Microhemorrhage in the Hyperplastic Thyroid Gland of the Rat

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A microhemorrhagic process was consistently observed in association with the induction of thyroid hyperplasia by dietary thiouracil in the rat. This process appeared to involve the extravasation of erythrocytes (RBCs) through hyperplastic capillary walls. Those sites of extravasation which were directly visualized involved endothelial openings of less than 1 μ. These openings were surrounded by endothelial cytoplasm containing a dense fibrous material and were associated with RBC constriction during passage. Extravasated RBCs were most often noted singly or in small groups, either in columns between follicular epithelial cells or embedded amongst the basal epithelial infoldings. Occasionally, extravasated RBCs were also observed within follicular lumens. Extravasated RBCs were usually intact ultrastructurally, but occasionally an apparent hemolytic process was observed, both for RBCs embedded amongst epithelial cells and for those within follicular lumens. The nature and etiology of this microhemorrhagic process are considered in relation to the hypervascularity of the gland, the possibility of capillary wall alterations, the presence of endothelial cell mitoses, and the localization of the process. (Am J Pathol 85:317–332, 1976)

ANIMALS FED A GOITROGEN such as thiouracil develop thyroid hyperplasia with a marked increase in the vascularity of the gland.[1-4] In ultrastructural studies of this phenomenon we have consistently observed focal areas of microhemorrhage in which varying numbers of erythrocytes (RBCs) were encountered amongst follicular epithelial cells and occasionally within follicular lumens. In the presence of microhemorrhage into a follicular lumen, phagocytic uptake of RBCs by the follicular epithelium was frequently noted. The present report describes the nature and pathway of microhemorrhage in the hyperplastic thyroid gland.

Materials and Methods

Induction of Hyperplasia

Male Fischer rats weighing 250 to 350 g were fed the Remington low-iodine diet (approximately 0.02 μg iodine g diet, Teklad Mills, Madison, Wisc.) containing 0.25% thiouracil. Tissues from 4 to 12 animals sacrificed after being maintained on this diet for each of the following periods of time: 0, 1, 2, 3, 4, 5, 7, 10, 14, and 100 days were processed for electron microscopy.

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Processing for Electron Microscopy

Perfusion Fixation

Rats were anesthetized with Nembutal, the thoracic cavity was entered, and (within 10 seconds) perfusion with fixative was begun through an 18-gauge needle inserted into the left ventricle. Perfusion was carried out for 3 minutes at 37 C, with drainage afforded by a large right atrial incision. Perfusate pressure was adjusted to vary from 140 mm Hg (for control rats) to 95 mm Hg (for rats fed thiouracil for 5 or more days). These pressures were determined in preliminary experiments to be just adequate to produce rapid (5 to 20 seconds) blanching of the thyroid vessels. The fixative consisted of 2% formaldehyde (freshly generated from paraformaldehyde), 2.5% redistilled glutaraldehyde (Ladd), and 1.5 to 3% dextran[5] (molecular weight, 35,000 to 50,000, Nutritional Biochemicals) in 0.04 M sodium cacodylate, pH 7.35.

Following perfusion fixation, all tissue processing was carried out at room temperature. Thyroid glands were carefully cut (to minimize tissue compression) into 1-mm cubes. These were then immersed in the fixative just described for an additional 1 to 3 hours, and postfixed in 1% osmium tetroxide buffered with 0.1 M cacodylate. Tissue cubes were rinsed with water and stained *en bloc* with 1.5% aqueous uranyl acetate. They were then dehydrated with a graded series of ethanols and propylene oxide, and embedded in Epon. Thick sections (1 μ) were stained with toluidine blue for light microscopic observations. Thin sections were stained for 4 to 5 minutes each with 5% uranyl acetate (in 25% methanol) and lead citrate and then carbon coated for examination with an A.E.I. EM 6B electron microscope.

Immersion Fixation

To evaluate the possibility that microhemorrhage was induced or influenced by the perfusion process, hyperplastic thyroid glands from 6 rats fed thiouracil for 5 days were fixed by immersion only. In addition (to rule out the possibility that microhemorrhage might be related to trauma during animal handling), care was taken with these animals to avoid any contact with or stress to the thyroid region. Both thyroid glands, together with a portion of trachea, were carefully excised *en masse* and immersed in the usual fixative solution for 1 to 3 hours. After postfixation in 1% osmium tetroxide, the thyroid glands were excised from the trachea and further processing was as described above.

Results

Light Microscopic Observations

Timing and Frequency

Microhemorrhage was observed most frequently in the thyroid of rats maintained on thiouracil for about 5 days. A square millimeter section of thyroid from such an animal typically showed several involved regions, each including from 1 to 100 extravasated RBCs (Figure 1). Microhemorrhage was relatively rarely observed in rats treated for less than 3 days or more than 10 days.

