ALLERGIC INFLAMMATION

III. The Fine Structure of Collagen Fibrils at Sites of Antigen-Antibody Interaction in Arthus-Type Lesions*

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Plates 53 to 62

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For many years it has been debated whether there is any alteration of the collagen fibers at the site of antigen-antibody interaction in allergic inflammation of the Arthus type. Some claimed that there is eosinophilic swelling, fragmentation, and disintegration of the collagen fibers. Others maintained that a protein material is deposited between the apparently unaltered fibers. The investigation of this problem seemed particularly well suited to electron microscopy.

The earliest changes in the Arthus type of allergic inflammation occur at the blood-tissue barrier (1, 2), and are followed by severe vascular changes, including thrombosis and hemorrhage (3–6). Thus if there are any connective tissue alterations due to direct antigen-antibody interaction in vascular connective tissue, they could not be distinguished from changes secondary to vascular alterations. Our first experiments were conducted on vascular connective tissue. However, following the publication by Germuth *et al.* (7), we reexamined the problem, using the avascular cornea, where the precise site of antigen-antibody interaction can be located and examined, and where complications due to vascular damage do not occur.

Materials and Methods

Immunization Procedure.—Twenty-two albino rabbits of both sexes weighing 2 to 3 kg were fed Purina rabbit chow and water ad lib. They were immunized with bovine serum albumin (BSA, Nutritional Biochemicals Corporation, Cleveland) or with cadmium-free horse ferritin (Nutritional Biochemicals Corporation).

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Group I: Fourteen of the twenty-two rabbits received 3 intravenous injections of ferritin (20 mg) at 48-hour intervals. This dosage was repeated after 3 weeks. Thereafter weekly injections of ferritin (50 mg) in 1 ml of complete Freund's adjuvant (Difco Laboratories, Inc., Detroit) were given intramuscularly. The last injection was given without adjuvant. The total immunization period lasted between 3 and 5 months. The antibody-nitrogen (Ab-N) level varied between 0.23 and 0.68 mg per ml of serum.

Group II: Four rabbits were given 16 intravenous injections of alum-precipitated BSA, given at the rate of 4 injections per week. The dose of antigen was increased gradually from 1.5 to 5 mg. Subsequently weekly injections of 50 mg BSA in 1 ml of complete Freund's adjuvant were administered intramuscularly. The last injection did not contain adjuvant. The period of immunization lasted 4 months. The antibody levels ranged between 0.15 and 0.4 mg Ab-N per ml of serum.

Group III: Four rabbits received weekly intramuscular injections of BSA in 1 ml complete Freund's adjuvant. The dose of BSA was 20 mg for 2 months and 30 to 50 mg for an additional month. Antibody levels were between 0.38 and 1.21 mg Ab-N per ml of serum.

Experiments on the Cornea.—The appropriate antigen was injected into the cornea of ten ferritin-immunized rabbits (group I); and of four BSA-immunized rabbits (group II). Care was taken to deposit it in the center of the cornea, between the connective tissue lamellae so as to form an area of opacity of approximately 3 to 5 mm in diameter. Between 1 and 5 mg (0.05 to 0.25 ml) of ferritin and between 1 and 3 mg (0.1 to 0.3 ml) of BSA were injected. Tissue was taken from three sites: from the center of the cornea, from the limbus, and from the part of cornea which contained the ring.

Experiments on the Cornea of Leukopenic Rabbits.—Three rabbits hyperimmunized with BSA (group III) were injected intravenously with nitrogen mustard (mustargen, Merck Sharp and Dohme, West Point, Pennsylvania). The first injection of nitrogen mustard (1.75 mg per kg body weight) was given 7 days after the last injection of BSA. Two days later a second dose of nitrogen mustard (1 mg/kg) was administered. On the day when the second dose of nitrogen mustard was given, the Ab-N was determined. The next day 3 mg (0.3 ml) of fluorescent isothiocyanate-labeled BSA (fluor-BSA) was injected into the cornea. Total white cell counts and differential counts were done daily. The first count was done before the injection of nitrogen mustard and the last one just before enucleation of the eye. All three animals showed an area of opacity at the site of injection, which became less opaque as the material diffused towards the periphery of the cornea. The area of opacity was fluorescent when examined in ultraviolet light. In two animals an opaque and intensely fluorescent ring was observed at the periphery of the cornea 48 hours after the injection. These animals had high antibody levels (over 1.0 mg Ab-N/ml). The corneal tissue containing the ring was excised 48 hours or 72 hours after the injection of antigen and processed as described below.

Control Rabbits.—Four normal rabbits were given injections of ferritin into the cornea as in the experimental group. Tissue was taken 24 and 48 hours after the injection.

Tissue Processing and Examination.—All tissues excised were fixed both in 10 per cent phosphate-buffered (pH 7.0) formalin and in 1 per cent veronal acetate-buffered osmium tetroxide (pH 7.4) containing 45 mg of sucrose per ml of fixative. The tissue was fixed in formalin for approximately 24 hours and after dehydration it was embedded in butyl methacrylate or paraffin for light microscopy. After fixation for 1 to 1½ hours in the sucrose-containing osmium tetroxide the tissues were placed for an additional hour into osmium tetroxide to which had been added calcium chloride (0.2 mg/ml) instead of sucrose. With the latter procedure tissue cohesion and preservation of cell membranes was improved. Following dehydration with graded alcohols the tissue was embedded in epon 812 or occasionally in selectron.

For light microscopy paraffin- and methacrylate-embedded sections were stained with hematoxylin-eosin or Masson's trichrom. Epon- or selectron-embedded material was stained with alcalinized Azure II. Sections of corneas injected with fluor-BSA were examined with a

fluorescent microscope and subsequently stained and examined with an ordinary light microscope. For electron microscopy, sections were stained with uranyl acetate, lead hydroxide, or phosphotungstic acid. An RCA EMU-3E electron microscope was used.

RESULTS

Corneal Lesions in Rabbits with Normal Leukocyte Levels.—Most animals developed an opaque ring at the periphery of the cornea (Fig. 1). This ring was white or yellow-white when BSA had been used as antigen, and yellow to yellow-brown when ferritin had been injected. In the animals given fluor-BSA, a brightly fluorescent ring was observed. At higher magnification, the fluorescent ring consisted of two concentric lines (Fig. 2).

Sections of corneas biopsied 12 hours after the injection of antigen showed on light microscopy an intensely acidophilic line of precipitate which corresponded to the ring seen with the naked eye (Fig. 3). At high magnification, the acidophilic line was seen to be composed of acidophilic dots or rodlets deposited between the collagen fibers (Fig. 4). Delicate acidophilic dots were also seen scattered between the collagen fibers central to the line of precipitate (Fig. 4). None were seen on the lateral or limbal side. There was some infiltration of the tissue by cells, most of which were polymorphonuclear leukocytes (heterophils).

Corneas biopsied 24 or 48 hours after the injection of antigen showed a marked infiltration of cells along a line corresponding to the ring seen in the gross (Fig. 5). The cell reaction was more intense in the superficial (towards the corneal epithelium) two-thirds of the cornea. Most of the cells were polymorphs. Between them remnants of the acidophilic line were still visible (Fig. 6). By 72 hours, the precipitate had disappeared.

An inflammatory reaction was observed in the limbal region in all the eyes examined, and became more intense in the later stages. The limbal venules were dilated and contained numerous red cells and polymorphs. The perivascular tissue was infiltrated by leukocytes (Fig. 7).

Sections taken from the center of the cornea contained scattered inflammatory cells, mainly polymorphs.

