Effects of Heterologous Antineutrophil Serum in Guinea Pigs

Hematologic and Ultrastructural Observations

David M. Simpson and Russell Ross

Two pools of rabbit anti–guinea pig neutrophil serum (ANS) were prepared using an intravenous (ANS I) or subcutaneous (ANS II) route of immunization with proteose peptone-stimulated peritoneal exudate neutrophils (PMNs) from albino guinea pigs. *In vitro*, both pools of ANS contained high titers of agglutinating antibodies to neutrophils and lower titers against lymphocytes and red cells. Agglutinins against all three cell types could be selectively removed by absorption. The *in vivo* hematologic effects of both the absorbed and unabsorbed antisera were examined after intraperitoneal administration, and the effects of ANS on neutrophils in blood, bone marrow, and peritoneal cavity were examined by light and electron microscopy of spleen, liver, lung, lymph node, buffy coat, bone marrow and pellets of peritoneal cells removed at various time intervals within 24 hours. Injection of either antisera caused a rapid decrease in circulating neutrophils and lymphocytes, which reached their lowest levels within 12 hours. Neutrophils that disappeared from the circulation were sequestered primarily in liver and spleen where they were phagocytized, as morphologically intact cells, by macrophages and then rapidly digested. Immature bone marrow neutrophils as young as early myelocytes were ingested by macrophages in the marrow at 6 hours or later after ANS. Neutrophils that were phagocytized were apparently opsonized by ANS since there was no ultrastructural evidence of neutrophil lysis in blood or bone marrow after ANS treatment. However, both lysed and ingested neutrophils were observed in the peritoneal cavity. Absorption of ANS with neutrophils removed the ability of the serum to produce neutropenia. However, absorption of ANS with lymphocytes did not alter the lymphopenia produced by the antiserum. The fate of lymphocytes leaving the peripheral circulation was not apparent. Lymphocytes did not accumulate in liver or spleen sinusoids and were not ingested by macrophages in these organs, as were the neutrophils. There was no evidence of paracortical depletion or extensive phagocytosis of lymphocytes in lymph nodes after ANS, as other investigators have reported after administration of antilymphocyte serum. (Amer J Path 65:79–102, 1971)

IT HAS BEEN KNOWN since the observations of Metchnikoff [1] that "antileukocyte serum" can be prepared by immunizing an animal with leukocytes from another species. In recent years, there has been increased investigation of the biologic properties of heterologous anti-

From the Department of Pathology, University of Washington School of Medicine, Seattle, Washington 98105.

Supported by grants AM 13970, GM 13453 and GM 00100 from the US Public Health Service.

Accepted for publication June 1, 1971.

Address reprint requests to Dr. Ross.

leukocyte sera due to increased utilization of these agents for various experimental and clinical purposes. Antineutrophil sera (ANS) have been utilized in several different species in investigations of the dynamics of myelopoiesis after systemic depletion of neutrophils,[2,3] in studies of the mechanism of the Arthus phenomenon [4] and in investigations of immune glomerulonephritis.[5]

Administering antineutrophil serum *in vivo* results in a rapid decrease in the number of circulating neutrophilic granulocytes (PMNs). The maximal effect of the antiserum occurs within 24 hours after a single injection, but some degree of neutropenia usually persists for up to a week after the administration of ANS.[2,6,7] It is possible to eliminate, transiently, virtually all circulating neutrophils with a single dose of a potent ANS. The fate of the neutrophils that disappear from the circulation within hours after ANS administration has not been determined unequivocally in previous investigations. It has been suggested that administration of antileukocyte sera results either in intravascular lysis of leukocytes [6,8] or in phagocytosis of leukocytes by reticuloendothelial macrophages after "opsonization" by antibody.[9,10] It has also been reported that intravascular agglutination and embolization of neutrophil thrombi to pulmonary capillaries occurs after intracardiac or intravenous injection of ANS.[11,12]

This paper reports observations concerning the fate of circulating guinea pig neutrophils and immature bone marrow neutrophils within a 24-hour period after intraperitoneal administration of ANS. The mechanism of action of ANS and its *in vivo* specificity are discussed in relation to the observations.

Materials and Methods

Collection of Neutrophils

Neutrophils to be used for immunization purposes were collected in peritoneal exudates from 500–600 g albino guinea pigs by a modification of the technic of Humphrey.[4] Exudates were stimulated by intraperitoneal injection of 40 ml of sterile 3% proteose peptone (Difco Labs, Detroit, Mich) in normal saline at 37 C; 15 hours later a second intraperitoneal injection of 15 ml of proteose peptone was given. Three hours after the second injection, the animals were exsanguinated by cardiac puncture and the peritoneal cavity was opened surgically. Approximately 40–50 ml of fluid present in the peritoneal cavity was removed with a pipet and mixed with an equal volume of heparinized normal saline. The peritoneal fluid from a single guinea pig contained from 5×10^8 to 10^9 total cells, 93–97% of which were neutrophils. Small numbers of macrophages, lymphocytes, erythrocytes and desquamated mesothelial cells were also present. The cells were sedimented by gentle centrifugation at $100 \times g$ for 10 minutes at 4 C. Erythrocytes were hemolyzed by briefly suspending the cells in 0.5% sodium citrate [2] and the cells were then washed twice in normal saline. After each washing, cells were collected

by centrifugation as described above and resuspended in fresh saline by gentle pipetting with a Pasteur pipet. After the final wash, the cells were resuspended in 4 ml of sterile isotonic saline and were again counted in a hemocytometer. The washing procedure was not deleterious to cell integrity since whole cells remained; they were used for injections without further treatment.

Preparation of ANS

Two pools of ANS were prepared using different immunization routes and schedules. Adult male New Zealand albino rabbits each weighing 3.5–4.5 kg were immunized with guinea pig neutrophils suspended in 0.85% saline either by intravenous injections without adjuvant (ANS I) or by subcutaneous injections with adjuvant (ANS II). For intravenous immunization, each of 2 rabbits received a series of six injections via the marginal ear vein at 4-day intervals; each injection consisted of approximately 3×10^8 neutrophils. One month later, a seventh injection of 3×10^9 neutrophils was given. For subcutaneous injections, each of 2 rabbits was injected three times at 1-week intervals with 4×10^8 neutrophils suspended in 2 ml of saline; at the first injection, 1 ml of Freund's complete adjuvant (Difco Labs) was mixed with the saline–cell suspension. At each weekly injection, the suspension was administered at four different subcutaneous sites, two anterior and two posterior, on the shaved backs of the rabbits.

The rabbits were exsanguinated by cardiac puncture 1 week after the final injections and the sera were pooled into two separate batches, ANS I and ANS II, according to the route of immunization. Serum was inactivated at 56 C for 30 minutes, absorbed as described below, sterilized by filtration through a double Millipore filter (0.45 and 0.22 μ pore size; Millipore Filter Corp, Bedford, Mass), dispensed in 2-ml vials and stored frozen at −20 C until further use.

Rabbit serum for controls was collected from previously untreated New Zealand rabbits and processed in the same manner.

Titration of Serum

In vitro activity of ANS against leukocytes was assessed by a modification of the leukoagglutination titration described by Steinberg and Martin.[13] Titers against neutrophils and lymphocytes were determined in serial twofold dilutions of ANS in 0.5 ml of 0.01 M phosphate-buffered normal saline (pH 7.2) set up in duplicate in small siliconized test tubes from 1:10 to 1:1280 dilution; 1.3×10^6 saline-washed neutrophils from peritoneal exudate or lymphocytes from minced mesenteric and cervical nodes were added to each tube as well as to control tubes containing appropriate dilutions of either NRS or saline. The tubes were then agitated and allowed to stand at room temperature for 1–2 hours prior to reading. Agglutination was assessed by phase-contrast microscopy at 250× after briefly shaking each tube and placing a drop of the cell suspension on a slide with a Pasteur pipet. The end point was taken as the highest dilution of ANS containing distinct clumps of cells, approximately 10 or more cells per clump, when compared to NRS control tubes which usually contained small aggregates of 3–4 cells as well as many isolated single cells.

ANS was examined for cytotoxic antibodies to neutrophils by adding 2.5×10^5 exudate neutrophils in 0.5 ml of Hanks' solution containing various dilutions of ANS or NRS to tubes containing 0.5 ml Hanks' solution with 10% inactivated autologous guinea pig serum and 10% fresh rabbit serum as a complement source. After incubation at 37 C for 30 minutes, a sample from each tube was diluted 1:10 with 0.2% trypan blue in saline and examined in a hemocytometer for cellular uptake of the dye.

Absorption of ANS

Red Cells. Hemagglutinins were eliminated in ANS by absorption with guinea pig red cells obtained by cardiac puncture and washed three times in normal saline prior to use. Two absorptions for 1 hour at room temperature with 5 ml of packed red cells/25 ml of ANS were found adequate to remove hemmagglutinating activity of both lots of serum.

Serum. Both pools of ANS were tested undiluted for precipitating antibodies to guinea pig serum by double diffusion in Ouchterlony agar-gel plates (Immunoplate, Hyland Labs) at room temperature for 48 hours. Antiserum antibody (present in ANS II only) was removed by absorbing with normal guinea pig serum (0.05 ml/ml of ANS) at room temperature for 1 hour with shaking, and the antigen-antibody complexes were then removed by centrifuging the serum at 30,000 *g* for 30 minutes at 4 C (Sorvall RC2-B Centrifuge). The ANS was then tested again by double diffusion to ensure that antibodies to serum had been removed.