Occurrence in Immersion-Fixed Tissue

The frequency, severity, and patterns of microhemorrhage observed in immersion-fixed tissue did not differ appreciably from those of perfusion-fixed tissue.

Character and Location

The most commonly observed pattern of microhemorrhage consisted of small clusters of extravasated RBCs embedded among the basal portions of the follicular epithelium (Figures 2 and 3). Slightly larger hemorrhagic accumulations often appeared as columns of RBCs, presumably in between epithelial cells, approaching the follicular lumen (Figures 2 and 4). When large numbers of extravasated cells were present, the formation of roughly circular pockets of tightly packed RBCs was frequently observed, in between or amongst distorted epithelial cells (Figures 2 and 3). Similar pockets were also observed which contained a material that stained like plasma and either lacked RBCs or included a variable number (Figure 2). When no RBCs were present, such structures had the appearance of a "pseudolumen."[4]

Microhemorrhage into follicular lumens occurred sporadically, as an apparent extension of the patterns noted above (Figures 1 and 4). Sections of such lumens showed variable RBC content—from a single cell to a tightly packed lumen. Occasionally, intraluminal RBCs appeared partially aggregated or clumped.

Polarization of Microhemorrhage

A very consistent feature of microhemorrhage was its localization toward the epithelial rather than the interstitial face of blood vessels (Figures 1–4). Although an occasional extravasated RBC was noted in the interstitium, the vast majority of RBCs were embedded amongst the follicular epithelial cells, having apparently traversed the follicular basal lamina.

Electron Microscopic Observations

Site of RBC Extravasation

Despite ultrastructural observations on a large number of regions with microhemorrhage, no evidence of sizeable vascular openings was obtained. In four rather similar instances, however, sites of apparent RBC extravasation were observed. In each case, one or more RBCs were in apparent transit across a capillary wall and were constricted at the level of the endothelium by an opening of less than 1 μ (Figures 5, 6, 8, and 9). A concentration of dense material was consistently present in the endothelial cell cytoplasm immediately adjacent to these openings (Figures 6, 7, and 9). This material consisted of approximately 7 nm diameter microfilaments extending radially from the vicinity of the opening, as well as granular elements of approximately the same diameter noted immediately adjacent to the opening.

It was not possible to determine unequivocally whether the sites of

apparent RBC extravasation observed involved penetration of RBCs between endothelial cells or whether penetration occurred directly through the (fenestrated) endothelial cytoplasm. In the case of the RBC shown in Figure 5, nonconsecutive serial sections from one side of the RBC neck (Figure 7) revealed no evidence of an intercellular junction in this region. The possibility of an intercellular localization for this RBC could not, however, be unequivocally ruled out in the absence of serial sections from the opposite side of the RBC neck.

The possibility that sites of RBC extravasation might be related to sites of endothelial mitoses (where the integrity of capillary walls might be impaired) was considered, and endothelial mitoses were noted occasionally throughout the tissue. Although some areas of microhemorrhage were noted in the vicinity of such mitoses, it was not possible to determine whether the endothelial cells directly involved in the four sites of apparent extravasation described above had recently undergone mitosis.

Epithelium-Embedded RBCs

The marked polarization of extravasated RBCs toward the follicular epithelium was confirmed by electron microscopic observations. Occasional extravasated RBCs had not penetrated the follicular basal lamina (Figure 5), but most had passed this structure and were embedded amongst the follicular epithelial cells. Many epithelium-embedded RBCs were clearly in the extracellular region between epithelial cells (Figure 8); however, some were deeply embedded amongst the basal epithelial infoldings, and whether they were extracellular or intracellular was unclear.

No evidence of epithelial uptake of these epithelium-embedded RBCs was observed; i.e., no actual pseudopod formation was observed in these regions, and there were no changes suggestive of early pseudopod development (Figures 8, 10, and 12). Similarly, no suggestion of intraepithelial RBC degradation was noted under these conditions unless evidence of concomitant luminal microhemorrhage was present. Therefore, it appeared unlikely that any of the epithelium-embedded RBCs were intracellular.

Larger pockets of epithelium-embedded RBCs (Figure 10) were easily seen to be extracellular. Such regions were characterized by distorted and attenuated (but apparently intact) epithelial cells. When tightly packed, RBCs in such regions were variously distorted by neighboring RBCs.

RBCs Within Follicular Lumens

Those RBCs which had entered follicular lumens (Figure 11) were most often ultrastructurally normal. Instances of irregularly lobulated or dis-

torted RBCs were, however, observed, generally in association with a region of tightly packed or clumped RBCs. No examples of RBCs at the precise point of entry into a follicular lumen were apparent during this study.

