of control cells were yellow-orange. Vacuoles that were clear at the time of preparation did not take the stain promptly, but within 30–45 min most of the toxic neutrophils contained neutral red vacuoles. The appearance and increase in neutral red vacuoles paralleled the increase in clear vacuoles in unstained preparations (Fig. 1 b). At the end of 3 hr many cells were almost totally degranulated. In neutrophils stained with neutral red, clear vacuoles did not

appear, suggesting that the dye was incorporated into newly forming vacuoles. Neutral red vacuole formation was also temperature dependent. The azurophilic granules of bone marrow cells stained deep red, but neutral red vacuoles did not form after incubation of bone marrow wet mounts at 37°C.

Prevention of vacuole formation: Because formation and degranulation of clear and neutral red vacuoles suggested alterations in lysosome activity, attempts were made to block these reactions by corticosteroids, chloroquine, and colchicine—compounds which "stabilize" lysosomes (28, 29). Response was measured by determining the proportion of vacuolated cells after incubation at 37°C for 1, 2, and 3 hr. Colchicine (Sigma Chemical Co.) in concentrations as low as 10 μg/ml, markedly decreased the formation of both clear (phase-lucent) and neutral red vacuoles (Fig. 1 *c*). Incubation of cells with 10^{-4} M hydrocortisone sodium succinate (Upjohn, Kalamazoo, Mich.) and 10^{-4} M chloroquine diphosphate (Sigma Chemical Co.) resulted in no detectable decrease in vacuole formation; in fact, chloroquine appeared to induce vacuoles.

TABLE I

Enzyme Activity in Control and Toxic Neutrophils*

Enzyme	Control	Toxic
Alkaline phosphatase	0.70	5.74 ($P < 0.001$)
Acid phosphatase	1.43	1.33 ($P > 0.05$)
β-glucuronidase	0.13	0.88 ($P < 0.05$)

* Activities expressed as units per 10^7 neutrophils.

Induction of vacuoles: Attempts to induce vacuoles in control cells were then made using techniques which "labilize" or "activate" lysosomes (28). Both clear and neutral red vacuolization with degranulation were easily induced by incubating cells at 37°C for 15 min-2 hr with 1 μg/ml of *Salmonella typhi* lipopolysaccharide (Difco, Detroit, Mich.) and following phagocytosis of a heat killed suspension of *Staphylococcus albus* (Fig. 1 *d*). Extensive clear and neutral red vacuolization was also noted in neutrophils collected from the inflammatory exudate of a modified 18 hr Rebuck skin window.

Enzyme Studies

The observation that toxic granules have an altered affinity for Wright stain, but are ultrastructurally similar to control granules, and the rapid vacuolization seen in toxic granules suggest that there might be changes in the membrane permeability or in the composition of neutrophilic granules during bacterial infections. To test this hypothesis, three lysosomal enzymes were selected to serve as indices of both content and activity, total activity reflecting content, and distribution reflecting "stability" of lysosomes. The

results are summarized in Table I and Fig. 2. Since phase microscopy revealed large numbers of granules sticking to nuclear fragments following homogenization, and because the distribution of enzymes between "nuclear" and "lysosomal" fractions varied from experiment to experiment, the distribution ratios were expressed as the relation of supernate (S) to nuclear plus lysosome (NL) portions.

Total alkaline phosphatase activity (Table I) was increased 8-fold in leuko-

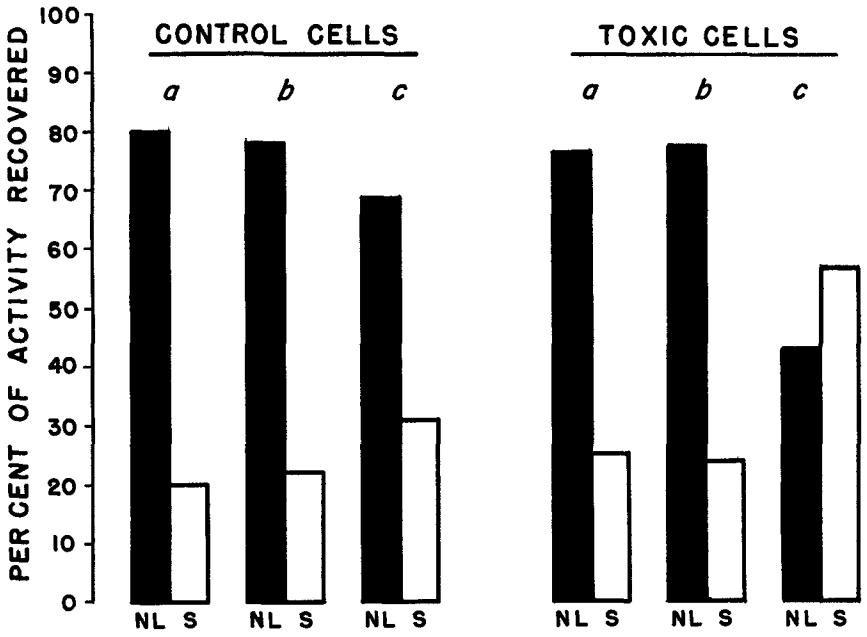

FIG. 2. Relative proportions of the total recoverable activity of alkaline phosphatase. (*a*), β-glucuronidase (*b*) and acid phosphatase (*c*) in the nuclear + lysosome (NL) and nonlysosome supernate (S) fractions of control and toxic neutrophils.

cytes from patients ($P < 0.001$); whereas, total acid phosphatase activity was not significantly different ($P > 0.05$). Both control and toxic leukocytes contained approximately 80% of the alkaline phosphatase and β-glucuronidase in the nuclear + lysosome fractions (Fig. 2), the difference not being significant. In contrast, 57% of the acid phosphatase activity in toxic neutrophils was in the nonlysosome fraction as compared with 31% in control cells; this difference was significant ($P < 0.01$).

DISCUSSION

The observations presented here clarify to some degree the nature of the cytoplasmic alterations seen in human blood neutrophils during serious bac-

terial infections. They show that Döhle bodies are aggregates of rough endoplasmic reticulum, consistent with the histochemical observations that Döhle bodies contain RNA (30). Structures resembling Döhle bodies have also been identified by light microscopy in the neutrophiles of pregnant females (31), in the May-Hegglin anomaly (32), and in patients receiving cyclophosphamide (33). In the only ultrastructural study of inclusions resembling Döhle bodies, which was of neutrophils in the May-Hegglin anomaly, Jordan and Larsen (34) described irregular cytoplasmic areas which lacked specific granules and which contained fine filaments, amorphous densities, and probably glycogen granules; they suggested that these structures may represent the Döhle bodies seen by light microscopy. Our studies do not corroborate this finding, at least with respect to those seen in leukocytes of infected patients. It is possible, however, that the inclusions in neutrophils of the May-Hegglin anomaly differ from those seen in bacterial infections. Rough endoplasmic reticulum usually reflects active protein synthesis; in the neutrophil it is present in significant quantities only in developing cells, where it participates in the synthesis of lysosomal constituents (35). Peroxidase, the only lysosomal enzyme studied by electron microscopy, could not be identified within Döhle bodies, but synthesis of other constituents of lysosomes can be postulated as in the 8-fold increase in alkaline phosphatase observed in toxic neutrophils.

"Toxic granules" have been observed in a variety of conditions and may represent morphologic expressions of diverse phenomena. In 1962, Gordin (36) reviewed the literature on toxic granules and concluded from morphologic observations that they were analogous to the cellular inclusions seen in so-called "cloudy swelling". Zucker-Franklin (37) studied the fine structure of neutrophils from joints of patients with rheumatoid arthritis, and suggested that toxic granules in those cells may be composed of phagocytized material. In studies of the ultrastructure of human leukocytes after chloroquine therapy, Fedorko (38) found membranous structures in chloroquine-induced toxic granules and suggested that they reflect either defective granule formation or "autophagy", probably the latter. Increased hemosiderin was demonstrated histochemically in toxic neutrophils by Koszewski et al. (39) who postulated that toxic granulation reflects increased metabolic activity of cells, not a phenomenon of denaturation of preexisting granules.