Lymphocytes. A 5-ml aliquot of ANS I was absorbed three times for 1 hour at room temperature with 3×10^8 lymphocytes each time. Lymphocytes for absorptions were collected from mesenteric and cervical guinea pig lymph nodes that were sliced into small fragments with razor blades and pressed through a sterile 60-mesh stainless-steel wire screen; the cells were washed three times in Hanks' solution prior to use. After the absorptions, the titer against lymphocytes was repeated to be certain that lymphocyte-agglutinating activity had been removed. This serum was then administered to 3 guinea pigs and blood counts determined over a 24-hour period.

Neutrophils. To determine if neutrophil-specific antibody was present in ANS and could be removed by absorption, a 3-ml aliquot of ANS I was absorbed three times for 1 hour at room temperature with saline-washed peritioneal exudate neutrophils (95% neutrophils, 5% mononuclear leukocytes); 8×10^8 cells were used for each absorption. The neutrophil-agglutinating titer of this aliquot of serum was then repeated to determine if activity had been removed, and the serum was also tested for activity *in vivo*.

Experimental Animals

A series of 30 adult, albino guinea pigs each weighing 400–500 g were used for initial hematologic and microscopic studies. Half of these animals received 3 ml/kg intraperitoneally of ANS I; the other animals received NRS at the same dose level. Total and differential counts of white cells were determined just prior to injection of serum and immediately prior to sacrifice at given time intervals within a 24-hour period after ANS or NRS administration. Blood samples for counts and smears were obtained by severing capillaries at the periphery of the ear after application of toluene for vasodilatation. At the time of sacrifice, the animals were anesthetized with ether and tissue specimens removed for light and electron microscopy.

Two other groups of 3 animals each were injected with ANS I that had been absorbed with either lymphocytes or neutrophils, and blood counts were followed for 24 hours.

Another group of animals was injected with ANS II and blood counts determined at various time intervals up to 3 days after serum injection. In addition, other guinea pigs that had been injected with ANS II were sacrificed at various time intervals up to 24 hours and their tissues fixed for light and electron micros-

copy. The results obtained after injection of ANS II were then compared with those obtained in animals receiving ANS I.

Tissue Preparation

At intervals of 15–30 minutes, 1, 2, 6, 12 and 24 hours after ANS or NRS injection, animals were anesthetized with ether after the blood counts were determined and specimens of middle and left lateral lobes of liver, spleen, mesenteric lymph nodes, right lung and femoral marrow were prepared for light and/or electron microscopy. In some animals, buffy coat from cardiac blood and cells in peritoneal washings were also prepared for electron microscopy.

Light Microscopy. Tissues fixed in 10% neutral-buffered formalin were embedded in paraffin, sectioned at 5 μ, and stained with either hematoxylin-eosin or periodic acid–Schiff (PAS). Blood and marrow smears were air-dried, fixed in methanol and subsequently stained with Wright's or May-Grünwald-Giemsa stains.

For quantitative studies of neutrophil accumulation in organs, the neutrophils were enumerated in PAS-stained sections of liver and spleen by counting 20 random oil-immersion fields in each organ. In spleen, only red pulp was counted since neutrophil sequestration did not occur in follicles.

Electron Microscopy. Tissues were fixed in one of two ways: (1) Specimens were immersed in a mixture of 2.5% glutaraldehyde–2% paraformaldehyde [14] in 0.1 M cacodylate buffer (pH 7.2) containing 0.25 mg/ml anhydrous $CaCl_2$ (approximately 950 mOsm/kg) at room temperature for 5–6 hours, washed in 0.1 M cacodylate buffer containing 7.5% sucrose at 4 C for 24–48 hours and subsequently postfixed in 2% OsO_4 in 0.2 M s-collidine buffer at 4 C for 1 hour. (2) Occasional specimens were fixed initially in 2% OsO_4 in 0.2 M s-collidine buffer (pH 7.3) at 4 C for 1 hour, washed briefly with 0.2 M s-collidine buffer and postfixed for 1 hour at room temperature in 10% phosphate-buffered formalin (pH 7.4).

After fixation, the tissues were dehydrated and embedded in Epon.[15] Thick sections (1 μ) for light microscopy were stained with azure II–methylene blue.[16] Thin sections were mounted on parlodian-coated grids reinforced with carbon, and double-stained with 3% uranyl acetate [17] and lead tartrate.[18] They were examined in an AEI-6B eletron microscope at 60 kV.

Results

Titration of Unabsorbed and Absorbed ANS

In the unabsorbed state, both ANS I and ANS II contained agglutinating antibodies to neutrophils, lymphocytes and red cells. The titer of ANS I against neutrophils was 1:320; of ANS II, 1:640. Both antisera agglutinated lymphocytes at dilutions up to 1:80. These agglutinins could be eliminated by absorbing aliquots of the serum with the respective cell types. Absorption of ANS with lymphocytes did not change the titer against neutrophils.

Hemmagglutinin titers were 1:80 in both pools of ANS and were eliminated after absorbing the sera with erythrocytes.

Cytotoxic Antibodies

Both antisera contained antibodies cytotoxic for neutrophils in the presence of complement. At low dilutions of ANS (1:20–1:500), essentially all neutrophils took up trypan blue into their nuclei. Even at ANS dilutions as high as 1:2000, approximately 50% of the neutrophils did not exclude trypan blue, whereas no more than 8–10% of the neutrophils in control tubes were stained with trypan blue at any dilution of NRS.

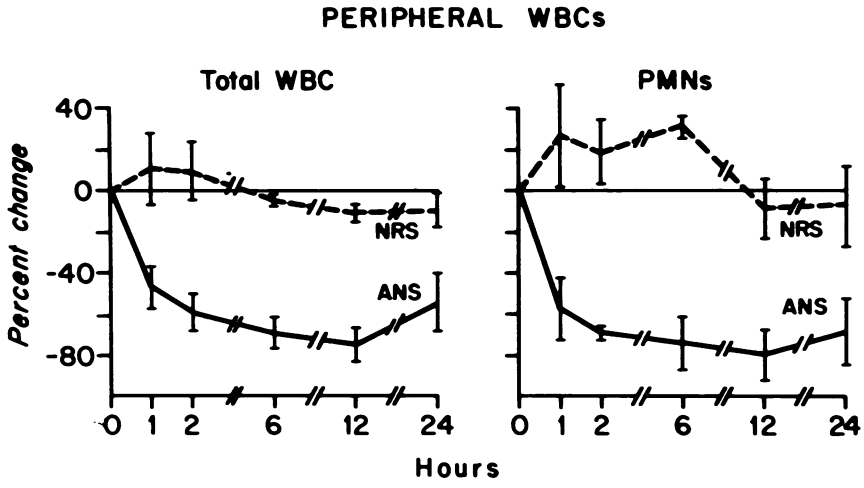
Hematologic Response to ANS and NRS

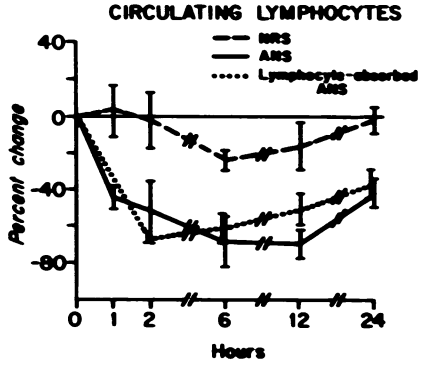
Neutrophils

A decrease in circulating neutrophils began within 15 minutes after ANS was injected intraperitoneally (Text-fig 1). A mean decrease of approximately 60% of the circulating neutrophils occurred within 1 hour. The lowest level of circulating neutrophils, approximately 80% below the starting level, was reached by 12 hours after antiserum injection. In contrast, peripheral neutrophils increased by approximately 30% at 6 hours after NRS injection, after which they tended to return to starting levels. In individual animals injected with ANS I, absolute neutrophil counts at 12 hours ranged from a low of 330 cells/cu mm to a high of 2500 cells/cu mm, representing decreases of 96 and 56%, respectively. In other animals receiving slightly greater doses of ANS (increases of 0.1–0.2 ml/dose), virtually absolute neutropenia could be produced within 12 hours after antiserum administration. No differences were observed in the peripheral blood response to ANS I and ANS II.

Variable numbers of immature nonsegmented neutrophils (band cells) appeared in the circulation between 6 and 24 hours after injection of ANS. This response varied from animal to animal and has been reported to vary with the dose of ANS.[3] The effect of ANS on platelets was not measured; however, large numbers of platelets were present in blood smears and qualitatively their distribution did not appear to be different from that of platelets from NRS-treated animals. Microhematocrits at 24 hours after ANS indicated that no anemia had occurred. Circulating monocytes appeared to be decreased by approximately 50–70% within 12 hours after ANS injection, but monocytes were difficult to quantitate due to the frequent appearance in smears of large cells with morphology indistinguishable from that of monocytes and large lymphocytes. Absolute numbers of monocytes were at or above original levels by 2 or 3 days after ANS.

The guinea pigs manifested few signs of reaction to ANS administration except for lethargy and decreased appetite within the first 6 hours.





TEXT-FIG 1—Changes in peripheral white blood cells after intraperitoneal administration of antineutrophil serum (ANS I) or normal rabbit serum (NRS), plotted as percent change from the preinjection level. Each point represents a mean derived from measurements from 3 animals and the vertical bars represent ±1 SE.

Thereafter, their appearance was similar to controls receiving NRS. At no time was shock observed.