Corneal Lesions in Leukopenic Rabbits.—The total white cell count was below 2000 in one rabbit and below 1500 per mm^3 in the other two leukopenic rabbits at the time their eyes were enucleated. Differential counts showed 0, 8, and 13 per cent granulocytes respectively.

The line of acidophilic precipitate was seen as in the animals with normal counts, except that it developed later and persisted longer. There was no precipitate at 24 hours, a moderately developed precipitate at 48 hours, and a well developed one at 72 hours (Fig. 8). The cells which infiltrated the precipitate were mainly mononuclears and they were more numerous in the superficial (epithelial side) two-thirds of the cornea. Higher magnifications showed palisades of acidophilic precipitate between the collagen fibers (Fig. 9), much as before.

Sections examined with the fluorescent microscope showed fluorescent material in the position of the acidophilic precipitate, and also fluorescent material in cells (Figs. 10 to 12). The fluorescence was more intense in the deeper third (towards the anterior chamber) of the cornea, where cellular infiltration was less marked.

Electron Microscopic Observations.—An accumulation of small electron-opaque precipitates corresponded to what was seen in the gross as a ring, and by light microscopy as an acidophilic line (Fig. 13). The tiny precipitates were very numerous at the site of the ring but were present in smaller numbers on either side of it. They were more numerous on the central side of the ring, than on the limbal side. On the central side, the precipitates decreased in number as the distances from the ring increased (Fig. 13). When ferritin was used as antigen, the precipitates were much more electron opaque (Fig. 14). At high magnification, ferritin could be identified in the precipitates (Figs. 15 and 16).

The collagen fibrils, when cut longitudinally, were often obscured by the precipitates containing ferritin, but intact fibrils crossing the precipitates could nevertheless be identified (Fig. 15). Adjacent fibrils often seemed separated from one another by the precipitates (Fig. 15). That the collagen fibrils crossed the precipitates could be seen best when the fibrils were cut obliquely (Fig. 16) or transversely (Fig. 17). The normal periodicity was preserved even in fibrils crossing large masses of precipitates (Fig. 18). Some contrast between collagen fibrils and precipitates was seen with all "staining" methods used, but phosphotungstic acid staining gave best contrast. In selectron-embedded tissue, the collagen fibrils were darker than the precipitates (Fig. 16), while in epon-embedded material the precipitates were dark and the fibrils showed a "negative staining"–like effect (Fig. 17). At the site of the intense polymorph infiltration, the collagen fibrils appeared normal, except that they were displaced mechanically by the cells. Fig. 19 shows collagen fibrils of an Arthus lesion of vascular connective tissue (sclera), for comparison with the collagen fibrils of the cornea.

The majority of the cells infiltrating the cornea in rabbits with normal leukocyte counts could indeed be identified as polymorphs. They were elongated between the bundles of collagen fibrils and had numerous delicate pseudopodia (Fig. 13). Many cells contained material similar to the precipitate between the collagen fibrils. The material was within sacs. At times cells seemed to be in the process of ingesting precipitates (Fig. 20). In leukocyte-depleted rabbits most of the cells in the lesion were histiocytes, which also seemed to phagocytose the precipitates (Fig. 21). Some of the precipitates were seen in membrane bound sacs along with the granules of the polymorph (Fig. 22).

Control rabbits in which ferritin was injected into the cornea showed a mild to moderate infiltration of polymorphs and there was no alteration of collagen fibrils.

DISCUSSION

Many agree that the severe injury produced in allergic inflammation of the Arthus type is due primarily to the vascular changes induced.

While many (8) have accepted the view of Rich and Follis (5) that the primary site of damage in the "sensitized" animal was the endothelium, recent studies (10, 1, 2) support Opie's view (4) that the primary change is a humoral one; *i.e.*, an interaction between the injected antigen and circulating antibody. This interaction takes place primarily in the lumen and wall of venules and initiates the vascular reaction which seems to be responsible for the progression of the lesion (11).

While the significance of the vascular alterations in allergic inflammation of the Arthus type is generally accepted, the question remains as to whether there is any alteration in the collagen fibrils induced directly by the antigen-antibody reaction as such. In the earliest studies carried out on the Arthus phenomenon (3) an alteration of collagen fibrils (Verquellung) was observed. Subsequently, similar changes were often described, mostly under the term "fibrinoid degeneration," though some have denied that such alterations occur (8, 9). The discrepancy is obvious in the two early attempts to study by electron microscopy the collagen fibrils in Arthus lesions. Wolpers (12) concluded that there was no alteration in the collagen fibrils, their fibrinoid-like appearance being due to deposition of fibrin between the fibrils, making them "sticky." On the other hand, Rich *et al.* (13) described swelling and loss of the normal periodicity of the fibrils. These studies were carried out on sonically and mechanically disrupted material.

Subsequently, Germuth and his coworkers (7) studied the alterations induced in the corneas of hyperimmune rabbits injected into the cornea with the appropriate antigen. The changes were limited to the line where antigen and antibody precipitated. "The collagen fibers that traversed the line of antigen-antibody precipitation appeared swollen, fragmented, and deeply eosinophilic. This was particularly apparent in the leucopenic animals, where the change was almost identical with that which has been called 'fibrinoid' degeneration" (7).

Since the original description of Neumann (14), the nature and origin of "fibrinoid" has been debated by many workers (15). Some claimed that it is an alteration of collagen fibrils and of ground substance and others believed that it was due to extravasated plasma proteins. This was one reason why we were interested in looking into this problem. However, our major aim was to find out whether there is any alteration of collagen fibrils at sites where antigen and precipitating antibody interact. We knew that there is a mild alteration of the blood-tissue barrier in the early phase of the Arthus reaction (1) and we had some indication that the progression of the lesions does not seem to be dependent on direct tissue "damage" by the interacting antigen and antibody (11). The corneal model of Germuth *et al.* (7) seemed particularly well suited for an electron microscopic analysis. Like Germuth and coworkers, we observed an acidophilic precipitate at the periphery of the cornea, invaded early by polymorphs, but persisting much longer in leukopenic animals. Already the light microscopic sections of plastic-embedded tissue indicated that the deposits lay, in a pallisade-like fashion, between the collagen fibers. However, the electron micrographs clearly showed

that the precipitates lay between the collagen fibrils. The fibrils showed no swelling and had normal periodicity.

Germuth and colleagues (7) postulated that traces of injected antigen reach the limbal vessels, where they interact with antibody, so rendering the vessels hyperpermeable. A massive outpouring of antibody and emigration of leukocytes follows. The line of precipitate was thought to occur when a critical concentration of antigen and antibody is reached. Electron micrographs, such as those shown in Fig. 13, prompt one to suggest further that antibody probably diffuses more centrally, beyond the line of precipitate, leading in a zone of antigen excess to small scattered precipitates, decreasing in number away from the line. In any case, all graduations of antigen-antibody complexes seem to form in both time and space, including complexes in antigen excess, which are said to be biologically active. There was no alteration of collagen fibrils at either side of the actual line or in the more central areas of the cornea.

The significance of polymorphs in the Arthus reaction has been stressed (16). Indeed, they play a major role in the vascular lesions (6, 10, 11). In the corneal lesions, there was persistence of the antigen-antibody precipitates in animals rendered leukopenic. As in the vascular lesions they seem to be concerned primarily with the removal of the antigen-antibody complexes. There was no evidence that the polymorphs, perhaps by releasing a proteolytic enzyme, altered the collagen fibrils. There was evidence that they ingested the precipitates, for phagocytized precipitates were seen, membrane enclosed; sometimes together with polymorph granules.

SUMMARY

The fine structure of collagen fibrils at sites of antigen-antibody interaction is described.