Our studies show that toxic granules are similar to the azurophilic granules seen by light microscopy only in neutrophil precursors. We could also visualize similar granules in mature control neutrophils by light microscopy, but only after "fixation" in glutaraldehyde or after prolonged exposure to Wright stain. Ultrastructurally, these azurophilic granules could be identified in both toxic and control neutrophils as the large dense granules. Furthermore, after fixation and processing for electron microscopy, we could not detect any consistent differences in the fine structure of the several types of granules in normal or

toxic neutrophils. However, it is possible that the techniques used here were not sensitive enough to demonstrate consistent alterations in the toxic granules, or that they may have induced equivalent changes in control neutrophils. In addition, we could not demonstrate significant differences in the number of large electrondense and small granules among toxic and control neutrophils, although admittedly sampling difficulties preclude definite conclusions in this regard. Nevertheless, our studies indicate that the toxic granules seen in blood neutrophils during infection do not represent (*a*) extraneous, phagocytized proteinaceous or particulate material (including bacteria or their products); (*b*) newly formed abnormal granules, or (*c*) autophagic bodies. However, we did observe a moth-eaten appearance in some granules of toxic neutrophils which resembled some of the granules described by Fedorko (38); and vacuoles, which probably were the result of autophagy, were occasionally present and were exaggerated by incubation.

The development, composition, and specific function of the azurophilic granule is not well understood. As usually visualized in this study, the human azurophilic granule in peripheral blood is large, oval or round, electron dense and peroxidase positive. Enomoto and Kitani (40) found peroxidase and acid phosphatase activity in similar granules of human neutrophils, although by their method the granules were less electron dense. Watanabe et al. (22) also considered the large dense granule to be the azurophil granule; in their study, however, the large elliptical crystal-containing granules were less dense than the azurophils and were considered to be a third type of granule. Rat neutrophil precursors contain large granules which are also peroxidase positive (41). In rabbits, the azurophilic granules contain acid phosphatase (42), and they evolve earlier and independently of the smaller, more lucent, specific granules (19). The latter, unlike the azurophilic granules, contain alkaline phosphatase (42). Histochemical studies of human bone marrow support the concept that azurophilic granules may contain acid phosphatase but not alkaline phosphatase (43) and we have observed peroxidase histochemically in neutrophil precursors which do not contain alkaline phosphatase. Bainton and Farquhar (19) suggested that the azurophilic granules in rabbits come from an area of Golgi zone distinct from that of the specific granules, and that the azurophilic granules diminish in total quantity during cell maturation. Unfortunately, similar detailed correlative histochemical and ultrastructural studies on developing human bone marrow cells are not yet available.

The induction of toxic granulation only after prolonged exposure to Wright stain or following fixation with 2.5% glutaraldehyde, which affects lysosome membrane permeability (44), suggests that alterations in the composition or membranes of these lysosomes in toxic neutrophils might be the basis of their staining characteristic. Some support for this is found in observations on the formation of neutral red and clear vacuoles and in the biochemical studies.

Acid phosphatase, an enzyme which may be confined to the azurophilic granule (40–42), is located mainly in the nonlysosomal fraction in toxic leukocytes, whereas it is mainly lysosomal in controls. Of the three enzymes determined, only acid phosphatase is predominantly nonlysosomal in location, suggesting that the permeability of the azurophilic granule may be specifically altered. This change in the localization of acid phosphatase may reflect either leakage of the enzyme into the cell hyaloplasm, or enhanced disruption of granules or "autophagic" structures by the procedures used to isolate the lysosomes and liberate the enzyme. A variety of external or internal influences might lead to more fragile secondary lysosomes. Alternatively, there may be selective enzyme permeability, altered latency of acid phosphatase, or new production of acid phosphatase located in organelles which are not sedimented at 21,000 *g*. The demonstration of similar levels of total acid phosphatase activity in control and toxic neutrophils argues against the last explanation.

The presence of clear vacuoles and the concomitant formation of clear and neutral red vacuoles in vitro within toxic neutrophils also seem to reflect alterations in lysosome permeability, although defective granule formation cannot be completely excluded. Several bits of evidence seem to favor the former explanation: (*a*) Neutral red and certain other cationic vital dyes not only stain lysosomes, but also may reveal their activity. If stimulated by light, lysosomes of cells stained with neutral red are activated and neutral red bodies are formed (45). Viral infections cause a similar reaction (44). (*b*) "Autophagic" bodies can be occasionally identified in toxic neutrophils and, if incubated with neutral red or endotoxin, their numbers are increased. (*c*) Both clear and neutral red vacuole formation can be inhibited by colchicine, a lysosome "stabilizer" (29). (*d*) Both clear and neutral red vacuoles can be induced by maneuvers known to "labilize" lysosomes, namely exposure to endotoxin, phagocytosis, and inflammation (28).

The presence of aggregates of rough endoplasmic reticulum (Döhle bodies) and azurophilia in some granules of toxic neutrophils may simply reflect cytoplasmic immaturity due to early release of cells from the bone marrow. If so, the azurophilic granule could have less affinity for Romanowsky stains as it matures. Although this may be the explanation, several findings suggest a more complex process. First, alkaline phosphatase is increased and this enzyme evolves late in the maturation of the neutrophil. However, since alkaline phosphatase may be an enzyme of the "specific" rather than the azurophilic granule, the elevation of this lysosomal enzyme could be unrelated to the toxic granule. Indeed, we have observed toxic granulation neutrophils of chronic myelocytic leukemia when the leukocyte alkaline phosphatase by histochemical determination was low. Second, toxic granules and Döhle bodies are apparently present with equal frequency in neutrophils with mature and immature nuclei; therefore, if the cytoplasm is immature, it would imply a dissociation between

the nuclear and cytoplasmic maturation. Third, vacuolization is not a characteristic of immature neutrophils.

Similarities exist between toxic neutrophils and certain cytoplasmic changes observed in stimulated monocytes and lymphocytes. Both monocytes and lymphocytes sometimes contain azurophilic granules; these are acid phosphatase positive and electron dense (46, 48). Upon stimulation, these granules increase in number (46, 49) and alter their neutral red reaction, and in lymphocytes, "labilize" acid phosphatase (49). By electron microscopy there may be an increase in rough endoplasmic reticulum, and hypertrophy of the Golgi apparatus (47, 50). "Toxic" monocytes and histocytes seen in the blood in certain chronic infections, and the atypical blood lymphocytes of certain viral infections may be the clinical counterparts of the in vitro stimulation of these cells. Thus, by analogy with monocytes and lymphocytes it is possible that the alteration in the cytoplasm of toxic neutrophils denotes stimulation rather than immaturity. Fedorko and Hirsch (35) suggested that new lysosome production in neutrophils proceeds at a rate faster than whole cell maturation, hence lysosome production may continue throughout the life-span of the cell.

The pathophysiologic consequences of changes in lysosome activity of toxic neutrophils are unknown. However, since neutrophils may participate in the inflammatory response in both a beneficial and deleterious way (9–13) the lysosomal and other cytoplasmic changes in toxic neutrophils may play some role in the host's response to severe bacterial infection.

SUMMARY

"Toxic" neutrophils from humans with severe bacterial infections, identified by the presence of Döhle bodies, "toxic" granules, and vacuoles were shown to differ from normal neutrophils both in ultrastructure and in lysosome activity.

Döhle bodies were identified as lamellar aggregates of rough endoplasmic reticulum. Toxic granules corresponded to the azurophilic granules usually identified by Romanowsky stains only in neutrophil precursors. By electron microscopy such granules were large, electron-dense, and peroxidase positive; they could usually be distinguished from the smaller, less dense, "specific" granules also present in control neutrophils, but in the latter they became visible by light microscopy only after prolonged staining or following fixation with glutaraldehyde. These observations suggest that toxic granules represent an abnormal staining reaction of the large dense granules in the toxic cells, and not phagocytized material, newly formed abnormal granules or autophagic bodies.