Effects of Absorption with Lymphocytes or Neutrophils

The activity of ANS was eliminated by absorbing with neutrophils. This was reflected in a depletion of the neutrophil-agglutinating activity of the serum *in vitro* as well as in the hemotologic response *in vivo*. The peripheral neutrophil counts of guinea pigs that received the neutrophil-absorbed ANS showed a substantial early increase, but by 12 hours

after ANS these counts were essentially the same as those of controls that received NRS.

The number of circulating lymphocytes decreased by nearly 70% in the first 24 hours after injection of both unabsorbed and lymphocyte-absorbed ANS. Even though the lymphocyte-agglutinating activity had been removed from the lymphocyte-absorbed ANS, no differences were observed in the hematologic responses of the two groups of guinea pigs (Text-fig 1). The decrease in circulating lymphocytes produced by ANS was relatively brief since lymphocyte counts were only approximately 20% below the original levels 48 hours after antibody injection. By 3 days, lymphocytes had nearly reached original levels.

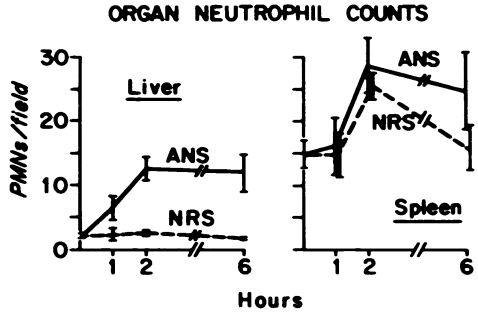
Effect upon Bone Marrow

Within 6–12 hours after ANS injection, the bone marrow demonstrated severe depletion of both mature segmented PMNs and band cells (Fig 1A). By 24 hours after ANS, the marrow exhibited a distinct shift to the left, in which myelocytes, metamyelocytes and band forms predominated. In contrast, no changes were observed in animals receiving NRS (Fig 1B).

Fate of PMNs in Liver, Spleen and Lymph Nodes

Mature neutrophils began to accumulate in the sinusoids of liver and spleen within minutes after ANS injection. One hour after ANS, a significantly greater number of PMNs was present within liver sinusoids of ANS-treated guinea pigs than of NRS-treated controls, which showed no rise in liver PMNs (Text-fig 2). Neutrophil sequestration in liver reached a peak within 2 hours after ANS injection and appeared to remain constant thereafter. In contrast, both ANS and NRS caused an apparent increased accumulation of PMNs in the spleen where neutrophils reached a maximum after 2 hours and appeared to decrease thereafter.

Neutrophils that accumulated in liver and spleen after ANS were phagocytized by Kupffer cells and red-pulp macrophages, respectively (Fig 2A and B). Phagocytosis of neutrophils by macrophages in paracortical sinuses of lymph nodes was occasionally observed after ANS injection, but neutrophil ingestion in lymph nodes varied widely and was not observed in tissue sections from every animal treated with ANS, in contrast to liver and spleen. Phagocytosis of neutrophils in lymph nodes was a minor phenomenon in terms of the total number of neutrophils ingested in individual animals, and is probably a reflection of the



TEXT-FIG 2—Graphs demonstrating the accumulation of neutrophils in liver and spleen after ANS I or NRS administration. Sequestration of neutrophils in liver after ANS administration begins early and increases rapidly. Both ANS and NRS administration result in neutrophil accumulation in the spleen. After NRS, neutrophils in spleen gradually decrease in number, although there was no evidence that they were phagocytized by splenic macrophages. In contrast, in ANS-treated animals neutrophils were phagocytized by macrophages and rapidly digested. Each point represents the mean from 3 animals and vertical bars represent ±1 SE.

small number of neutrophils normally circulating through the lymph nodes.

Even though circulating lymphocytes decreased substantially after ANS injection, lymphocytes were never observed to accumulate in spleen or liver sinusoids, as did neutrophils, nor were lymphocytes phagocytized by macrophages in spleen, liver or bone marrow. Only in several cases was a macrophage containing an ingested lymphocyte observed in lymph nodes from animals receiving ANS not absorbed with lymphocytes, and in these cases phagocytosis of lymphocytes was not extensive. No depletion of small lymphocytes was observed in paracortical areas of lymph nodes, as has been described in animals treated with antilymphocyte serum.[19-21]

Neutrophils within phagocytic vacuoles in macrophages could be readily identified in 1-μ sections soon after phagocytosis had occurred (Fig 2A and B). As digestion of PMNs proceeded, identification of neutrophils within macrophages became increasingly difficult, especially in paraffin sections. Consequently, the curves demonstrating neutrophil accumulation in liver and spleen after ANS injection (Text-fig 2) probably underestimate the true magnitude of this process, especially at the time (2–6 hours) when digestion of ingested neutrophils is well advanced and partially digested cells can only be identified ultrastructurally.

Macrophages in the spleen usually contained several neutrophils (Fig 2B and 8). Numerous PMNs in various stages of digestion were often observed within a single-membrane-bounded vacuole, and spleen macrophages with as many as five or six ingested neutrophils were commonly

observed within 2–6 hours after ANS. On the other hand, Kupffer cells commonly contained one or two neutrophils which usually were present in individual membrane-bounded structures (Fig 2A and 9).

Within 24 hours after ANS, phagocytosis and digestion of neutrophils was essentially complete, although occasional macrophages still contained ingested neutrophils in a relatively early stage of digestion. After 24 hours, both splenic macrophages and Kupffer cells often contained whorls of membranes, presumably residual bodies representing the end stage of neutrophil digestion (Fig 10).

Phagocytosis of Neutrophils in Bone Marrow

Many immature neutrophils were ingested by macrophages within the marrow of ANS-treated animals (Fig 3 and 4). Neutrophil precursors as immature as early myelocytes were observed within phagocytic vacuoles in macrophages between and adjacent to marrow sinusoids. The ingested cells could be identified as neutrophil precursors by the presence of azurophil and, in more mature cells, specific granules in their cytoplasm (Fig 3). Often azurophil granules in ingested immature cells were in the process of condensation after budding from Golgi vesicles as "nucleoid" forms, as described in progranulocytes of rabbit marrow by Bainton and Farquhar.[22] Prior to digestion, many of the ingested immature cells contained abundant rough endoplasmic reticulum, mitochondria, large oval nuclei and specific granules in variable numbers according to the stage of cell maturity. No phagocytosis of cells was observed in marrow from NRS-treated animals. Phagocytosis of neutrophil precursors in bone marrow was most prominent 12 hours after ANS and was not observed earlier than 2 hours after injection of the antiserum. No phagocytosis of cells other than those of the neutrophil series was observed.

Ultrastructural Changes in Neutrophils

Prior to or immediately after ingestion, some neutrophils in spleen and liver from ANS-treated animals had an altered appearance consisting of cytoplasmic pooling or clumping of glycogen. These glycogen accumulations were particularly evident after aldehyde fixation in cacodylate buffer since they did not tend to be stained under these circumstances and appeared as large areas of low density within the cytoplasm (Fig 5 and 6). Glycogen in PMNs of NRS-injected guinea pigs frequently existed as discrete deposits up to 1 μ in diameter or was evenly dispersed throughout the cytoplasm as small granules of low density (Fig 7), and was never observed in accumulations as large as those occasionally seen in neutrophils from ANS-treated animals.

Digestion of phagocytized neutrophils occurred rapidly and was initially manifested by a coalescence of nuclear chromatin into a homogeneous, dense mass. Condensation of cytoplasm caused progressive crowding of azurophil and specific granules (Fig 9). The neutrophil plasmalemma generally appeared to be intact during these early degradative changes and was closely adapted to the vacuolar membrane. As digestion progressed, whorls of membranes appeared concomitant with the disappearance of the cytoplasmic granules; simultaneously, nuclear debris tended to become dispersed throughout the vacuole (Fig 10).

Effects of ANS on Neutrophils in the Peritoneal Cavity

Ultrastructural examination of the cell population present in the peritoneal cavity 6 hours after injection of ANS revealed the presence of numerous damaged neutrophils. Injection of the serum caused an increased number of neutrophils to appear in the peritoneal cavity. Many of these cells were clumped in groups of three to five cells, some of which demonstrated obvious lytic changes consisting of absence of portions of the plasma membrane, escape of granules from the cell, dispersion of nuclear contents and decreased density of the cytoplasmic matrix. Free neutrophil granules and membranous debris were present in the intercellular spaces. Other cells present in the exudate included macrophages and eosinophils, all of which appeared intact. Some of the macrophages contained ingested neutrophil debris.