Following injection of antigen (BSA or ferritin) into the center of the cornea of hyperimmune rabbits, an acidophilic ring of precipitate forms at the periphery of the cornea, where antigen and antibody interact in optimal proportions. The precipitate is soon removed by infiltrating polymorphs, but persists longer in leukopenic animals.

Electron microscopic examination of the cornea showed no alteration of the collagen fibrils in the area of antigen-antibody precipitation or in the remaining cornea. Contrary to many claims there was no swelling, fragmentation, or disintegration of the fibrils and they had a normal periodicity.

Polymorphs infiltrated the precipitates and phagocytosed the antigen-antibody complexes. There was some ultrastructural evidence that degradation of the complexes took place in the polymorphs.

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least we wish to thank Dr. A. C. Ritchie for valuable help and criticism in preparation of this manuscript.

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EXPLANATION OF PLATES

PLATE 53

FIG. 1. Eye of hyperimmunized rabbit injected 24 hours earlier into the cornea with ferritin. The arrow points to a ring of antigen-antibody precipitate at the periphery of the cornea. × 2.3.

FIG. 2. The arrows indicate two parallel fluorescent lines at the periphery of the cornea. The rabbit had received 72 hours previously an intracorneal injection of fluor-BSA. The limbal vessels (*ves*) are seen as black dots and lines. × 12.

FIG. 3. Twelve-hour-old corneal lesions produced with ferritin. A line of acidophilic (red in section) precipitate (*ppt*) is seen deposited into the dense collagenous (*col*) matrix. A few leukocytes (*leuk*) have begun to infiltrate the precipitate. Masson's trichrome. × 700.

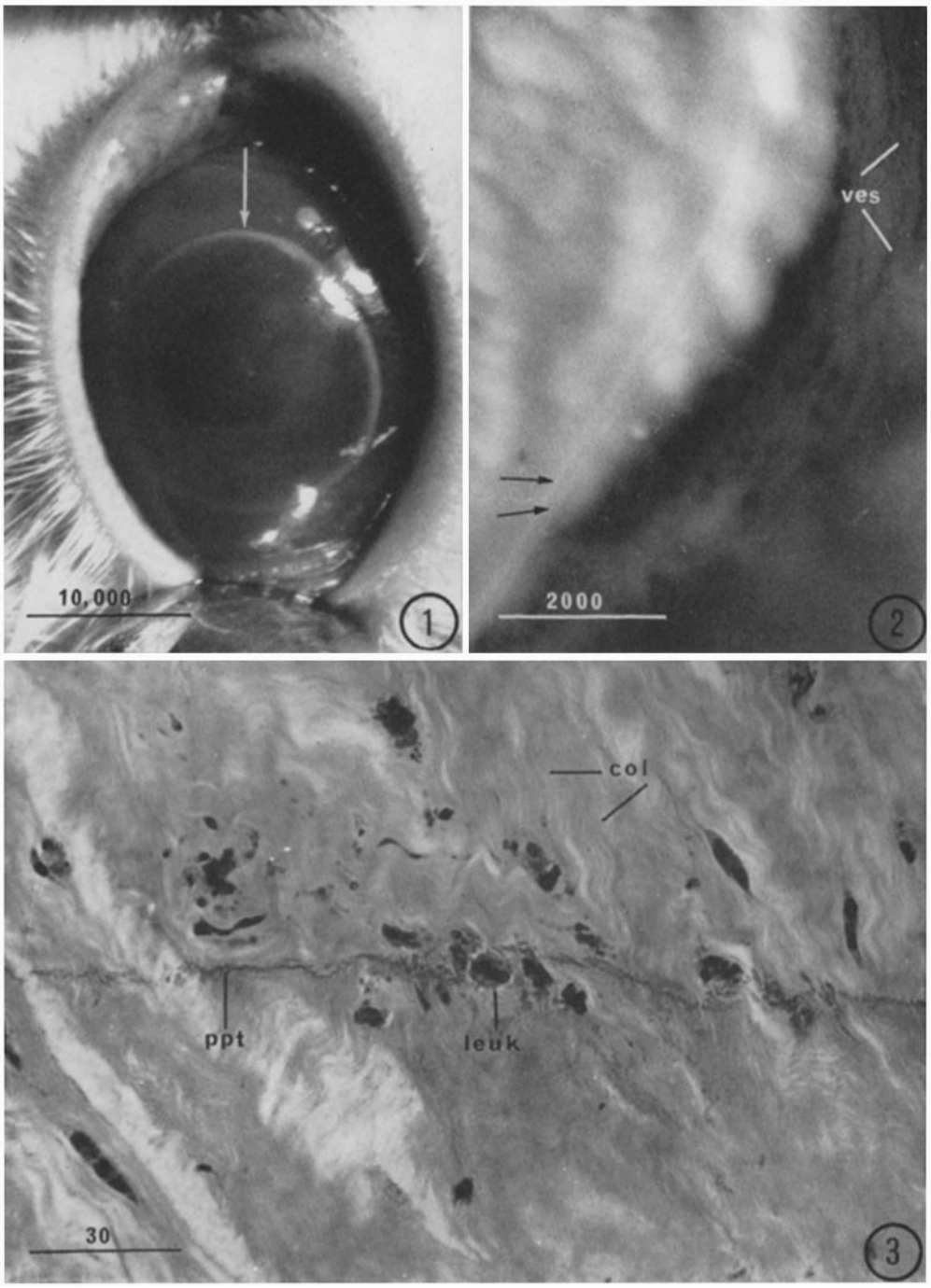

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PLATE 54

FIG. 4. Higher magnification of Fig. 3. Note that the line of precipitate consists of dark (red in section) pallisading dots and rodlets. The limbal side is on the top and the central side of the cornea in the bottom of the picture. The arrows point to tiny black (red in section) precipitates on the central side (compare with Fig. 13). × 1760.

FIG. 5. Twenty-four–hour–old lesion produced with BSA. Infiltration of the line of precipitate by cells. The arrow points to an area where the line is still visible. Azure II. × 176.

FIG. 6. Higher magnification of Fig. 5. The majority of the infiltrating cells are polymorphonuclear leukocytes (heterophils). The arrows indicate bits of precipitate between the infiltrating cells. × 700.