Alkaline phosphatase activity was significantly greater in toxic neutrophils than in normal ones; 80% of the activity of both was located in the lysosome fraction. Beta glucuronidase was normal. Total acid phosphatase was normal, but the percentage located in the nonlysosome fraction of toxic neutrophils was increased, suggesting that lysosomes were "labilized."

Formation of neutral red vacuoles in supravitally stained preparations, an index of lysosome activity, occurred more rapidly in toxic neutrophils. This reaction paralleled degranulation and the formation of clear vacuoles in unstained wet mounts and could be blocked by colchicine, a lysosome stabilizer, or enhanced by procedures which activate lysosomes. "Autophagic" vacuoles were observed by electron microscopy in some toxic neutrophils.

These observations are discussed in relation to the concept that the "toxic" neutrophils in severe bacterial infection reflect cellular immaturity and/or stimulation or degeneration.

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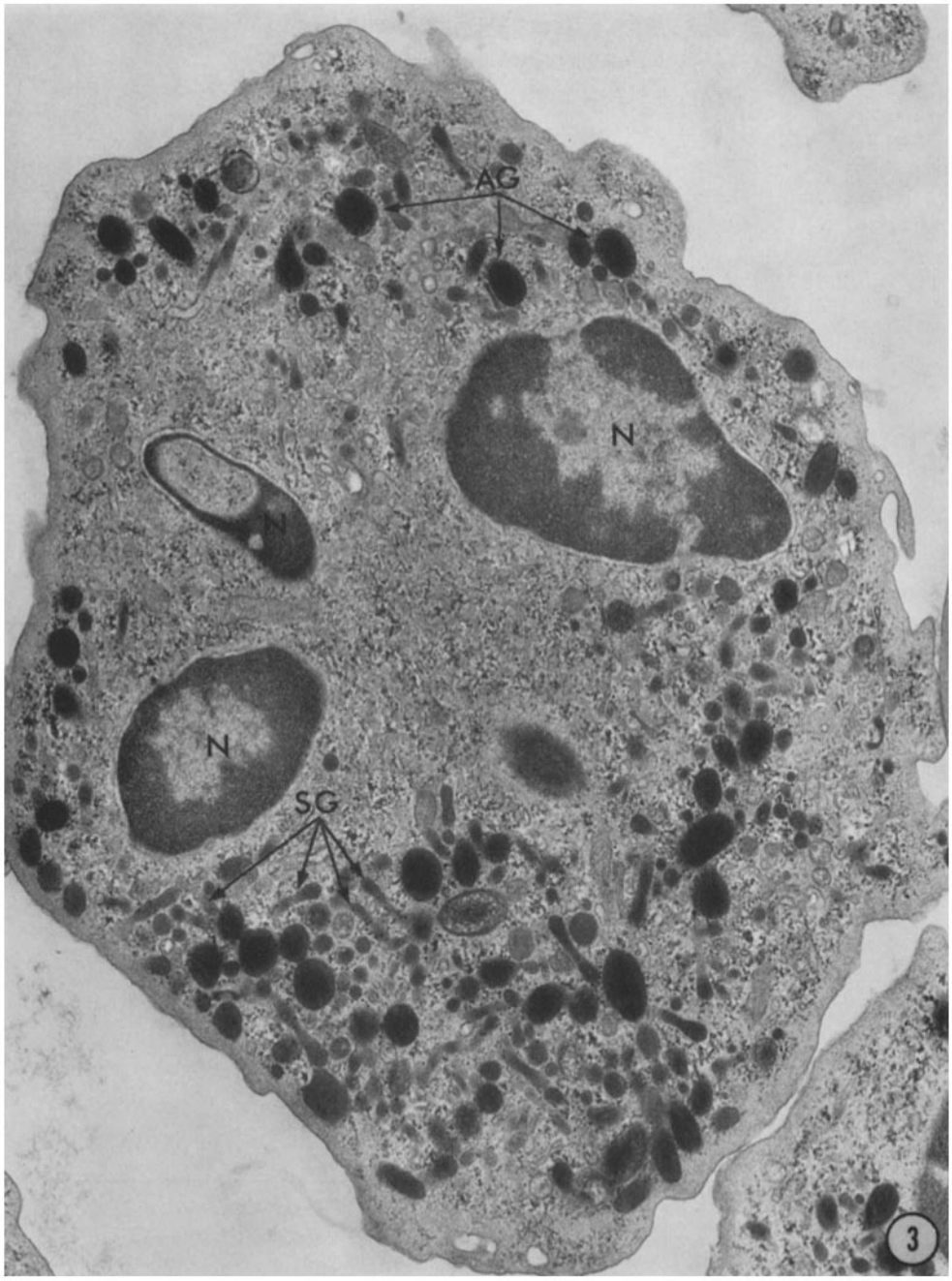
BIBLIOGRAPHY

1. Metchnikoff, E. 1893. Lectures on the comparative pathology of inflammation. F. A. Starling and E. H. Starling, translations. Kegan, Paul, Trench, Trubner, and Co. Ltd., London.
2. Ponder, E., and R. V. O. Ponder. 1942. The cytology of the polymorphonuclear leukocyt in toxic conditions. *J. Lab. Clin. Med.* **28:** 316.
3. Wintrobe, M. H. 1962. The Leukocytes. *In* Clinical Hematology. Lea & Febiger, Philadelphia, 214.
4. Döhle, H. 1911. Leukocyteneinschlüsse bei Scharlach. *Zentralbl. Bacteriol. Parasitenk. Infektionskr. Hyg. Abt. Orig.* **61:** 63.
5. Zieve, P. D., M. Haghshenass, M. Blanks, and J. R. Krevans. 1966. Vacuolization of the neutrophil. An aid in the diagnosis of septicemia. *Arch. Intern. Med.* **118:** 356.
6. Kugel, M. A., and N. Rosenthal. 1938. Pathologic changes in polymorphonuclear leukocytes during the progress of infections. *Am. J. Med. Sci.* **183:** 657.
7. de Duve, C., B. C. Pressman, R. Bianetho, R. Wattiaus, and F. Appelmans. 1955. Tissue fractionation studies. VI. Intracellular distribution patterns of enzymes in rat-liver tissue. *Biochem. J.* **60:** 604.
8. Cohn, Z. A., and J. G. Hirsch. 1960. The isolation and properties of the specific cytoplasmic granules of rabbit polymorphonuclear leucocytes. *J. Exp. Med.* **112:** 983.
9. Cohn, Z. A., and J. G. Hirsch. 1960. The influence of phagocytosis on the intracellular distribution of granule-associated components of polymorphonuclear leucocytes. *J. Exp. Med.* **112:** 1015.
10. Janoff, A., and B. W. Zweifach. 1964. Production of inflammatory changes in microcirculation by cationic proteins extracted from lysosomes. *J. Exp. Med.* **120:** 747.
11. Herion, J. C., J. K. Spitznagel, R. C. Walker, and H. C. Zeyer. 1966. Pyrogenicity of granulocyte lysosomes. *Am. J. Physiol.* **211:** 693.
12. Thomas, L. 1964. Possible role of leucocyte granules in the Shwartzman and Arthus reactions. *Proc. Soc. Exp. Biol. Med.* **115:** 235.
13. Janoff, A., G. Weissmann, B. W. Zweifach, and L. Thomas. 1962. Pathogenesis of experimental shock. IV. Studies of lysosomes in normal and tolerant animals subjected to lethal trauma and endotoxemia. *J. Exp. Med.* **116:** 451.