Discussion

The hematologic effects of ANS demonstrated in this study are in general agreement with the observations of previous investigators. The neutropenia resulting from a single injection of ANS reaches a maximum in 24 hours and may persist up to 7 days or longer, during which a gradual reappearance of neutrophils occurs.[2,4,6,7,23,24] The shift to the left in bone marrow and the appearance of immature neutrophils in the circulation after a single injection of ANS have also been documented previously.[2,6,12] Intravenous or intracardiac routes of ANS administration generally produce similar effects, although the neutropenia is immediate in onset and may be accompanied by shock.[3,11,23]

Effects on Neutrophils

Opsonization

The mechanism by which ANS promotes phagocytosis of neutrophils *in vivo* is of interest since it has not been clear whether the participation of complement is necessary for the opsonizing effect of the antiserum

upon neutrophils or whether cytotoxic injury occurred prior to ingestion. We observed no morphologic evidence of cytotoxicity of ANS on circulating neutrophils prior to ingestion, except for the occasional cytoplasmic accumulations of glycogen described above. The significance of these extensive glycogen deposits is unknown; however, such large accumulations of glycogen were not observed in neutrophils in NRS-treated guinea pigs. The neutrophils that demonstrated the extensive glycogen clumping were always within spleen or liver sinusoids. They were usually adjacent to macrophages, or in some phase of ingestion or digestion by macrophages (Fig 5 and 6). Glycogen accumulation in the neutrophils may be a manifestation of altered metabolic activity which has been demonstrated to occur *in vitro* following ANS treatment of neutrophil suspensions.[25]

The ultrastructural appearance of neutrophils prior to ingestion suggests that intravascular ANS acts as an opsonin and promotes phagocytosis of neutrophils without cytotoxic damage. Apparently, the extent of this opsonizing effect of ANS *in vivo* has not been previously recognized. It has been reported that ANS-treated neutrophils are phagocytized by macrophages *in vitro,* but this reaction was said to require complement.[9] Several investigators have demonstrated that large numbers of lymphocytes are ingested by macrophages in liver, spleen, and lymph nodes after ALS treatment[21,26,27]; the lymphocytes undergoing phagocytosis appear by ultrastructural characteristics to be uninjured cells.[26] In addition, other investigators have demonstrated an opsonization of ascites tumor cells by cytotoxic isoantibody both *in vitro*[28] and *in vivo*[29]; in both of these studies, the tumor cells appeared uninjured by the antibody, as assessed by ultrastructural criteria[29] and by ability to exclude trypan blue.[28] Thus, fundamental differences seem to exist in the effects of antibodies on cells *in vitro* and *in vivo,* and the extent of participation of complement in these events is poorly understood.[30]

Neutrophil thrombi have been reported to occur in lung capillaries after intracardiac or intravenous injection of ANS.[11,12] Also, thrombi of degenerating lymphocytes have been observed in postcapillary venules after intraarterial infusion of ALS.[8] We saw no evidence of intravascular leukocyte agglulination or thrombus formation after intraperitoneal injection of ANS.

Cytotoxicity

Antibody cytotoxic for neutrophils in the presence of complement was clearly present in these preparations of ANS as demonstrated by the failure of neutrophils to exclude trypan blue in the cytotoxicity tests. On this basis, one might expect to see injured or lysed neutrophils within

the circulation, or debris from injured neutrophils being ingested by reticuloendothelial macrophages within several hours after ANS injection when large numbers of these cells are disappearing from the peripheral circulation. In this regard, other investigators [31] have recently described the ultrastructural features of the complement-dependent lysis of human neutrophils in whole blood incubated *in vitro* with rabbit anti-human neutrophil antiserum.

Although we observed degenerating and lysed neutrophils in electron micrographs of peritoneal cells obtained 6 hours after intraperitoneal ANS injection, we did not observe similarly injured neutrophils or debris from degenerating neutrophils in intravascular sites at any time interval in animals given ANS intraperitoneally. A possible explanation for the lack of intravascular neutrophil lysis may be the decreased complement levels that have been reported to occur in the blood after both ANS and ALS injection. Previous investigators have claimed that complement is transiently depleted by up to 50% within 4 or 5 hours after antiserum injection.[32,33] Our observations indicate that the phagocytic activity reaches a peak by approximately 6 hours after antiserum injection. If complement is essential to the mechanism for clearance of circulating neutrophils after ANS treatment, it is possible that the affected cells may maintain some degree of transient resistance to a surface injury from small numbers of antibody-complement complexes, possibly through repair of a membrane defect, or the affected neutrophils may be ingested so rapidly that cytolysis does not become manifest prior to removal of the cells from the circulation. Alternatively, complement might be necessary for adherence and ingestion but not involved in cytolysis.

Effects on Immature Neutrophils

The observation that immature neutrophils as young as early myelocytes are ingested by macrophages in bone marrow is of interest since it suggests that at this stage of development the cell has acquired one or more of the unique surface antigens characteristic of the mature segmented neutrophil. We did not observe any degenerating or lysed mature or immature neutrophils in the bone marrow. Thus, it would appear that ingested bone marrow neutrophils are also opsonized by the antiserum.

Effects on Lymphocytes

A moderate to marked depletion of circulating lymphocytes has been consistently observed after ANS administration,[2,3,6,7,11,23,24] even after the injection of sera that have been exhaustively absorbed with erythrocytes, spleen cells, and liver and kidney cells.[4] Most investigators have

not emphasized this observation, presumably because it is either poorly understood or because it is thought to result from cross-reacting antibodies. The results of agglutination and cytotoxicity tests generally confirm that, indeed, cross-reactivity of ANS with lymphocytes occurs *in vitro,* although the titers against lymphocytes are much lower than those obtained against neutrophils.

Although we observed a maximum mean depletion of nearly 70% of the circulating lymphocytes by 12 hours after ANS injection, several observations tend to suggest that the cross-reactivity of ANS with lymphocytes occurs to only a minor extent after intraperitoneal injection *in vivo.* The strongest evidence against extensive cross-reactivity is the observation that lymphocyte depletion occurred to same extent in guinea pigs injected with lymphocyte-absorbed ANS as in those injected with unabsorbed antiserum. The lymphocyte-absorbed ANS was treated extensively with lymphocytes and the lymphocyte-agglutinating activity was removed, so it seems unlikely that lymphocyte antibody remained. Further evidence against extensive cross-reactivity against lymphocytes is the fact that no lymphocytes were observed in macrophages at any of the time intervals examined in spleen, liver or bone marrow, even in animals receiving ANS not absorbed with lymphocytes, despite the fact that large numbers of neutrophils were ingested in all of these organs throughout the 24-hour period after ANS injection. In contrast, injection of heterologous antilymphocyte serum (ALS) has been shown to result in phagocytosis of large numbers of lymphocytes by macrophages in spleen, liver and lymph nodes.[21,26] In addition, when lymphopenia occurs after ALS administration, its duration is usually of the order of 2–4 weeks.[19,34] The lymphopenia after ANS is of much shorter duration since lymphocyte levels were approximately at original levels by the third day after antiserum administration in this study. Hence, our data suggest that either little cross-reactivity of ANS against circulating lymphocytes occurred after intraperitoneal injection *in vivo,* or that cross-reactivity against lymphocytes is of a different nature in that it is manifested by neither phagocytosis nor sustained lymphopenia as observed after injection of antilymphocyte serum.[19,26,34]

Injecting NRS into guinea pigs has also been shown to cause a transient decrease in circulating lymphocytes lasting 24–48 hours.[7,35] In this study, a maximum decrease of 24% in circulating lymphocytes occurred by 6 hours after NRS injection; original levels were almost restored by 24 hours. Even though the lymphocyte depletion after ANS is usually much greater than that seen after NRS injection, the general shapes of the disappearance and reappearance curves are similar. The lymphocyte decrease after NRS injection may possibly be a manifestation of an im-

mune response to the serum or a reaction to a form of stress; it seems likely that the lymphocyte decrease after ANS may result, at least in part, from the same factors.

Specificity of the Antiserum

Although numerous investigators have previously demonstrated that heteroimmune sera can be produced not only against neutrophils but also against lymphocytes,[36] mast cells,[37] platelets[38,39] and macrophages,[40] the biologic effects of these anisera have never been fully characterized. It is presumed that the primary effect of these antisera results from antibodies formed against cell-specific antigenic sites on the immunizing cells. In most instances, however, the effects of heterologous antisera do not seem to be limited to the single cell type used as immunogen, either *in vitro* or *in vivo*.

As pointed out by Medawar,[41] immunization of an animal with xenogeneic leukocytes presumably causes the production of antibodies to a wide variety of intracellular and surface antigenic sites, including histocompatibility antigens and cell-specific antigens. It seems likely that the effects of a given antileukocyte serum *in vivo* would depend to a large extent on the concentration of leukocyte-specific antibodies in relation to the concentration of irrelevant antibodies. Despite the presence of irrelevant antibodies in antileukocyte sera, the apparent marked *in vivo* specificity of the ANS used in this study is striking. The cells that were phagocytized by hepatic and splenic macrophages were exclusively neutrophils. There was no evidence of intravascular degeneration of any cell type, including neutrophils. Thus the effects of ANS *in vivo* appear to result primarily from opsonization of circulating neutrophils, with the resulting neutropenia due to phagocytosis of opsonized cells largely in liver and spleen. A possible explanation for the striking selectivity of ANS *in vivo* may relate to the route of injection. After intraperitoneal injection, it seems probable that many of the irrelevant, cross-reacting antibodies may be removed by reacting with the resident leukocytes and other cells and organs in the peritoneal cavity. By the time the antiserum reaches the circulation it may have had the benefit of an "absorption" *in vivo*, and the antibody molecules that remain would be primarily active against neutrophils. This hypothesis is supported by the experiments of Shigeno and colleagues[42] who were able to make a lymphocyte-specific antiserum by absorption of heterologous ALS *in vivo* after intraperitoneal injection into mice syngeneic with the mice from which the immunizing lymphocytes were originally obtained; this procedure removed cytotoxic H-2 antibody and presumably would remove other alloantibodies present in ALS.