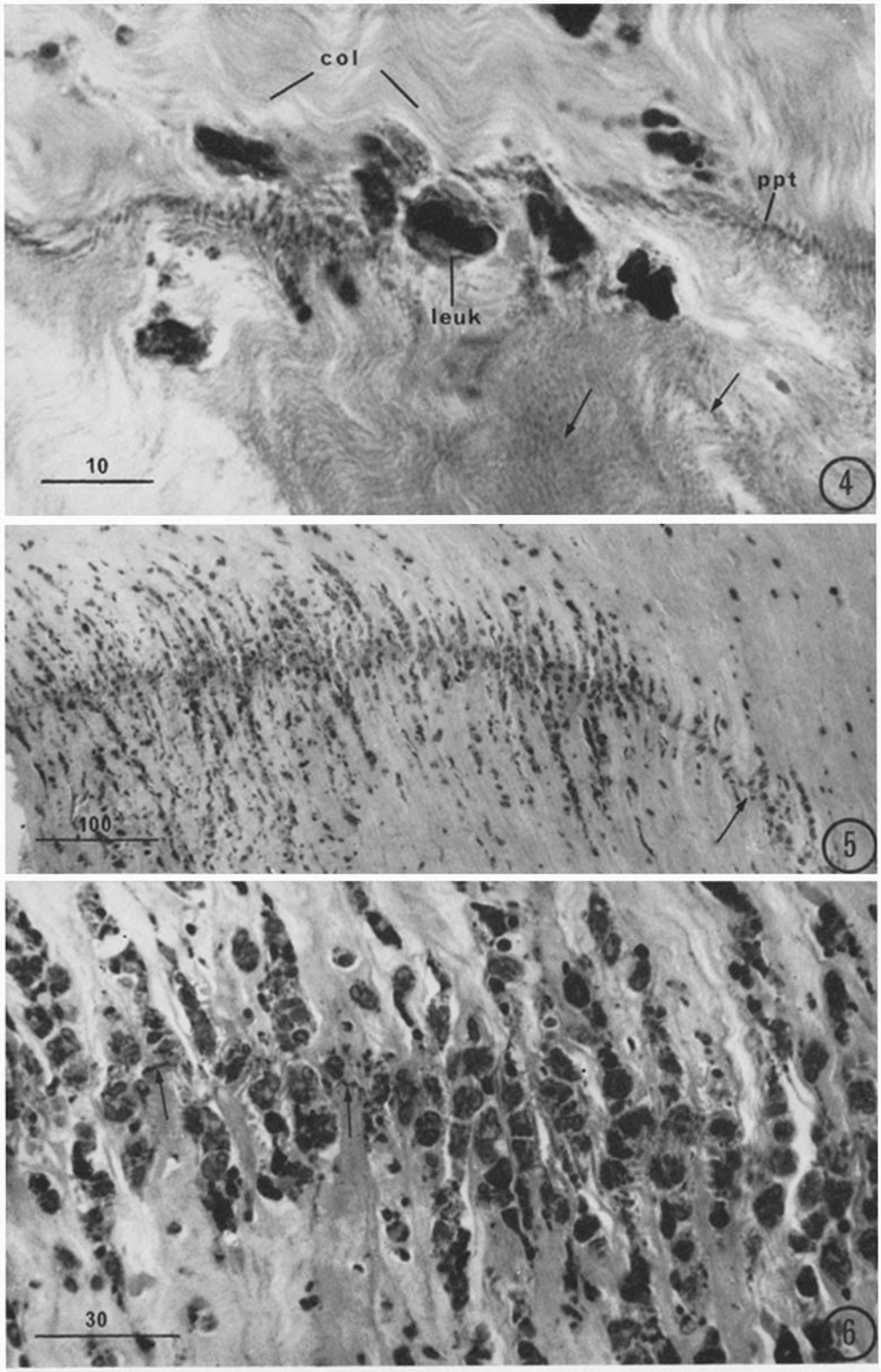

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PLATE 55

FIG. 7. Limbal region 48 hours after injection of ferritin into the center of the cornea. The epithelium (*ep*) is to the left and two venules (*ven*) are seen in the center. The venules are congested and contain numerous leukocytes. The latter are seen also in the surrounding connective tissue. Masson's trichrome. × 268.

FIG. 8. Seventy-two–hour–old corneal lesion of leukopenic animal. A line of precipitate (*ppt*) is seen in the center. There is invasion of the precipitate (left two-thirds) by cells. *ep*, epithelium; *bow*, Bowman's membrane; *des*, Descement's membrane. Masson's trichrome. × 268.

FIG. 9. Higher magnification of Fig. 8. Note that the line of precipitate consists of elongated, often spindle-shaped dark (red in section) structures (arrows), deposited between the light grey (blue in section) collagen fibers. × 1920.

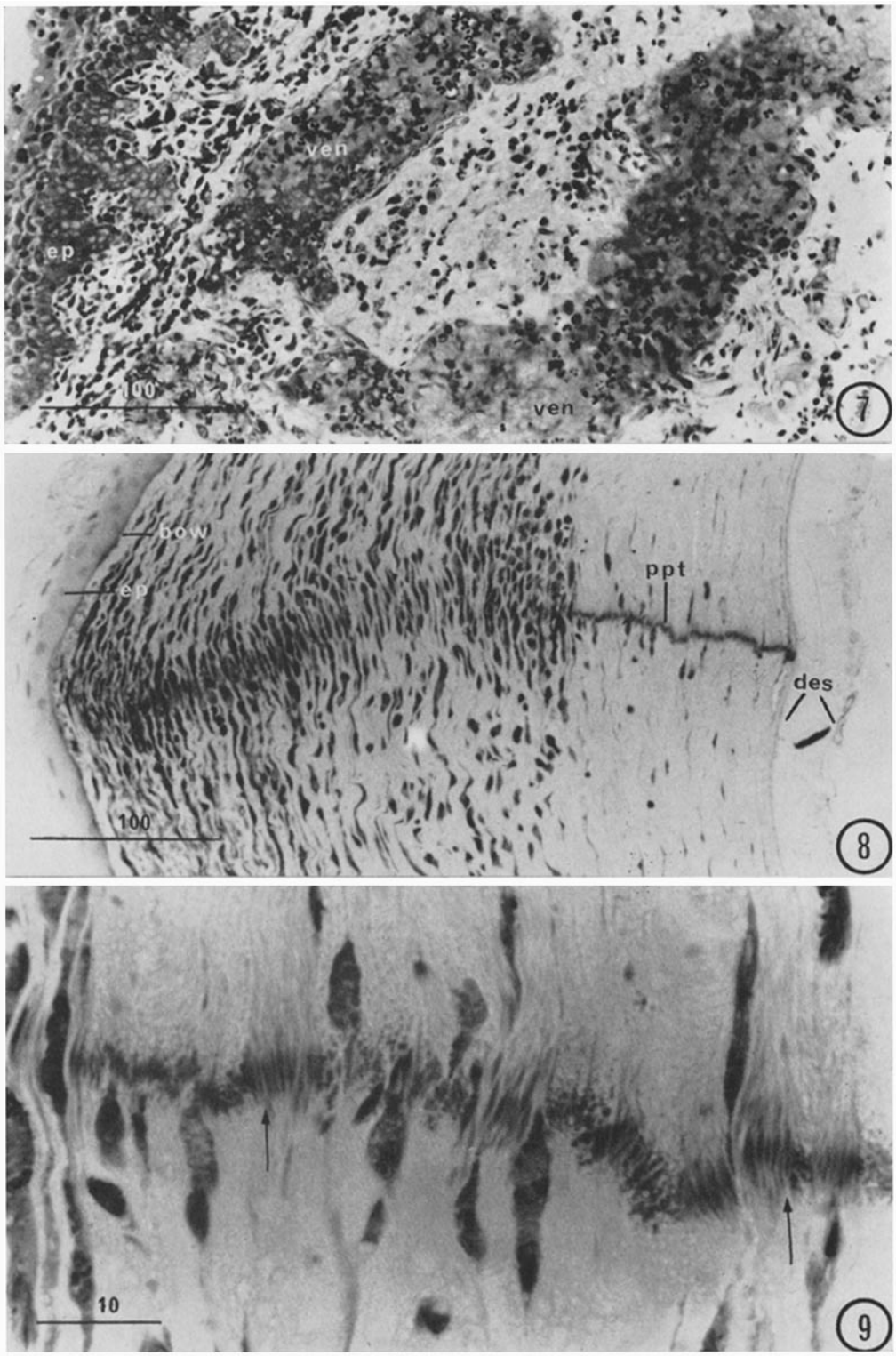

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PLATE 56

FIGS. 10 and 11. Low and high magnification of 48-hours' corneal lesion in leukopenic rabbit. Note fluorescence (yellow-green in section) of the line of precipitate (*ppt*) and of cells (dots and clumps), indicating the presence of BSA and probably anti-BSA. *des*, Descement's membrane. Fig. 10, × 364. Fig. 11, × 1600.