14. Quie, P. G., J. G. White, B. Holmes, and R. A. Good. 1967. *In vitro* bactericidal capacity of human polymorphonuclear leucocytes; diminished activity of chronic granulomatous disease of childhood. *J. Clin. Invest.* **46:** 688.
15. White, J. G. 1966. The Chediak-Higashi syndrome: A possible lysosomal disease. *Blood.* **28:** 143.
16. Anderson, D. R. 1966. Ultrastructure of normal and leukemic leukocytes in human peripheral blood. *J. Ultrastruct. Res. Suppl.* **9:** 1.
17. Palade, G. E. 1952. A study of fixation for electron microscopy. *J. Exp. Med.* **95:** 285.
18. Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixation of high osmolarity for use in electron microscopy. *J. Cell Biol.* **27:** 44A.
19. Bainton, D. E., and M. Farquhar. 1966. Origin of granules in polymorphonuclear leukocytes. *J. Cell Biol.* **28:** 277.
20. Breton-Gorius, J. 1966. Structures periodiques dan les granulations eosinophiles et neutrophiles des leucocytes polynuclèares due sang de l'homme. *Nouv. Rev. Fr. Hematol.* **6:** 195.
21. Trump, B., and R. E. Bulger. 1966. New ultrastructural characteristics of cells fixed in a glutaraldehyde-osmium tetroxide mixture. *Lab. Invest.* **15:** 368.
22. Watanabe, I., S. Donahue, and N. Hoggatt. 1967. Method for electron microscopic studies of circulating human leukocytes and observations on their fine structure. *J. Ultrastruct. Res.* **20:** 366.
23. Graham, R. C., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: Ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14:** 291.
24. Fallon, H. J., E. Frei, J. D. Davidson, J. S. Trier, and D. Burk. 1962. Leukocyte preparations from human blood: Evaluation of their morphologic and metabolic state. *J. Lab. Clin. Med.* **59:** 779.
25. Folette, J. H., W. H. Valentine, and J. S. Lawrence. 1952. The beta glucuronidase content of human leucocytes in health and disease. *J. Lab. Clin. Med.* **40:** 825.
26. Fishman, W. H., Springer, B., and Brunetti, R. 1948. Application of an improved glucuronidase assay method to the study of human blood β-glucuronidase. *J. Biol. Chem.* **173:** 449.
27. Bessis, M., and J. Thiery. 1961. Electron microscopy of human white blood cells and their stem cells. *Int. Rev. Cytol.* **12:** 199.
28. Weissman, G. 1965. Lysosomes. *N. Engl. J. Med.* **273:** 1084, 1143.
29. Malawista, S. E., and P. T. Bodel. 1967. The dissociation by colchicine of phagocytosis from increased oxygen consumption in human leukocytes. *J. Clin. Invest.* **46:** 786.
30. Oski, F. A., J. L. Naiman, D. M. Allen, and L. K. Diamond. 1962. Leukocytic inclusions-Döhle bodies-associated with platelet abnormality (May-Hegglin Anomaly). Report of a family and review of the literature. *Blood.* **20:** 657.
31. Abernathy, M. R 1966. Döhle bodies associated with uncomplicated pregnancy. *Blood.* **27:** 380.
32. Hegglin, R. 1945. Simultaneous constitutional changes in neutrophils and platelets. *Helv. Med. Acta.* **12:** 439.

33. Itoga, T., and J. Laszlo. 1962. Döhle bodies and other granulocytic alterations during chemotherapy with cyclophosphamide. *Blood.* **20:** 668.
34. Jordan, S. W., and W. E. Larsen. 1965. Ultrastructural studies of the May-Hegglin anomaly. *Blood.* **25:** 921.
35. Fedorko, M. E., and J. G. Hirsch. 1966. Cytoplasmic granule formation in myelocytes. An electron microscope radio-autographic study on the mechanism of formation of cytoplasmic granules in rabbit heterophilic myelocytes. *J. Cell Biol.* **29:** 307.
36. Gordin, R. 1952. Toxic granulation in leukocytes. *Acta Med. Scand. Suppl.* **143:** 270.
37. Zucker-Franklin, D. 1966. The phagosomes in rheumatoid synovial fluid leukocytes: a light, fluorescence, and electron microscope study. *Arthritis Rheum.* **9:** 24.
38. Fedorko, M. 1967. Effect of chloroquine on morphology of cytoplasmic granules in maturing human leucocytes. An ultrastructural study. *J. Clin. Invest.* **46:** 1932.
39. Koszewski, B. J., H. Vahabzadeh, and S. Willrodt. 1967. Hemosiderin content of leukocytes in animals and man and its significance in the physiology of granulocytes. *Am. J. Clin. Pathol.* **48:** 474.
40. Enomoto, T., and T. Kitani. 1966. Electron microscopic studies on peroxidase and acid phosphatase reaction in human leukocytes. *Acta Haemat.* **29:** 554.
41. Yamada, E. 1966. Electron microscopy of the peroxidase in the granular leucocytes of rat bone marrow. *Arch. Histol.* **27:** 131.
42. Wetzel, B. K., S. S. Spicer, and R. G. Horn. 1967. Fine structural localization of acid and alkaline phosphatase in cells of rabbit blood and bone marrow. *J. Histochem. Cytochem.* **15:** 311.
43. Ackerman, G. A. 1964. Histochemical differentiation during neutrophil development and maturation. *Ann. N. Y. Acad. Sci.* **113:** 537.
44. Allison, A. C., and L. Malluci. 1965 Histochemical studies of lysosomes and lysosomal enzymes in virus-infected cell cultures. *J. Exp. Med.* **121:** 463.
45. Allison, A C., and M. R. Young. 1964. Uptake of dyes and drugs in living cells. *Life Sci.* **3:** 1407.
46. Cohn, Z. A., and B. Benson. 1965. The differentiation of mononuclear phagocytes: morphology, cytochemistry, and biochemistry. *J. Exp. Med.* **121:** 153.
47. Cohn, Z. A., J. G. Hirsch, and M. E. Fedorko. 1966. The *in vitro* differentiation of mononuclear phagocytes. IV. The ultrastructure of macrophage differentiation in peritoneal cavity and in culture. *J. Exp. Med.* **123:** 747
48. Brittinger, G., R. Hirschhorn, S. D. Douglas, and G. Weissman. 1968. Studies on lysosomes. XI. Characterization of a hydrolase-rich fraction from human lymphocytes. *J. Cell Biol.* **37:** 394.
49. Hirschhorn, R., G. Brittinger, K. Hirschhorn, and G. Weissman. 1968. Studies on lysosomes. XII. Redistribution of acid hydrolases in human lymphocytes stimulated by phytohemagglutinin. *J. Cell Biol.* **37:** 412.
50. Elves, M. W., J. Gough, J. A. Chapman, and M. C. G. Israels. 1964. Electron microscopy studies of lymphocytes. Transformation under the influence of phytohemagglutinin. Lancet **1:** 306.