References

1. Metchnikoff E: Etudes sur la resorption des cellules. Ann Inst Pasteur 13:737, 1899
2. Lawrence JS, Craddock CG, Jr, Campbell TN: Antineutrophilic serum, its use in studies of white blood cell dynamics. J Lab Clin Med 69:88–101 1967
3. Patt HM, Maloney MA, Jackson EM: Recovery of blood neutrophils after acute peripheral depletion. Amer J Physiol 188:585–592, 1957
4. Humphrey JH: The mechanism of Arthus reactions. II. The role of polymorphonuclear leukocytes and platelets in reversed passive reactions in the guinea pig. Brit J Exp Path 36:283–289, 1955
5. Cochrane CG, Unanue ER, Dixon FJ: A role of polymorphonuclear leukocytes and complement in nephrotoxic nephritis. J Exp Med 122:99–116, 1965
6. Cajano A, Maurea C [Anticell and antiorgan sera.] Haematologica. 34:1133–1179, 1950
7. Finch SC, Cajano A, Ross JF: Leukocyte response to leukocyte nuclear and cytoplasmic antisera. J Lab Clin Med 46:871–878, 1955
8. Anderson ND, Wood S Jr: Intravascular thrombosis and leukocyte destruction *in vivo* by heterologus antilymphocyte serum. Fed Proc 29:145–149, 1970
9. Finch SC, Ross JF, Ebaugh FG: Immunologic mechanisms of leukocyte abnormalities. J Lab Clin Med 42:555–569, 1953
10. Miescher P: The antigenic constituents of the neutrophilic leukocyte with special reference to the L.E. phenomenon. Vox Sang 2:145–158, 1957
11. Moeschlin S, Meyer H, Israels LG, Tarr-Gloor E: Experimental agranulocytosis: its production through leukocyte agglutination by antileukocytic serum. Acta Haemat 11:73–94, 1954
12. Miescher P: Leukocyte antibodies, The Physiology and Pathology of Leukocytes. Edited by H Braunsteiner, D Zucker-Franklin. New York, Grune & Stratton, Inc, 1962, pp 232–235
13. Steinberg RA, Martin RA: Factors influencing leukoagglutination by antileukocytic sera. J Immun 51:421–426, 1945
14. Karnovsky MJ: A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol 27:137A, 1965, abstr
15. Luft JH: Improvements in epoxy resin embedding methods. J Biophys Biochem Cytol 9:409–414, 1961
16. Richardson KC, Jarrett L, Finke EH: Embedding in epoxy resins for ultrathin sectioning in electron microscopy. Stain Techn 35:313–323, 1960
17. Watson ML: Staining of tissue sections for electron microscopy with heavy metals. J Biophys Biochem Cytol 4:475–478, 1958
18. Millonig G: A modified procedure for lead staining of thin sections. J Biophys Biochem Cytol 11:736–739, 1961
19. Taub RN, Lance EM: Histopathological effects in mice of heterologous antilymphocyte serum. J Exp Med 128:1281–1297, 1968
20. Turk JL, Willoughby DA: Central and peripheral effects of antilymphocyte sera. Lancet 1:249–251, 1967
21. Tyler RW, Everett NB, Schwarz MR: Effect of antilymphocyte serum on rat lymphocytes. J Immun 102:179–186, 1969
22. Bainton DF, Farquhar MG: Origin of granules in polymorphonuclear leukocytes: two types derived from opposite faces of the Golgi complex in developing granulocytes. J Cell Biol 28:277–301, 1966

23. Chew WB, Stephens DJ, Lawrence JS: Antileucocytic serum. J Immun 30: 301–318, 1936
24. Stuart AE: Properties of antileukocytic serum. Brit J Exp Path 43:614–620, 1962
25. Kritzman J, McCarthy JS: Effects of heterologous antileukocyte serum on leukocyte metabolism. J Immun, 92:299–304, 1964
26. Everett NB, Schwarz MR, Tyler RW, Perkins WD: Observation relative to the mechanism of action of antilymphocyte serum. Fed Proc 29:212–219, 1970
27. Martin WJ, JFAP Miller: Cell to cell interaction in the immune response. IV. Site of action of antilymphocyte globulin. J Exp Med, 128:855–874, 1968
28. Bennett B, Old LJ, Boyse EA: Opsonization of cells by isoantibody *in vitro*. Nature 198:10–12, 1963
29. Journey LJ, Amos DB: An electron microscope study of histiocytic response to ascites tumor homografts. Cancer Res, 22:998–1001, 1962
30. Möller G, Möller E: Immune cytotoxicity and immunological enhancement in tissue transplantation, Antibodies to Biologically Active Molecules. Proceedings of the Second Meeting of Federation of European Biochemical Societies. Edited by B. Cinader. Oxford, Pergamon Press, 1967, p 362
31. Glick AD, Horn RG, Collins RD, Bryant RE: An electron microscopic study of human polymorphonuclear leukocyte injury induced by rabbit antiserum. Exp Molec Path 12:275–285, 1970
32. Waksman BH, Arbuys S, Arnason B: The use of specific "lymphocyte" antisera to inhibit hypersensitive reactions of the "delayed" type. J Exp Med 114:997–1022, 1961
33. Willoughby DA, Coote E, Turk JL: Complement in acute inflammation. J Path 97:295–305, 1969
34. Agnew HD: The effect of heterologous antilymphocytic serum on the small lymphocyte population of rats. J Exp Med 128:111–119, 1968
35. Chew WB, Lawrence JS: Antilymphocytic serum. J Immun 33:271–278, 1937
36. Ciba Foundation Study Group No. 29. Antilymphocytic Serum. Edited by GEW Wolstenholme, M O'Connor. Boston, Little, Brown and Company, 1967
37. Valentine MD, Bloch KJ, Austen KF: Mechanisms of immunologic injury of rat peritoneal mast cells. III. Cytotoxic histamine release. J Immun 99:98–110, 1967
38. Humphrey JH: Origin of blood platelets. Nature 176:38, 1955
39. Henson PM: Release of vasoactive amines from rabbit platelets induced by antiplatelet antibody in the presence and absence of complement. J Immun 104:924–934, 1970
40. Unanue ER: Properties and some uses of anti-macrophage antibodies. Nature 218:36–38, 1968
41. Medawar PB: Biological effects of heterologous antilymphocyte sera, Human Transplantation. Edited by FT Rapaport, J Dausset. New York, Grune & Stratton, 1968, pp 501–509
42. Shigeno N, Hammerling U, Arpels C, Boyse, EA, Old LJ: Preparation of lymphocyte-specific antibody from anti-lymphocyte serum. Lancet 2:320–323, 1968

The authors would like to thank Mrs. Claire Haney and the personnel of the Electron Microscopy Laboratory for skilled technical assistance. We also thank Dr. David Lagunoff for helpful suggestions and for critically reading the manuscript.

Fig 1 and 2 are 1 μ sections of Epon-embedded tissue stained with Azure II–methylene blue.

Fig 1—Bone marrow from animals receiving either (**A**) ANS I or (**B**) NRS, 12 hours previously. Note the decrease in segmented neutrophils and band cells in **A** compared with **B**. Arrows in **A** point to areas where macrophages have phagocytized neutrophil precursors. Arrows in **B** point to several of the mature neutrophils (× 520).

Fig 2—Liver and spleen 6 hours after ANS I administration. **A**—the presence of numerous neutrophils in the hepatic sinusoids. Some are free in the sinusoids while others have been ingested by Kupffer cells and are in various stages of degradation (arrows). **B**—Numerous neutrophils are characteristically found in phagocytic vacuoles in spleen red-pulp macrophages (*arrows*) at this time interval. Note that some cells within phagocytic vacuoles have been digested beyond recognition while other cells in the same vacuole are clearly recognizable as neutrophils which were presumably recently phagocytized (× 1320).

Fig 3–10 are electron micrographs of tissue fixed in glutaraldehyde-paraformaldehyde and then in osmium, with the exception of Fig 8 and 10, which were fixed in osmium. All were stained with uranyl acetate and lead.

Fig 3—Early neutrophil myelocyte within a phagocytic vacuole of a bone marrow macrophage (*M*) from a guinea pig injected 6 hours previously with ANS I. Note in the myelocyte the preponderance of large, dense azurophil granules characteristic of this stage of development of the cell and the abundance of rough endoplasmic reticulum (*er*). Very little degradation of the myelocyte has occurred and the plasma membrane appears to be intact at most areas around the periphery of the phagocytic vacuole (× 14,250).

Fig 4—Bone marrow macrophage containing at least two partially digested neutrophil precursors 6 hours after ANS administration. The ingested cell above the macrophage nucleus contains several partially formed azurophil granules and material resembling strands of incompletely digested endoplasmic reticulum. The cell below the macrophage nucleus appears to be a somewhat more mature neutrophil in a more advanced stage of degradation (× 10,900).

Fig 5—This micrograph illustrates a neutrophil apparently being phagocytized by a macrophage in a hepatic sinusoid 2 hours after ANS administration. A large accumulation of cytoplasmic glycogen (*g*) in the neutrophil is evident adjacent to a portion of the cell that has already been enclosed by processes from the macrophage. Several azurophil granules adjacent to the glycogen accumulations have small surface blebs oriented toward the glycogen deposit (*arrows*)(× 9500).

Fig 6—This micrograph demonstrates a splenic red-pulp macrophage (*M*) that has ingested a neutrophil 1 hour after ANS. No degradative changes are yet apparent in the neutrophil, but the cell demonstrates an extreme accumulation of cytoplasmic glycogen (*g*) (× 14,000).