FIG. 12. This shows a fluorescent micrograph of the cornea shown in Figs. 8 and 9. The photograph was taken prior to staining with Masson's trichrome. The acidophilic line of precipitate shown in Figs. 8 and 9 is seen here as a fluorescent line (*ppt*). The arrows point to fluorescent material in cells. × 1000.

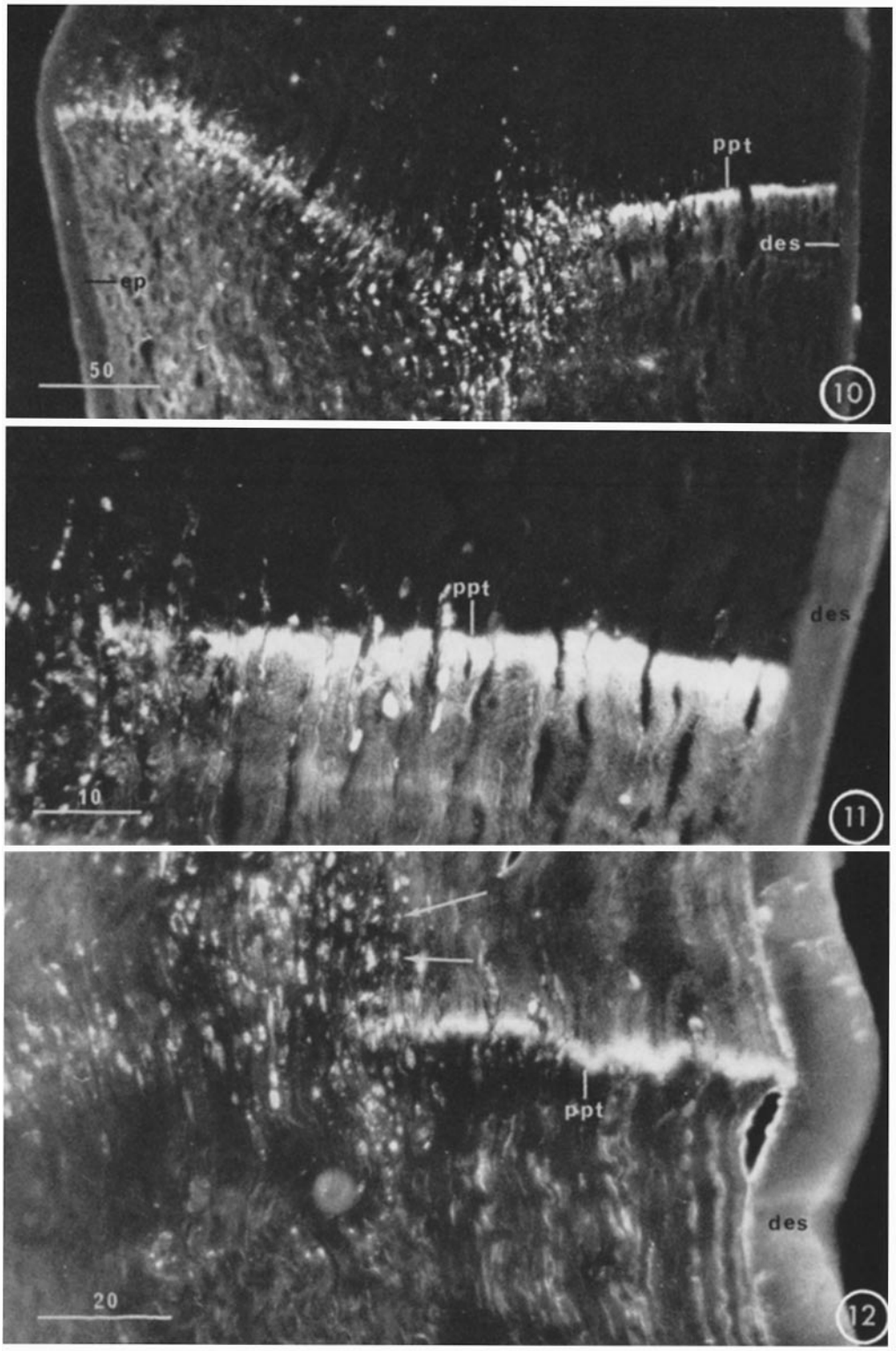

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PLATE 57

FIG. 13. Low power electron micrograph of 24-hour-old corneal lesion produced with BSA. The "line" of precipitate (*ppt*) is made up of small electron-opaque precipitates, which do not stop abruptly, but become less dense (arrows) towards the center of the cornea (bottom of micrograph). The limitation towards the limbus (top) is more definite. The infiltrating leukocytes (*leuk*) are elongated and have many pseudopodia. *col*, collagen. × 3780.