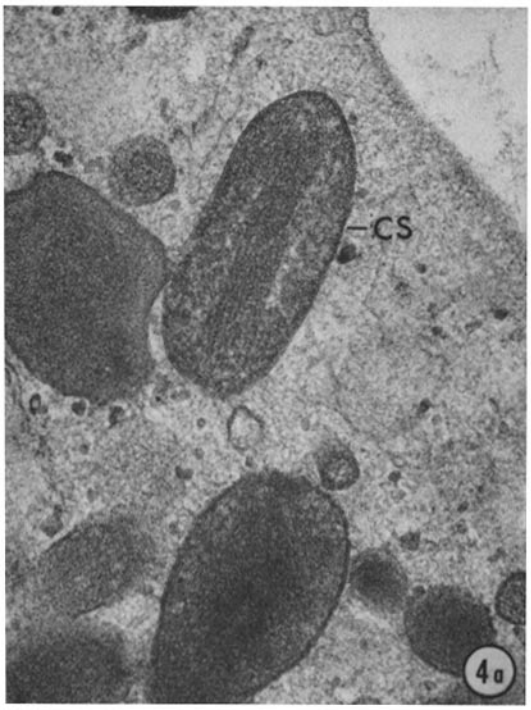


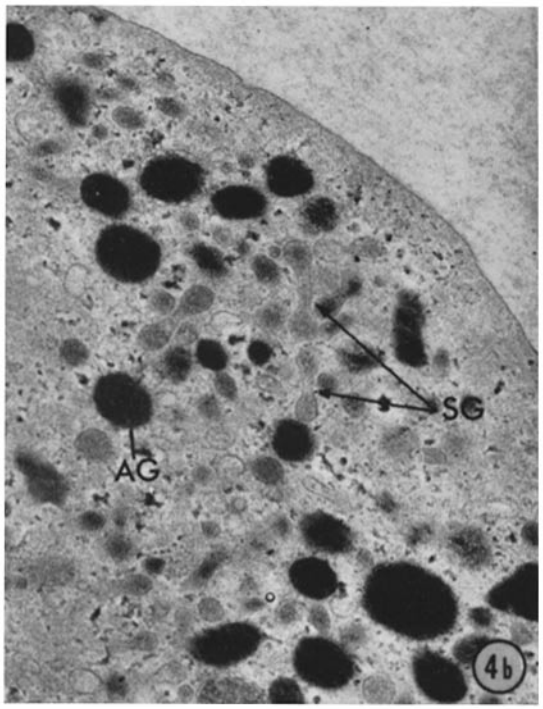
Unless specified otherwise, all electron micrographs are from sections stained with uranyl acetate and lead citrate. All magnifications are approximate.

FIG. 3. Electron micrograph of a peripheral mature blood neutrophil from a bacteremic patient; this neutrophil is indistinguishable from that of a control. Note the types of granules that can be seen with our routine fixation procedure: Large, round or oval, dense granules (*AG*); small, round or elongated, sometimes dumbbell-shaped, less dense, specific granules (*SG*). *N*, nucleus. × 20,000.

FIG. 4. (*a*) Detail of a large elliptical granule with a central crystalloid (*CS*) in a control neutrophil. Watanabe et al. (22) considered this a third type of granule; in this preparation all granules were of the same electron density. × 57,000. (*b*) Detail of a portion of a toxic neutrophil clearly showing the difference between the large, dense, (azurophilic) granules (*AG*) and the specific granules (*SG*). *N*, nucleus. × 28,000.

FIG. 5. Electron micrograph of a "toxic" neutrophil exhibiting a lobulated nucleus (*N*), rough endoplasmic reticulum (*RER*), large dense and small specific granules; *C*, centriole; *G*, golgi complex. × 28,000.


CS
4a


SG
AG
4b

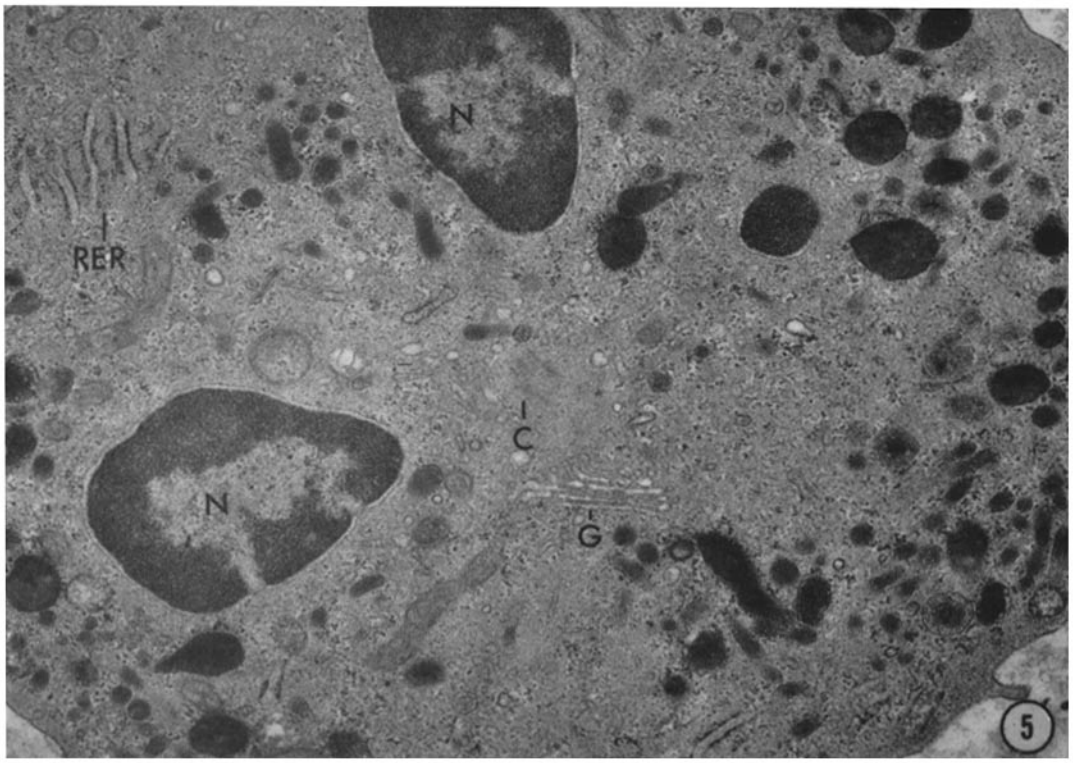
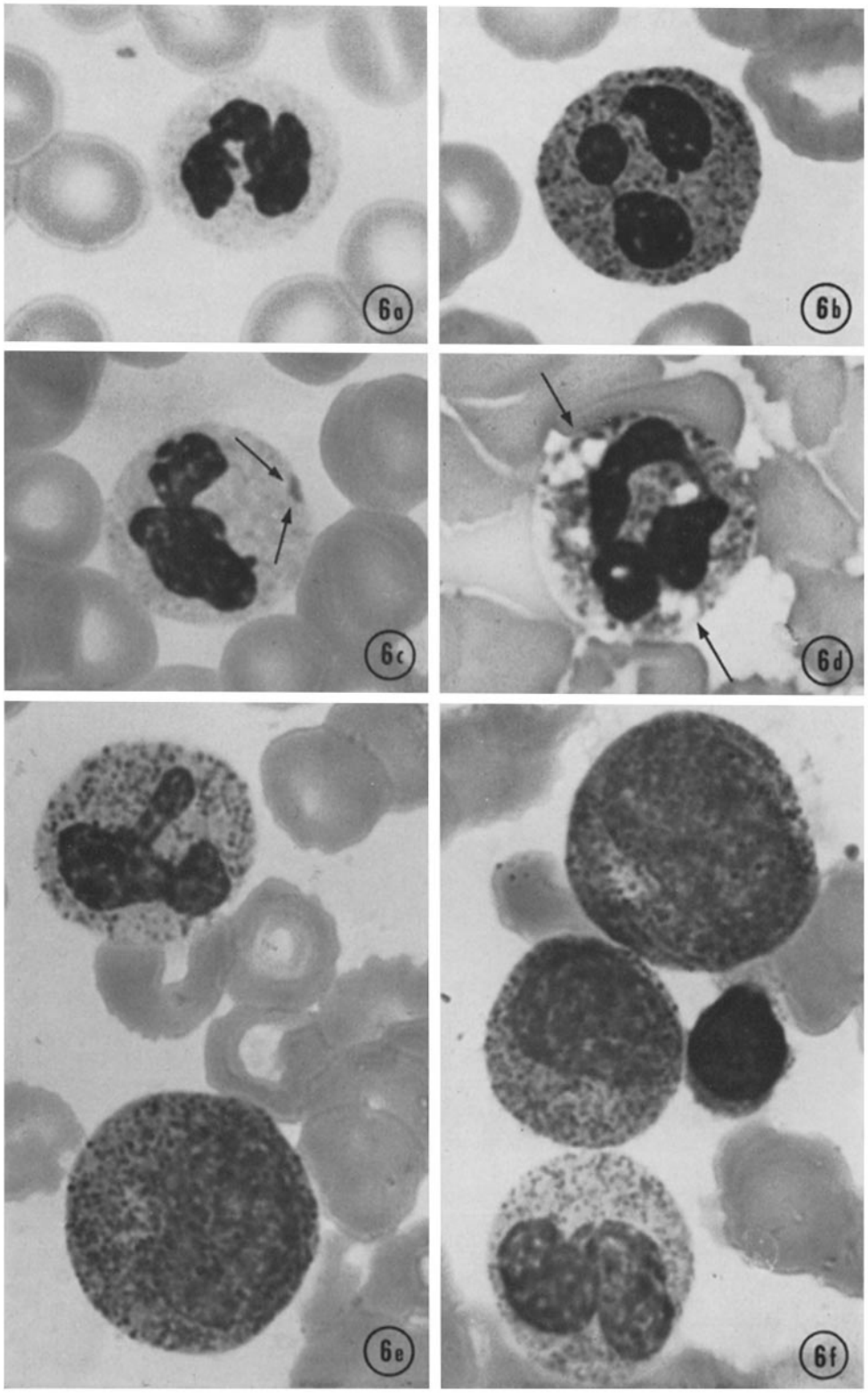