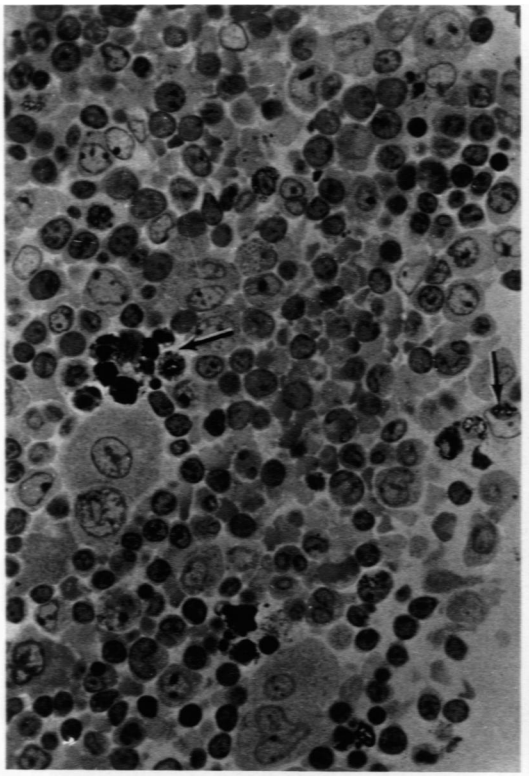
Fig 7—A neutrophil from a control guinea pig 6 hours after the intraperitoneal injection of normal rabbit serum (NRS). This neutrophil, which is present within a hepatic sinusoid, illustrates the characteristic features of the mature gunea pig neutrophil—*eg*, two different populations of granules, a segmented nucleus and scattered small deposits of glycogen. Glycogen deposits several times this size are often found in control neutrophils, but accumulations as large as those seen in Fig 5 and 6 were not observed in controls (× 10,000).

Fig 8—A spleen macrophage engorged with neutrophils in different stages of digestion within a single phagocytic vacuole 12 hours after ANS administration. The macrophage nucleus (*N*) is present at the cell periphery and is closely adapted to the phagocytic vacuole. A thin rim of macrophage cytoplasm surrounds the vacuole and arrows delineate the vacuole periphery. (× 7500).

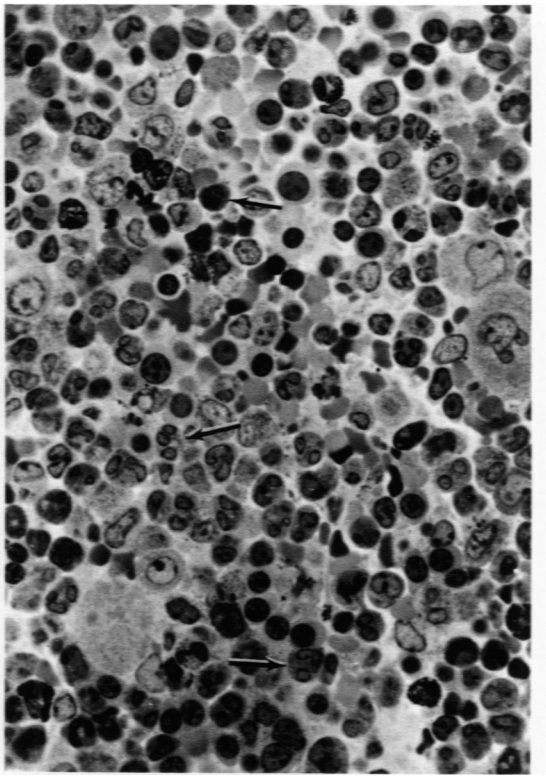
Fig 9—Six hours after ANS, a Kupffer cell containing a single, partially digested neutrophil is seen in this illustration. Note the crowding of neutrophl granules after cytoplasmic condensation and the decreased density of many of the granules (× 9300).

Fig 10—This phagocytic vacuole in a Kupffer cell illustrates an advanced stage of degradation of a cell presumed to be a neutrophil. Cytoplasmic granules and plasma membrane have disappeared and numerous whorls of membranous material are seen throughout the vacuole. Numerous vacuoles containing cells in this stage of degradation were seen in Kupffer cells and spleen macrophages 12 to 24 hours after ANS administration (× 22,100).