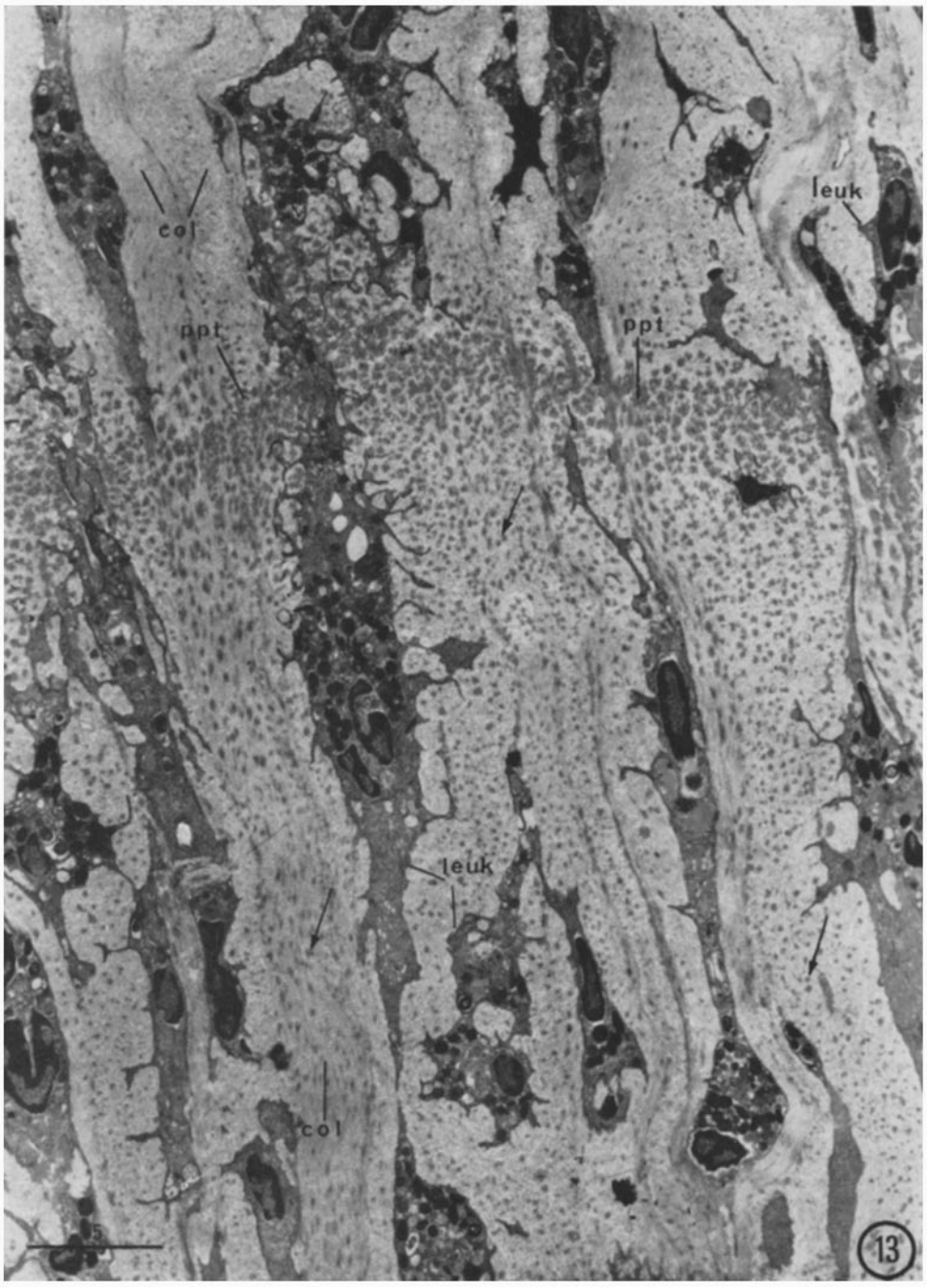

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PLATE 58

FIGS. 14 and 15. Low and high power views of ferritin containing precipitates (*ppt*) between the collagen fibrils (*col*). The latter are often separated by the precipitates (top), but often seem to traverse them (lower half). The periodicity of the fibrils is well preserved. Fig. 14, × 6500. Fig. 15, × 72,000.

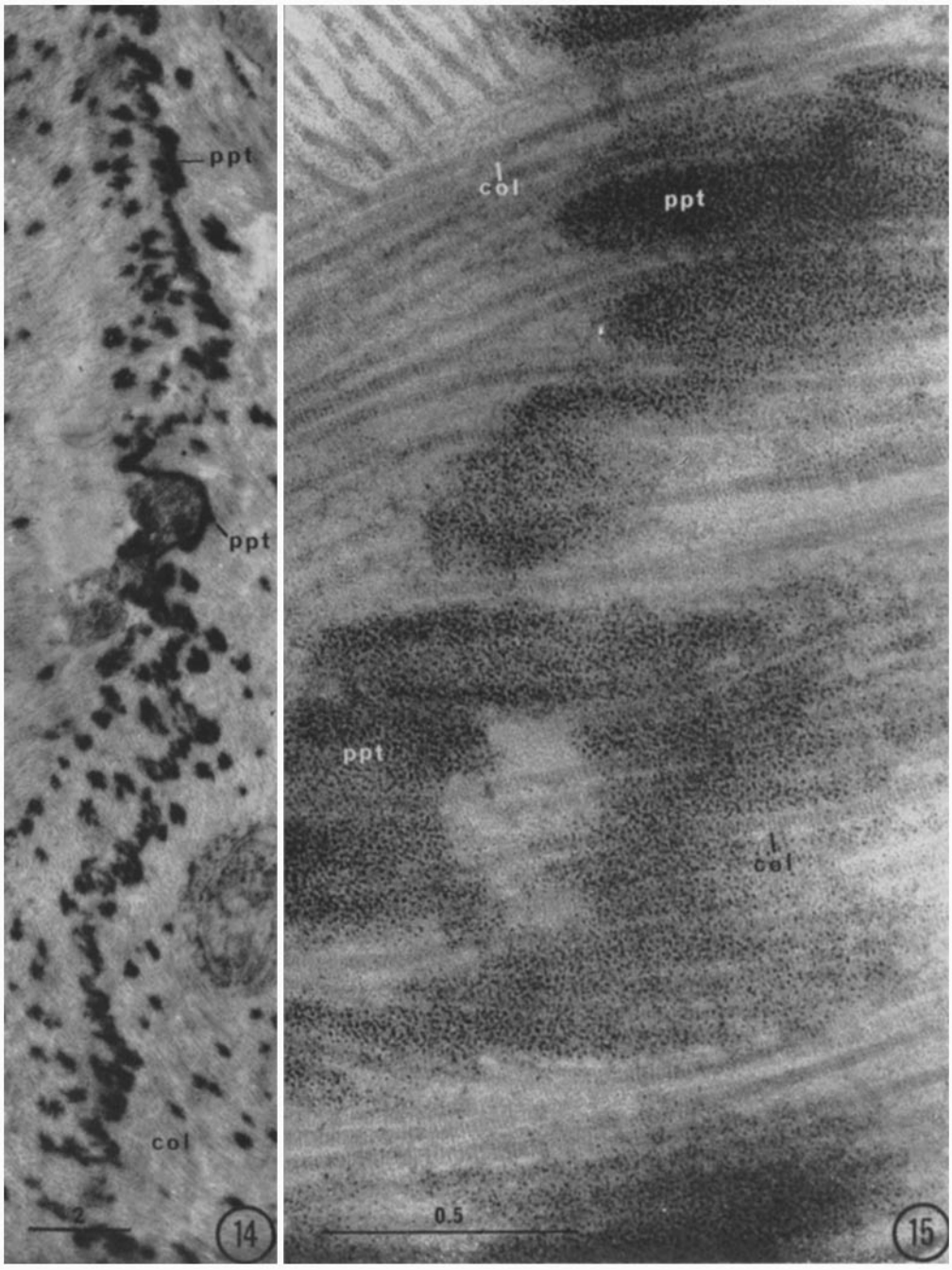

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PLATE 59

FIG. 16. This micrograph shows ferritin containing precipitates (*ppt*) and collagen fibrils (*col*). The latter are cut obliquely and look normal both within and outside the precipitates. × 107,000.

FIG. 17. The cross-sectioned collagen fibrils (arrows) are surrounded by the electron-opaque precipitates of BSA–anti-BSA. Note that many collagen fibrils are outlined as a ring and are separated from the precipitates by a narrow space. × 64,000.