N
RER
C
N
G
5

FIG. 6. (*a*) Normal blood neutrophil Wright-stained for 4 min. × 2000. (*b*) Normal blood neutrophil Wright-stained for 60 min. Granules are deeply stained (cf. part *a*) and appear similar to those visible in toxic cells. × 2120. (*c*) Döhle body (arrows) within cytoplasm of a blood neutrophil, Wright-stained for 4 min. × 2120. (*d*) Cytoplasmic vacuolization (arrows) in toxic blood neutrophil, Wright-stained for 4 min. × 2120. (*e*) Toxic granulation in mature neutrophil and metamyelocyte from blood smear of a bacteremic patient. Wright-stained for 4 min. × 2120. (*f*) Bone marrow smear from patient with toxic neutrophils in peripheral blood. Azurophilic granules of neutrophil precursors resemble toxic granules in mature neutrophil. Wright-stained for 4 min. × 2120.


6a
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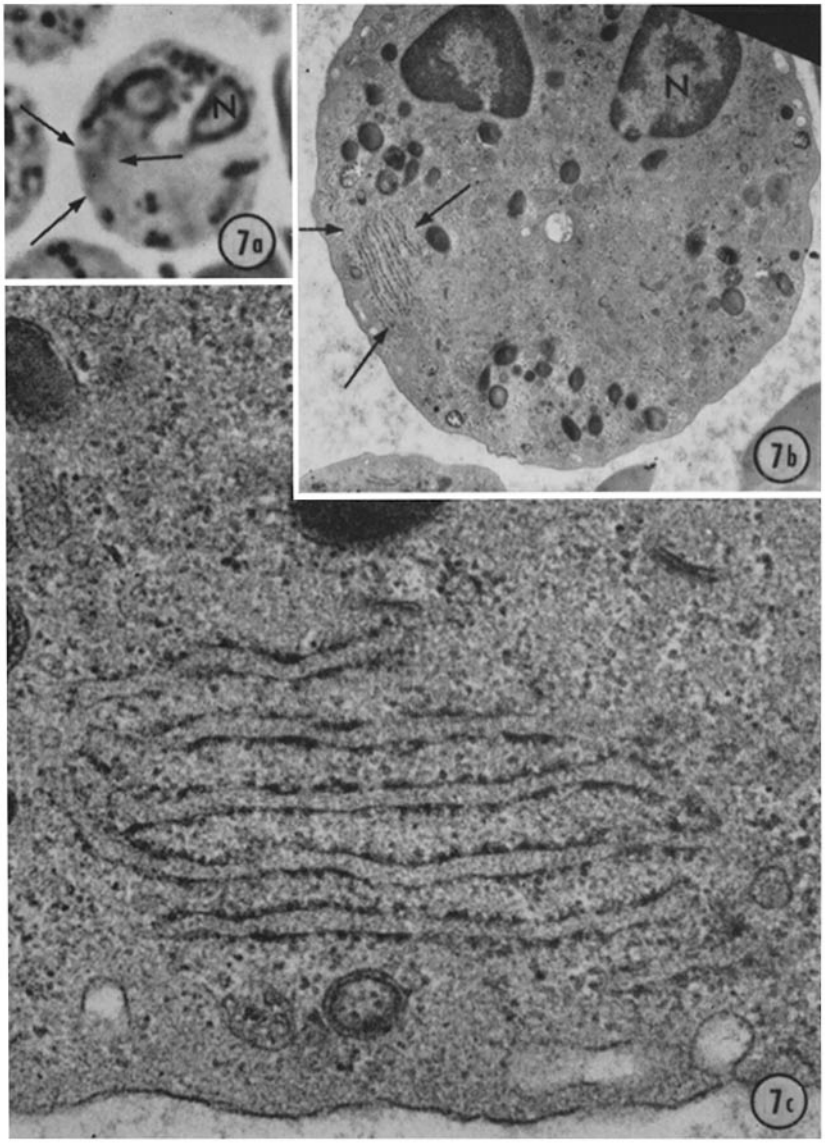
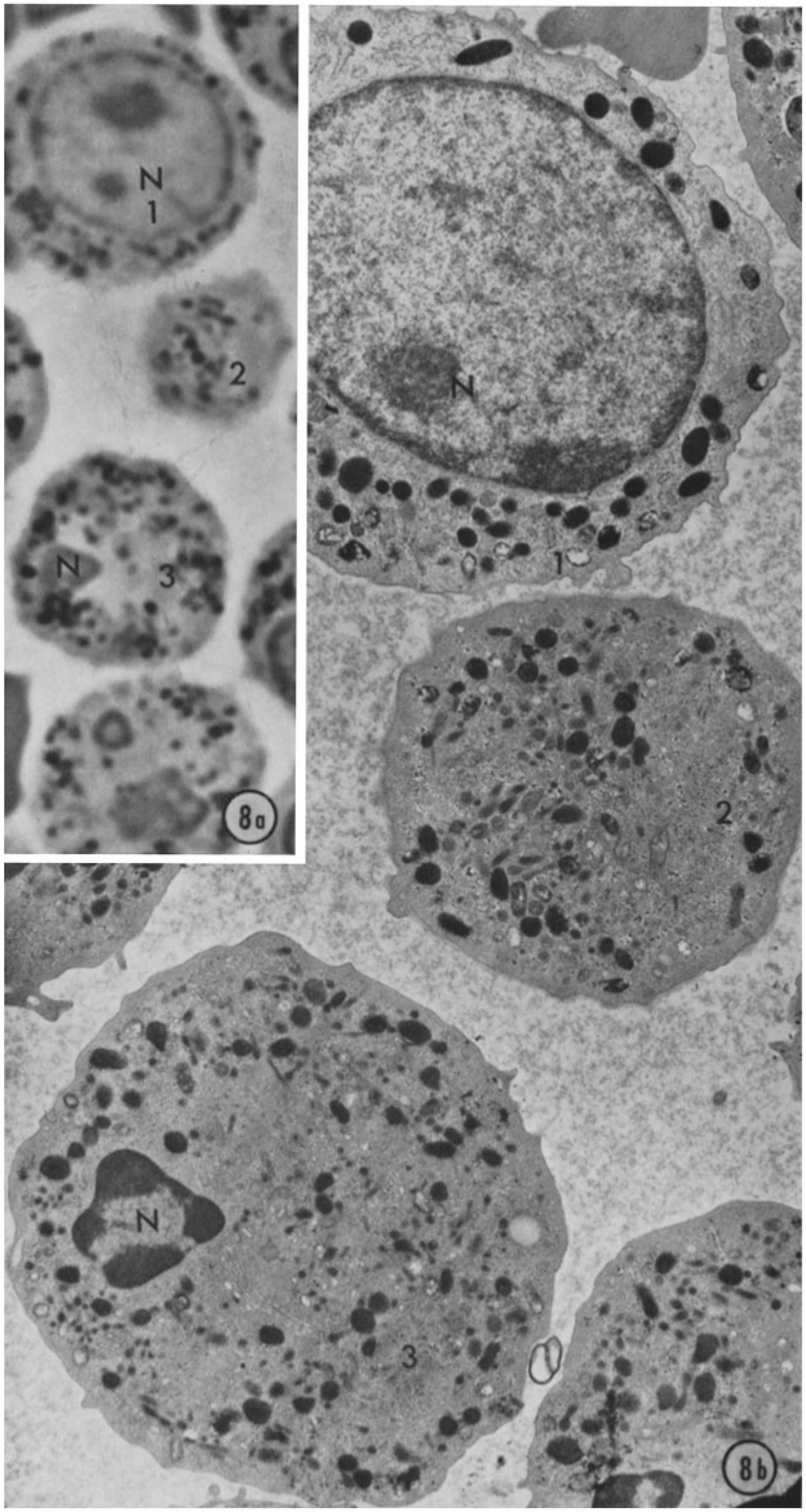
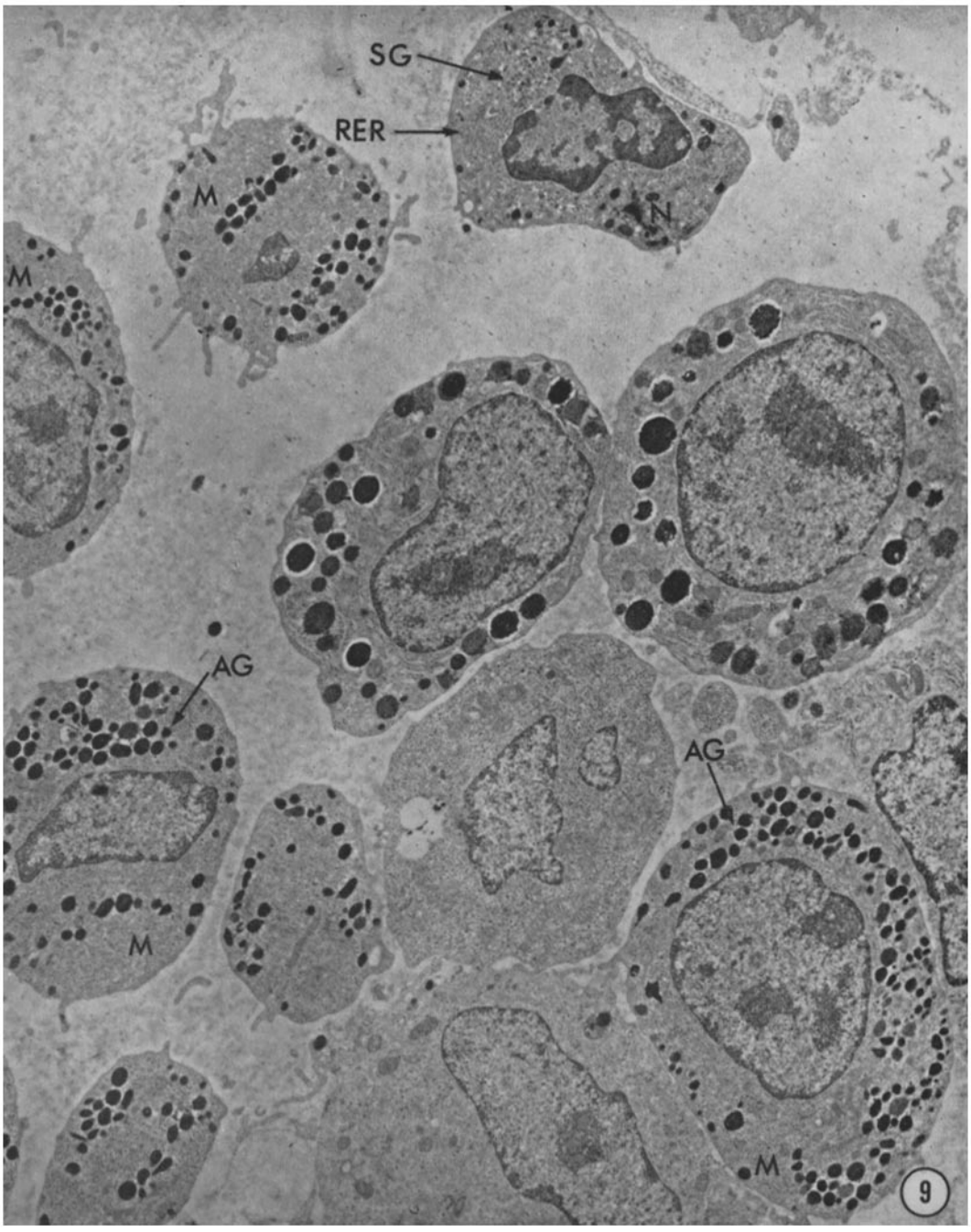