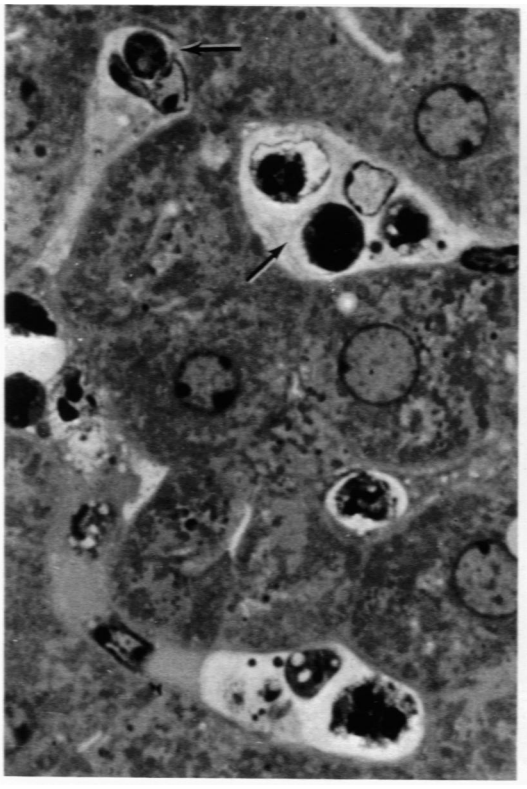
1A



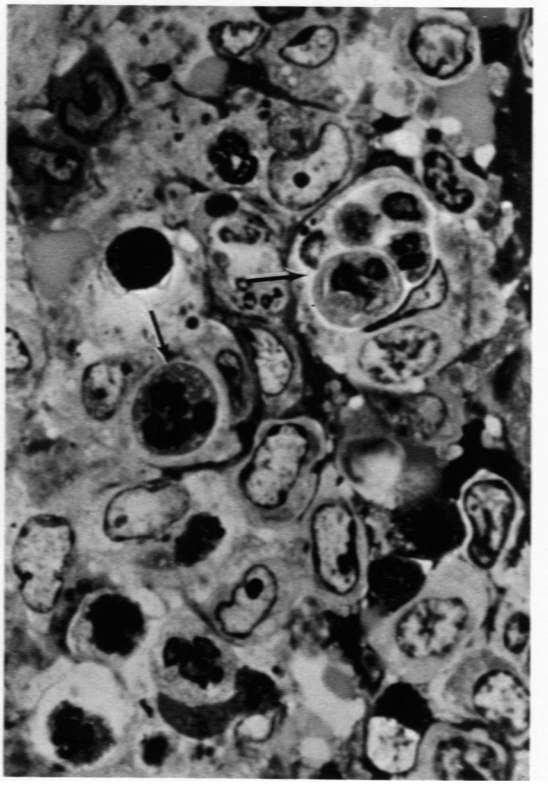
1B

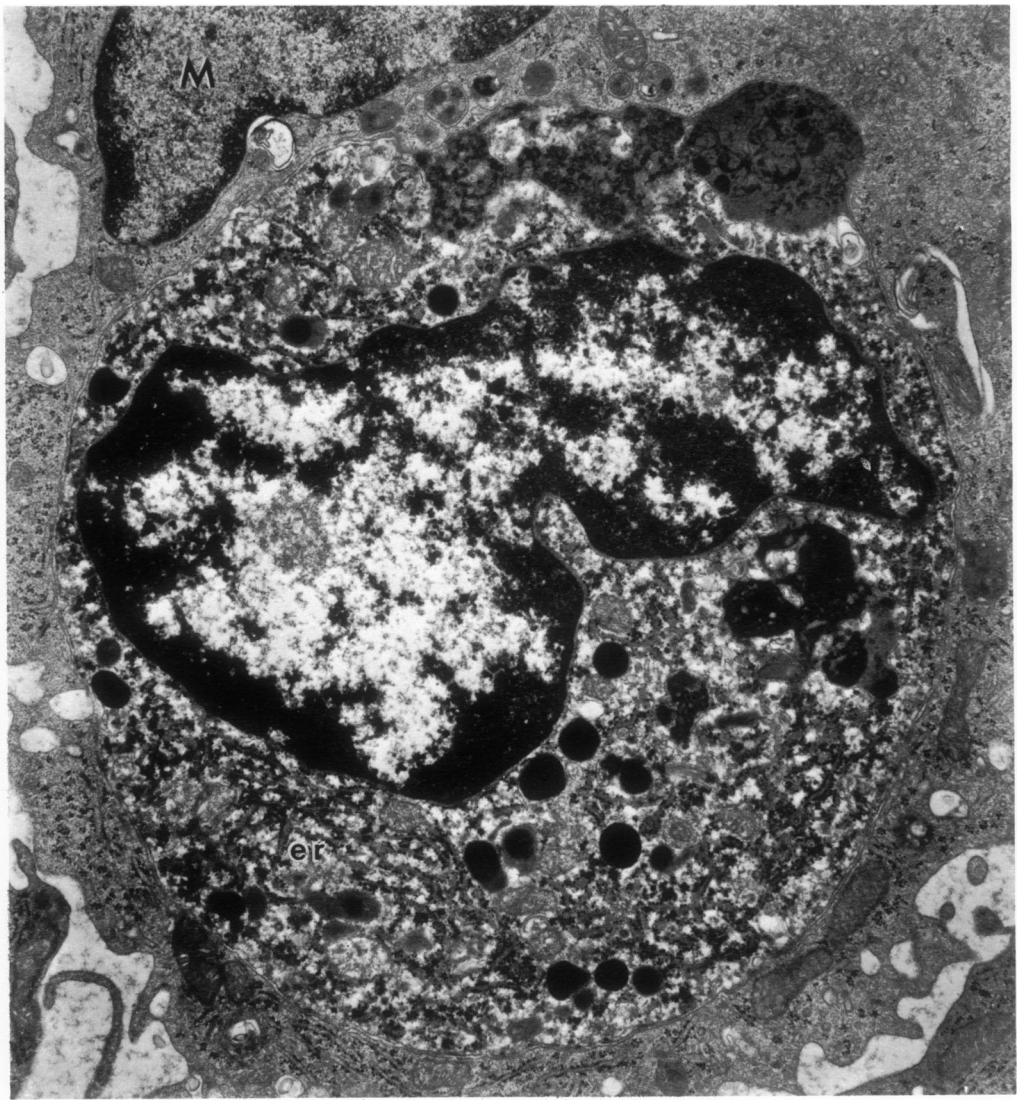


2A

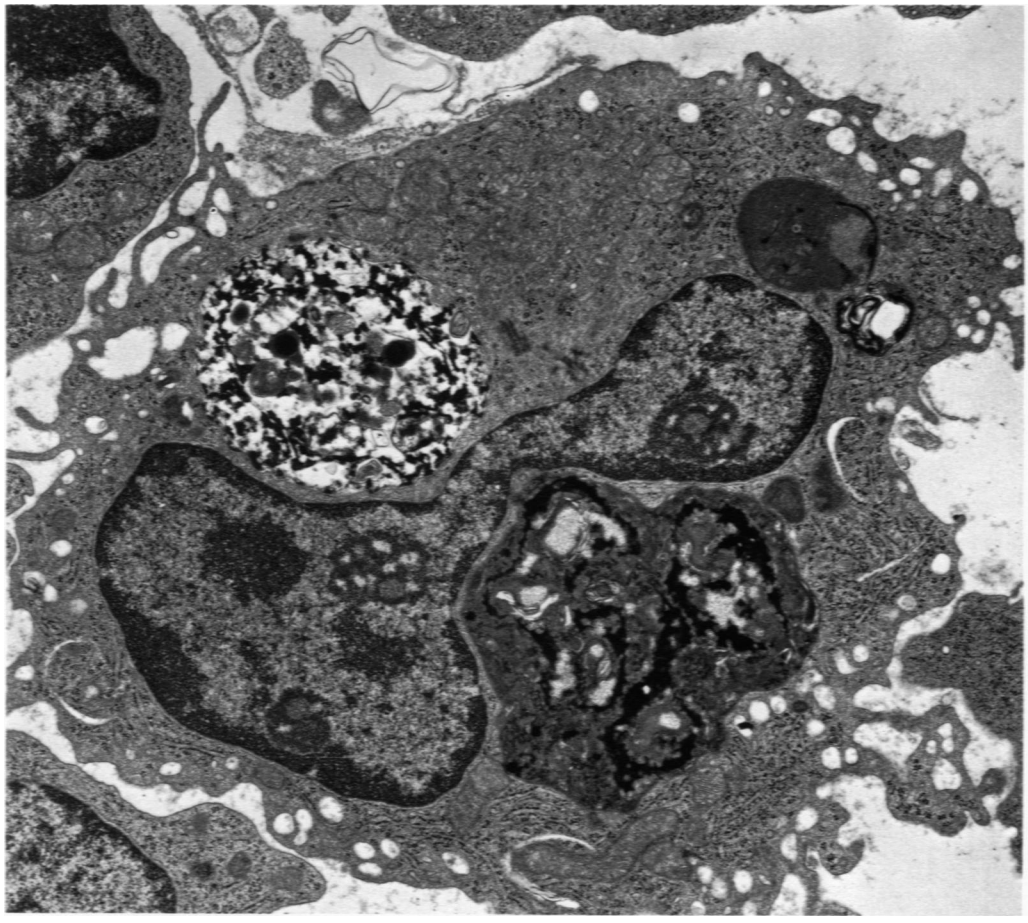


2B

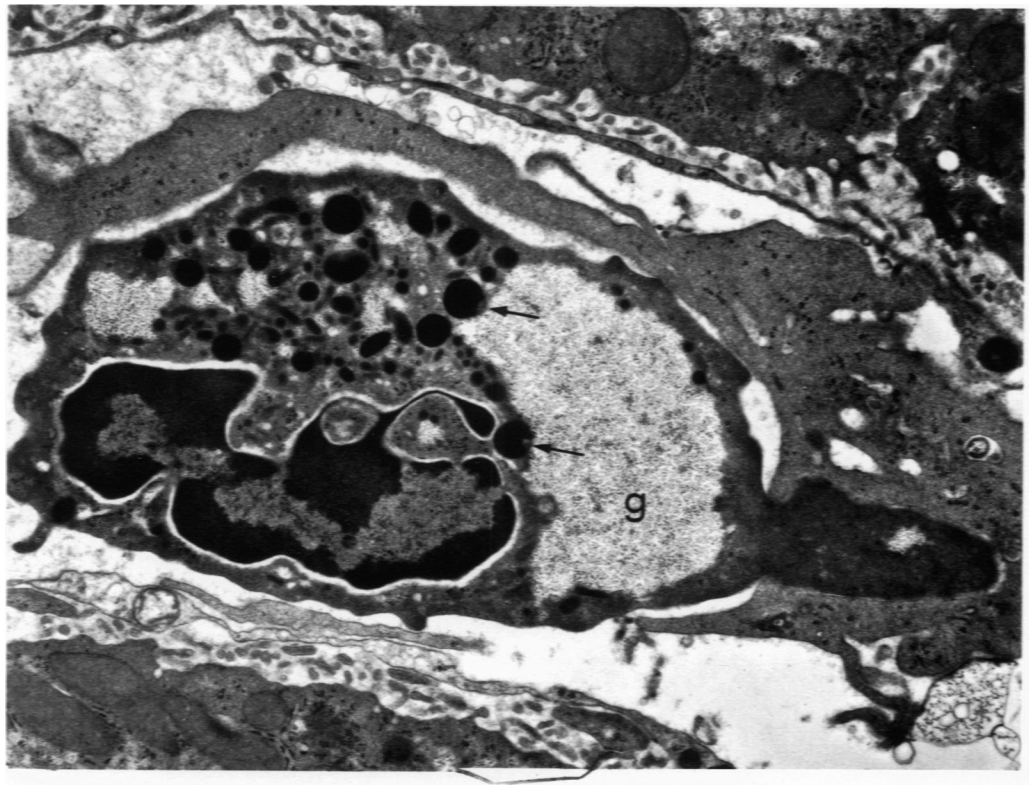