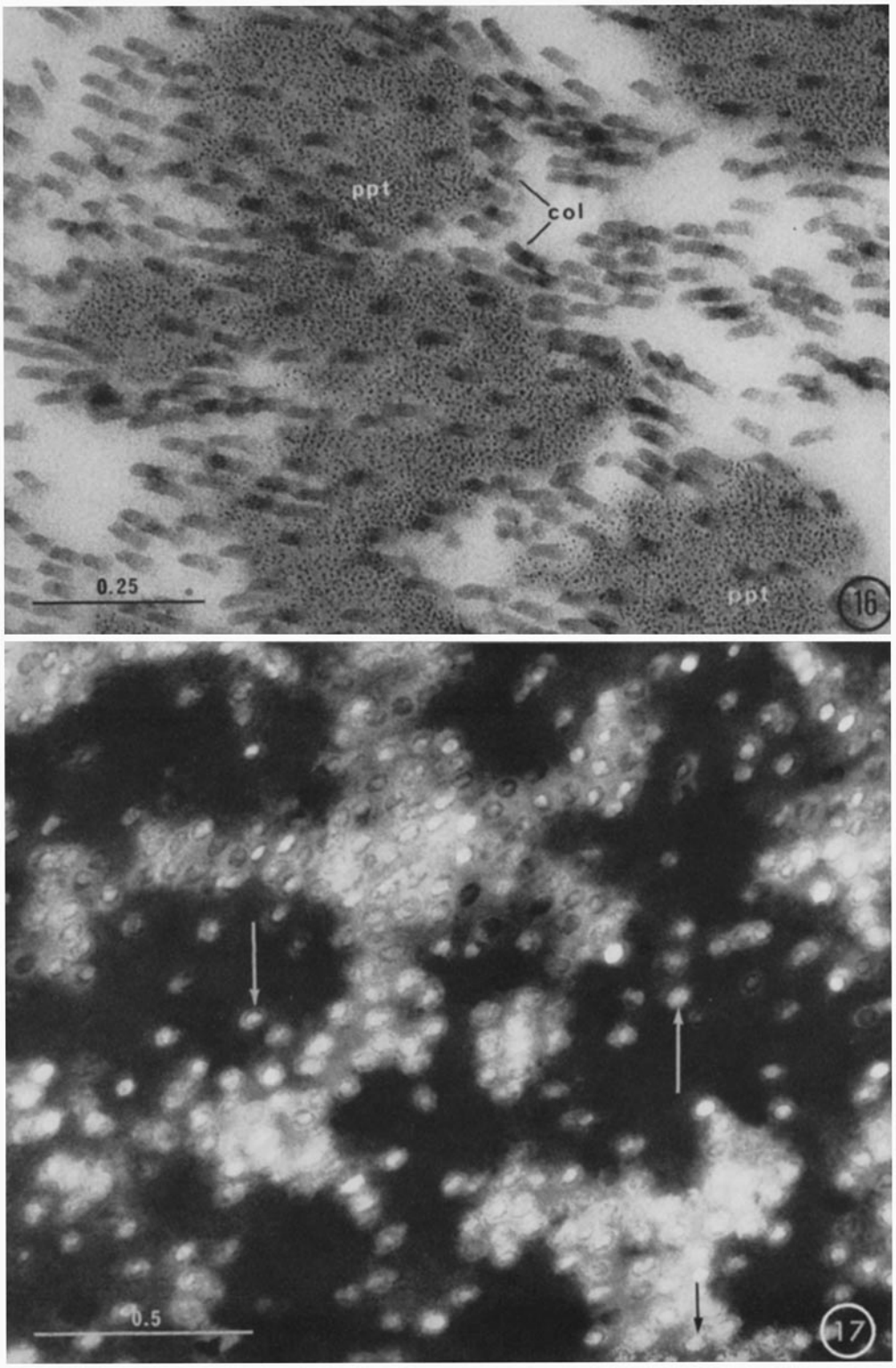

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PLATE 60

FIG. 18. This micrograph shows several collagen fibrils in longitudinal section. Their periodicity is well preserved, even the fibril (*col*) traversing a large mass of BSA–anti-BSA precipitate (*ppt*). Seventy-two–hour–old corneal lesion of leukopenic rabbit. × 76,000.

FIG. 19. Scleral Arthus lesion. For comparison with corneal lesion a hyperimmune rabbit was injected with BSA into the sclera and the tissue fixed 24 hours later. The normal appearing collagen fibrils (*col*), which are more robust than those of the cornea, are separated by precipitates of BSA–anti-BSA. × 57,000.

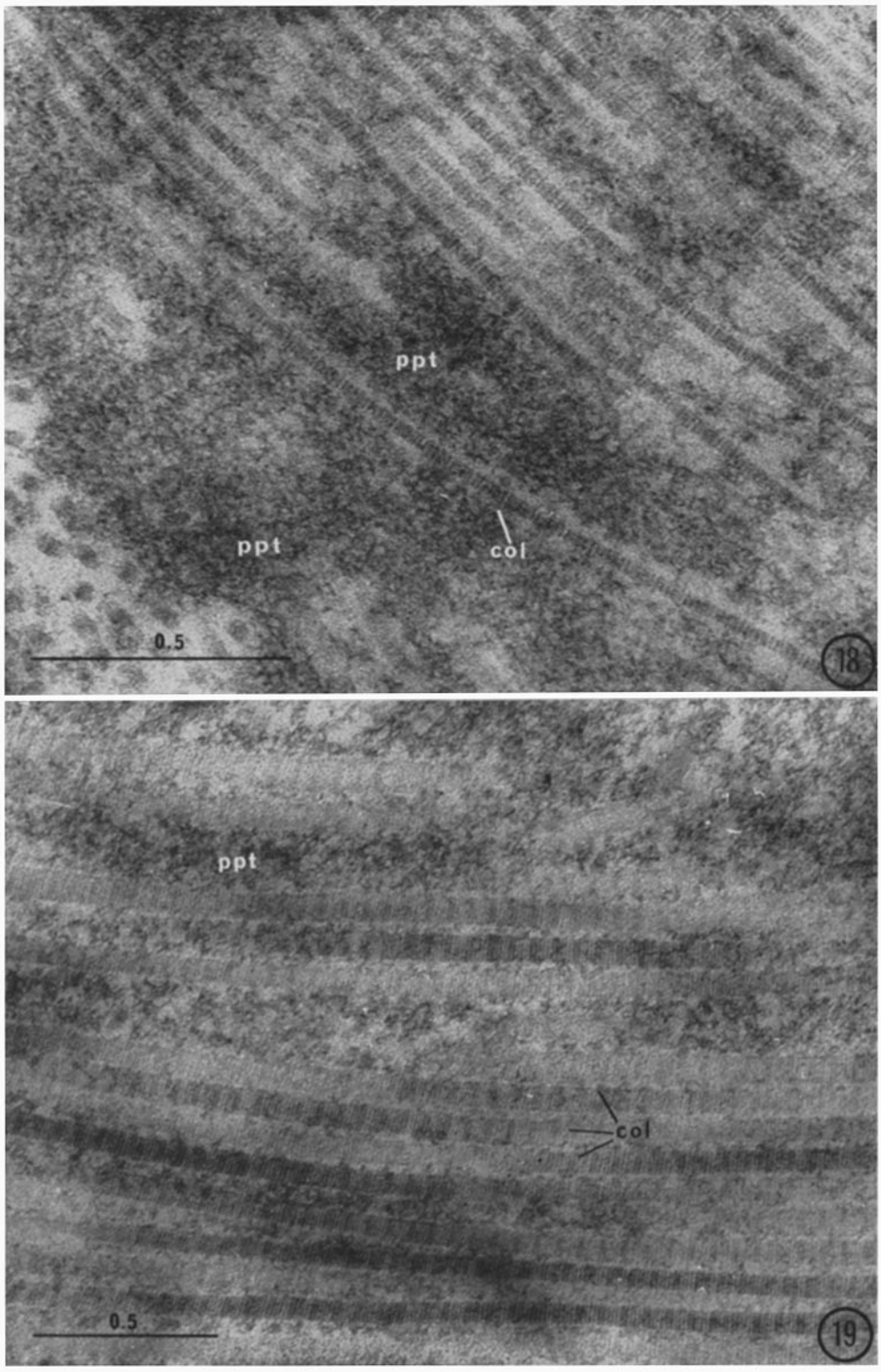

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PLATE 61

FIG. 20. Cytoplasm of leukocyte (*leuk*) in a 24-hour-old corneal lesion. Precipitates (*ppt*) of ferritin-antiferritin are seen outside the cell and some are probably being phagocytosed. *col*, collagen fibrils; *gr*, granule. × 50,000.

FIG. 21. Histiocyte (*his*) in 72-hour-old corneal lesion of leukopenic rabbit. Precipitates (*ppt*) of BSA–anti-BSA are seen within the cell and between the collagen fibrils (*col*). × 23,500.