FIG. 7. (*a*) Light micrograph of Wright-stained thick epon section of toxic neutrophil. Döhle body is indicated by arrows. *N*, nucleus. × 3800. (*b*) Electron micrograph of same toxic neutrophil in part (*a*) as seen in adjacent thin section. Döhle body identified as aggregate of rough endoplasmic reticulum. (arrows). Contents of some large granules appear heterogeneous and stippled. *N*, nucleus. × 9000. (*c*) Higher magnification of the rough endoplasmic reticulum in part (*b*). × 53,000.

FIG. 8. Light micrograph of Wright-stained thick epon section from patient with toxic neutrophils showing azurophilic granules within the cytoplasm. Cells 1, 2, and 3 identified by electron microscopy in adjacent thin section shown in part (*b*). Cell 1 is a mononuclear granulated cell that could not be classified further. *N*, nucleus. × 4000. (*b*) Electron micrograph of cells 1, 2, and 3. Note distribution of large dense granules similar to that of azurophilic granules. *N*, nucleus. × 9000.


N
1
2
N
3
8a
N
1
2
N
3
8b


SG
RER
M
N
M
AG
AG
M
M
9

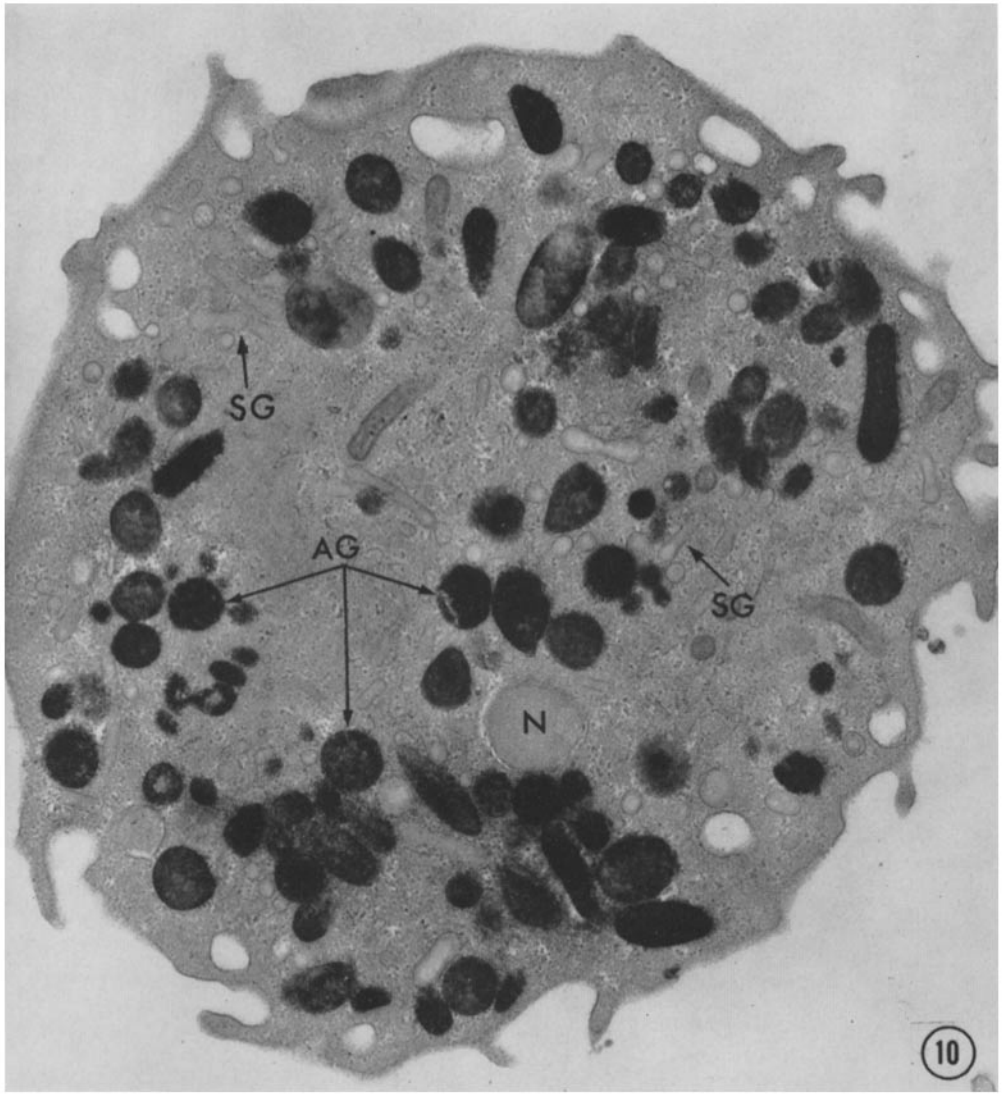
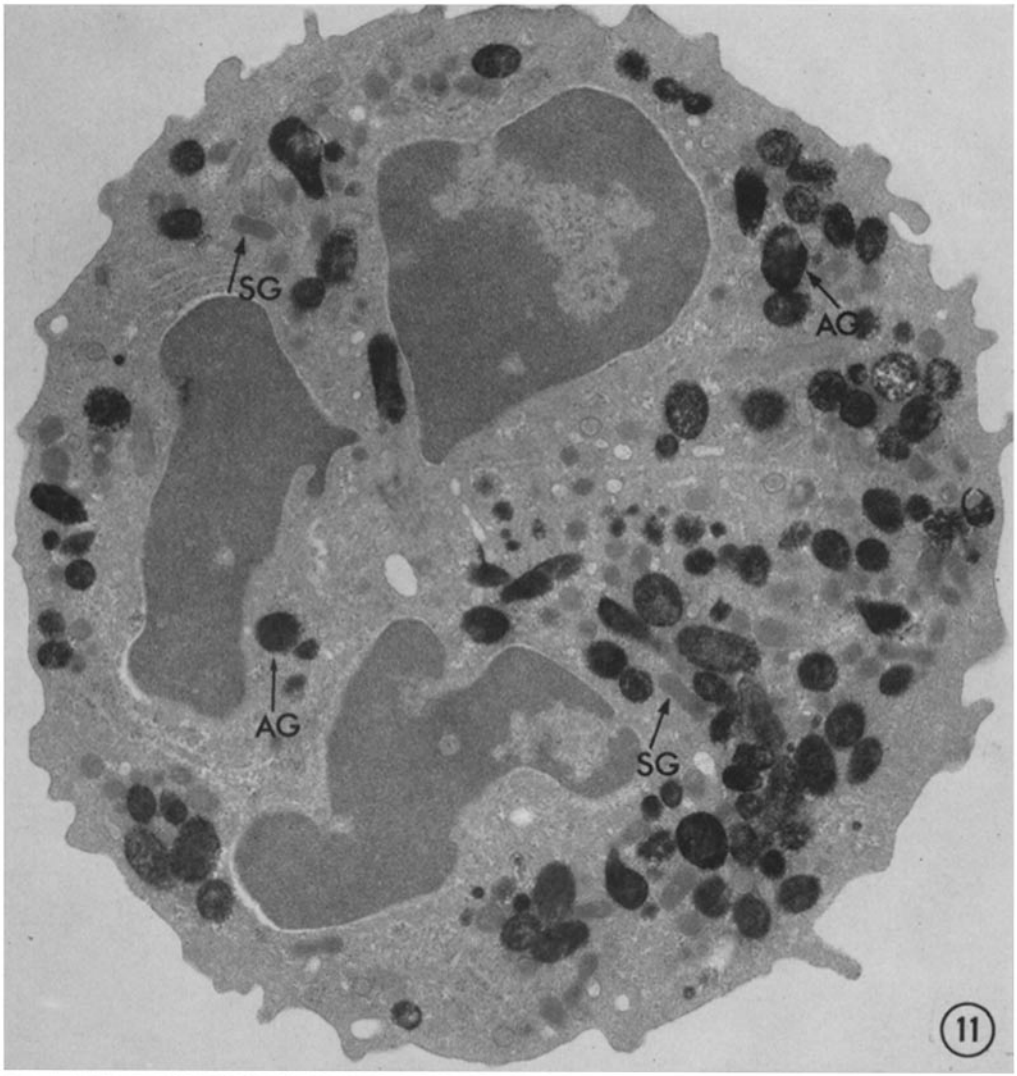


FIG. 10. Electron micrograph of control neutrophil reacted for peroxidase. Large round or oblong granules (*AG*), corresponding with large granules of sections not reacted for peroxidase, are peroxidase positive; most smaller and dumbbell-shaped granules (*SG*) are peroxidase negative. *N*, nucleus. Stained with lead only. × 24,000.

FIG. 9. Electron micrograph of bone marrow from patient with bacteremia. Cells labeled *M* are immature neutrophil precursors. Note numerous large dense granules (*AG*). Cell labeled *N* is more mature neutrophil showing a group of specific granules (*SG*) and an aggregate of rough endoplasmic reticulum (*RER*). × 6000.


SG
AG
AG
SG
11

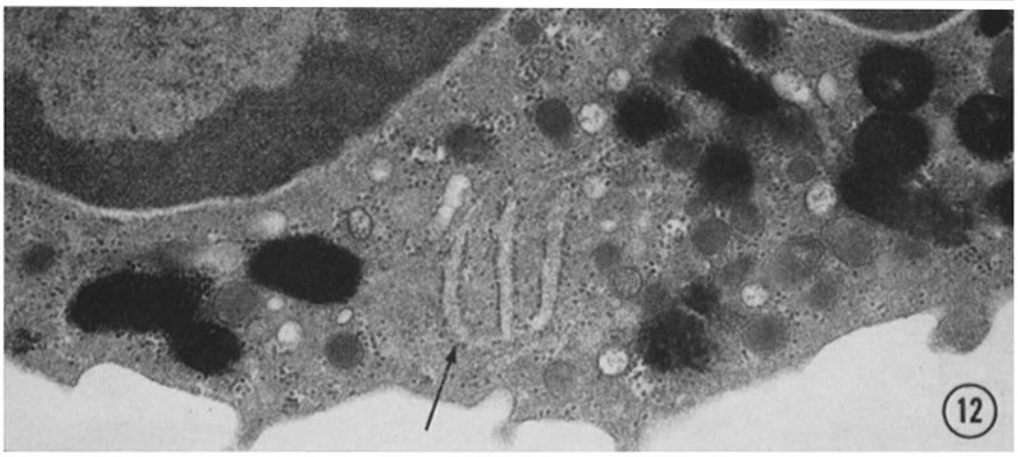

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FIG. 11. Electron micrograph of toxic neutrophil reacted for peroxidase. Large dense azurophilic granules (*AG*) are peroxidase positive and most specific granules (*SG*) are peroxidase negative. Lead stain. × 21,000.

FIG. 12. Toxic neutrophil reacted for peroxidase. The arrays of rough endoplasmic reticulum (arrow) are peroxidase negative. Lead stain. × 33,000.