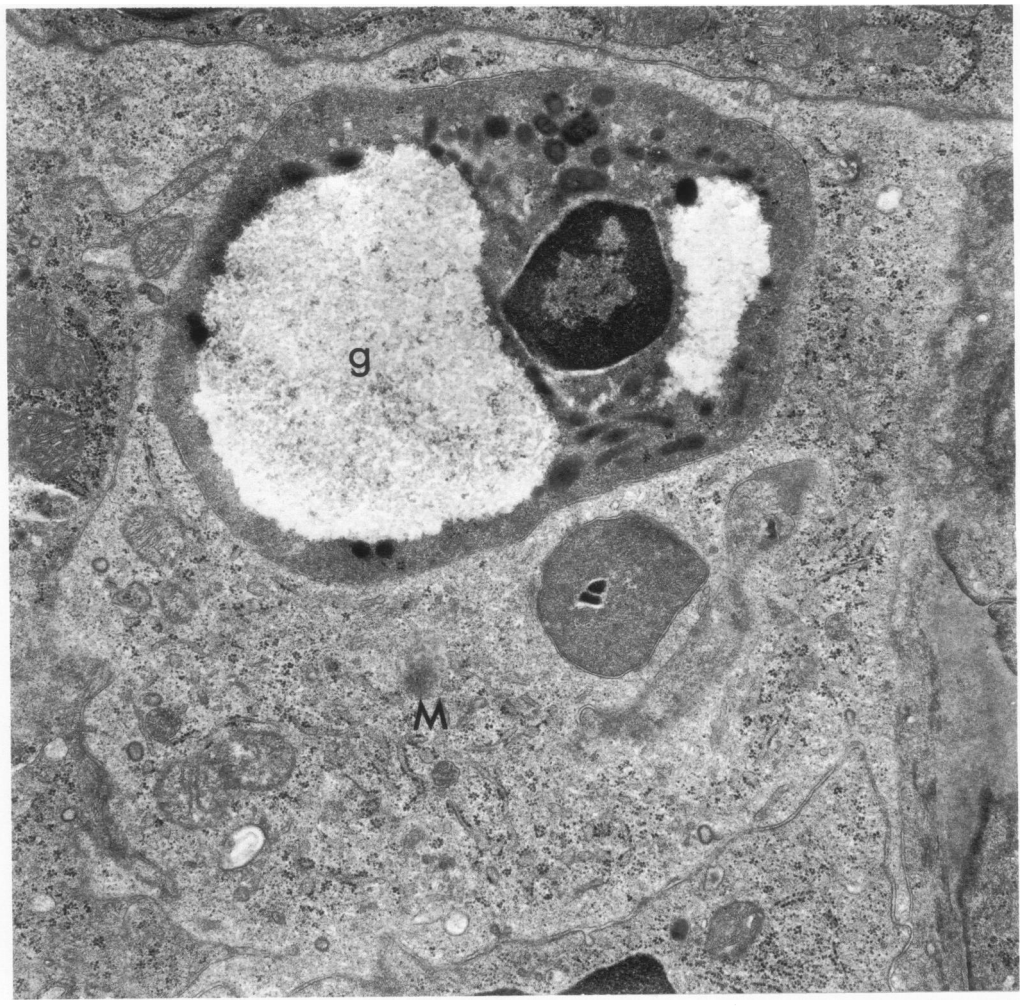
3



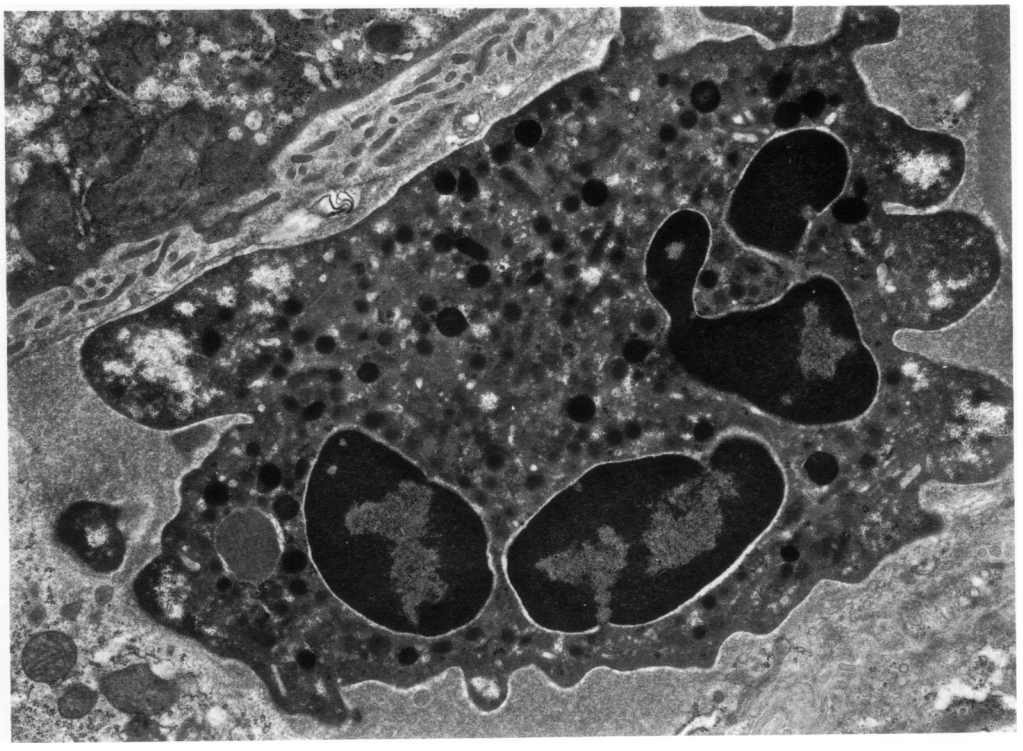
4



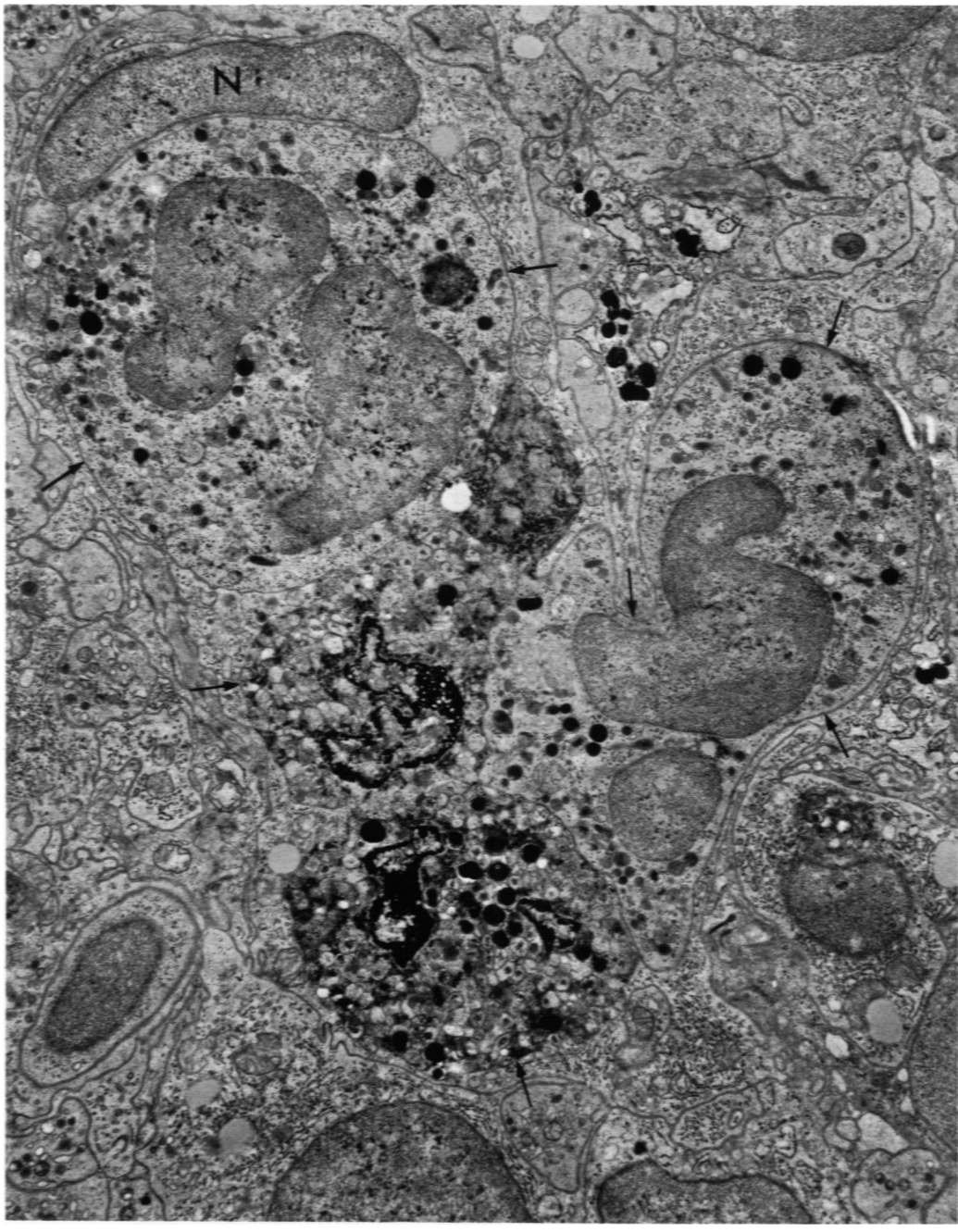
5



6

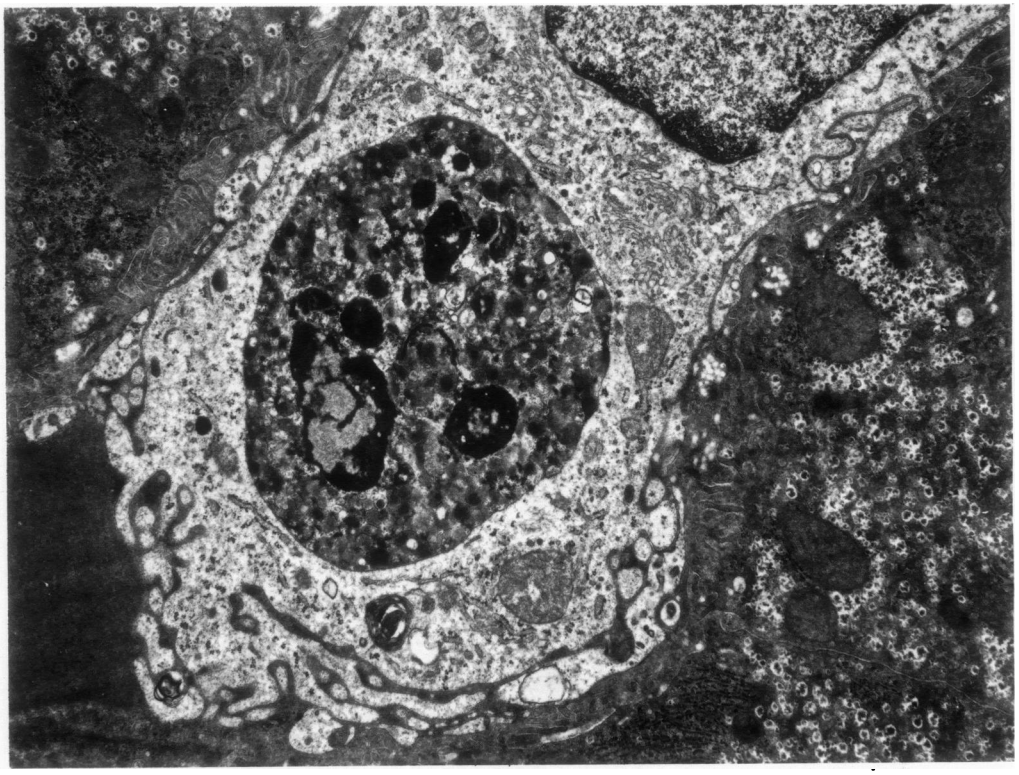


7



8

9



10