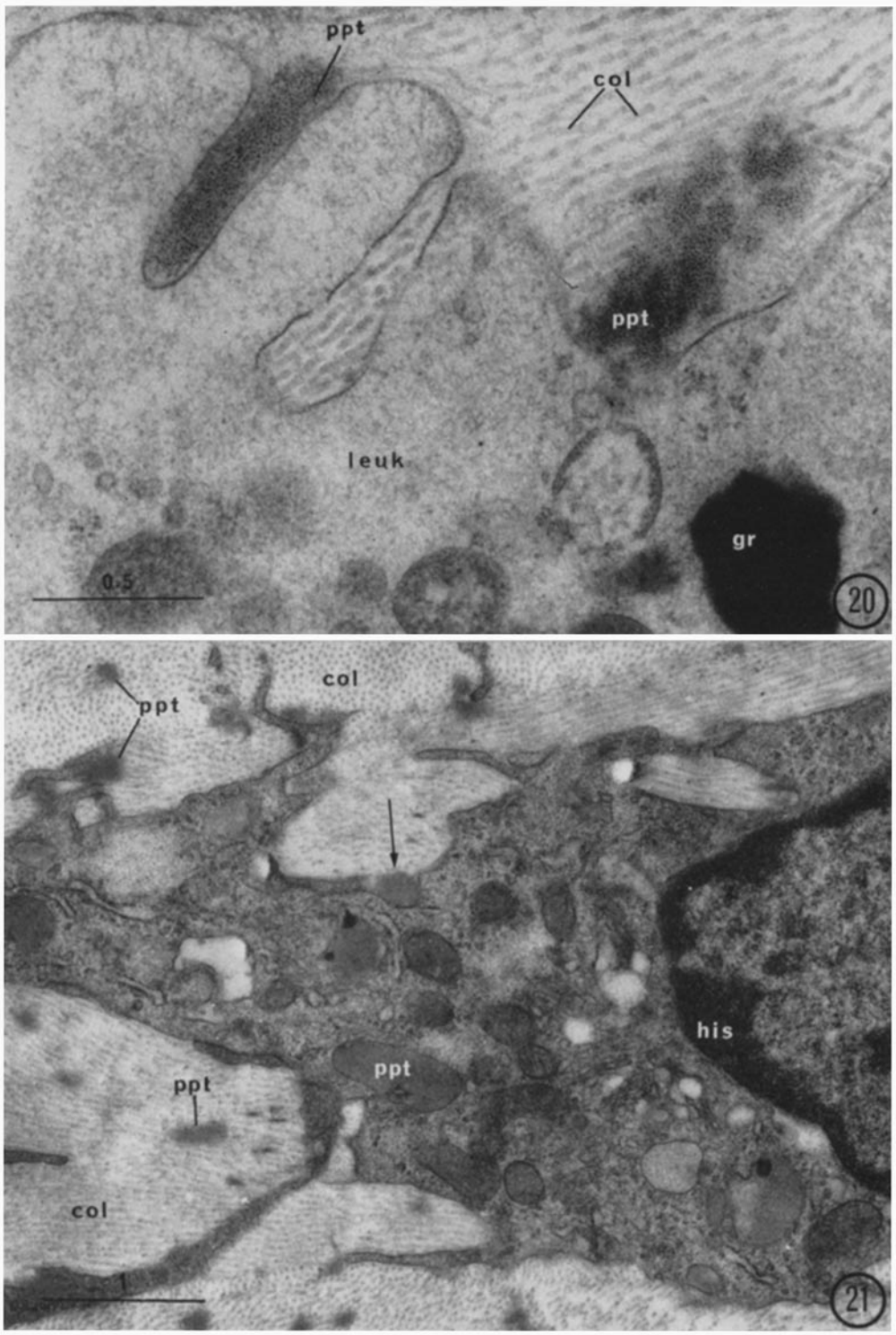

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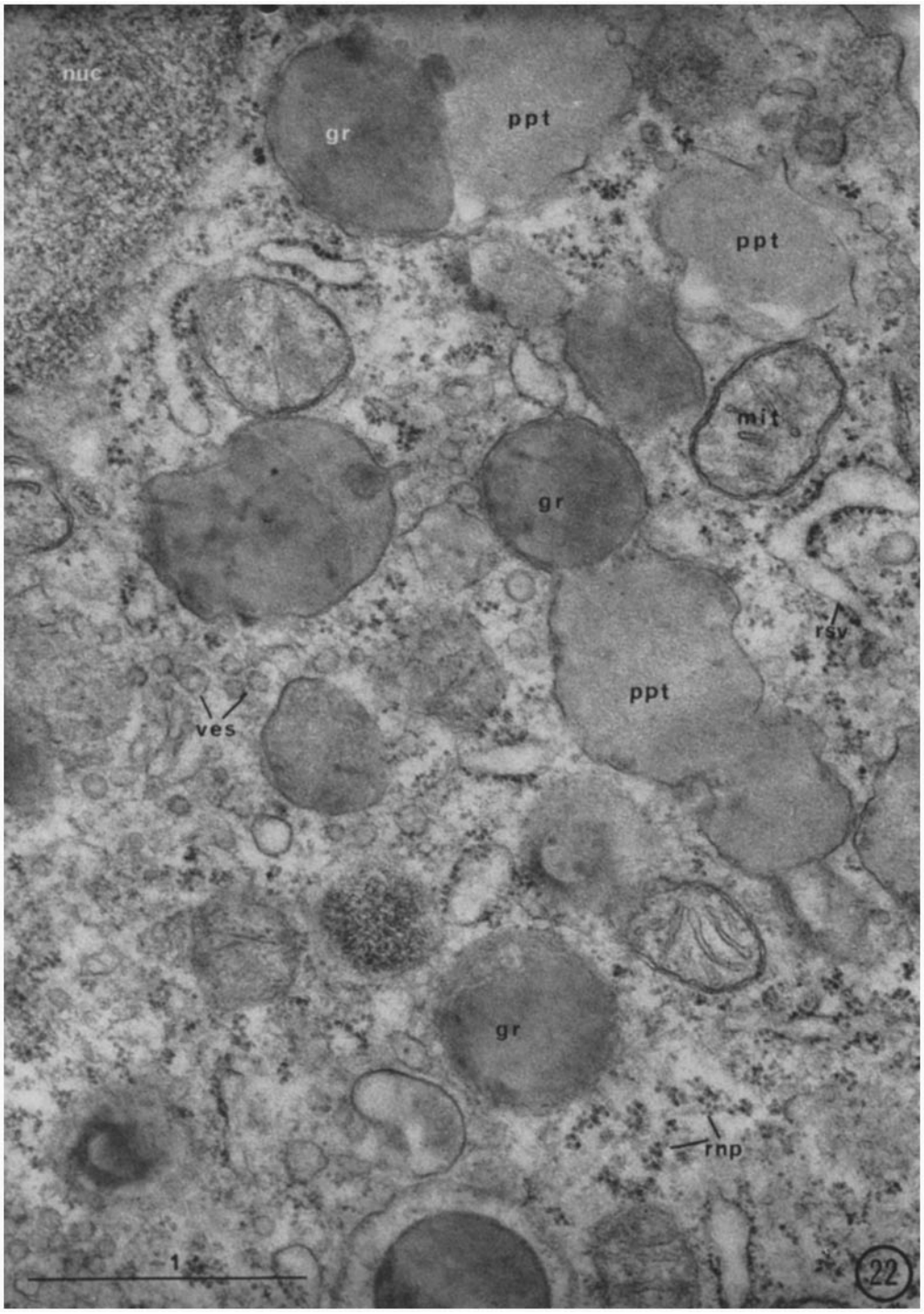
PLATE 62

FIG. 22. Polymorph from 24-hour-old lesion. The precipitates (*ppt*) of BSA–anti-BSA are in membrane enclosed sacs, which also contain leukocyte granules (*gr*). *nuc*, nucleus; *mit*, mitochondrion; *rnp*, ribonucleoprotein granules (ribosomes); *rsv*, rough surfaced vesicle. × 40,000.



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