Fate of Extravasated RBCs

It was evident from the time periods in which microhemorrhage was observed that the majority of extravasated RBCs disappeared in less than 1 week; however, the mechanisms involved in their disposal were not entirely clear. Of those RBCs embedded among epithelial cells, it is possible that some entered nearby follicular lumens. It is also possible that some were removed via nearby lympatic channels.[6] In addition, some epithelium-embedded RBCs appeared to undergo a hemolytic process (Figure 13). This process was characterized by the appearance of a homogeneous dense material around RBCs and in nearby regions between epithelial cells. This dense material generally resembled RBC contents. The RBCs involved were irregularly rounded in shape, often with a somewhat decreased matrix density (which frequently varied slightly from cell to cell). Although focal membrane lesions were generally not observed in these cells, a thin pale zone was sometimes noted just inside the cell membrane (Figure 13).

The fate of many RBCs within follicular lumens appeared to be removal by erythrophagocytosis and degradation within 3 to 5 days. This was evidenced by the disappearance of RBCs from follicular lumens (after longer periods of thiouracil stimulation) and the concomitant appearance of RBC phagosomes and degradative stages within epithelial cells. In addition, some intraluminal RBCs also appeared to undergo a form of hemolysis (Figure 11). This differed from the process just described for epithelium-embedded RBCs and was characterized by small, raised disruptions in the RBC surface through which flocculent RBC contents appeared to emerge. Similar flocculent material was sometimes also visible nearby, apparently free within the lumen. Disruptions of the RBC membrane varied from approximately 20 to 300 nm in diameter and were distributed around a sizeable, but variable, portion of the RBC perimeter. The involved RBCs generally had a normal overall shape and did not appear rounded in section. Occasionally such RBCs exhibited a mottled cellular matrix, but evidence of severe hemolysis or true RBC-ghost formation was not observed.

Evidence for the removal of extravasated RBCs by macrophages or leukocytes was not obtained in the present study. Such cells were, in fact, very rarely observed.

Dense Extracellular Patches

Sometimes associated with epithelium-embedded RBCs or with RBCs within follicular lumens were small round or elongate patches of very electron dense, extracellular material (Figures 12 and 13). Although a clear banding pattern was not observed in this material, it appeared similar to deposits of fibrin observed in other systems [7,8] by virtue of its marked electron density and patchy fibrous character.

Discussion

It is clear from the present study that microhemorrhage is a common and consistent finding in the thiouracil-stimulated rat thyroid gland. This, therefore, appears to be a useful system for the study of RBC extravasation associated with vascular hyperplasia. Of particular interest are questions related to the etiology of this process and the site of RBC extravasation.

Etiology of Microhemorrhage

In considering the etiology of microhemorrhage in hyperplastic thyroid tissue, several possible mechanisms seem worthy of discussion: a) The possibility that microhemorrhage might be related to neck trauma during animal handling, or to the vascular perfusion used for fixation, seems ruled out by our finding of the usual frequency and patterns of microhemorrhage in immersion-fixed thyroids from atraumatically handled animals. b) Microhemorrhage may be at least partially related to the hypervascularity associated with thiouracil administration. It is possible that increased intrathyroidal blood flow or blood pressure may interact with a particular weak spot in the vascular system to contribute to the development of microhemorrhage. c) It is possible that changes in blood vessel walls are induced during thyroid stimulation which might result in the production of weak spots. A possible indication of such changes noted during the present study were the findings of occasional clumps of platelets, RBCs, or leukocytes adherent to vessel walls. The frequency of encountering such aggregates generally paralleled the frequency of microhemorrhage observed. However, it was not possible to determine whether a group of adherent cells were actually signaling a change in the vessel wall or were simply reacting to alterations at an old site of extravasation. A vessel wall change, if present, might reflect a region of increased vascular fragility and/or might act as a focus for local thrombus formation. Microhemorrhage might thus ensue from local vascular injury. d) Another possible mechanism for the production of vascular weak spots is by mitosis of endothelial cells. Endothelial mitoses could result in extravasation of RBCs in at least two ways. First, it is possible that cells which

have recently completed division are unusually fragile, such that mechanical injury (perhaps at the site of a thin fenestrated process) is more likely. Secondly, since it is known that the interpolar axis of endothelial division is tangential to (rather than perpendicular to) the vessel,[9,10] it is possible that during telophase sealing of the vascular lumen becomes incomplete. Extravasation of RBCs might then ensue between the daughter cells, in the region of the midbody. Such an apparent increase in vascular permeability—at the level of the midbody—was actually demonstrated by Stehbens [10] in regenerating endothelium using silver nitrate staining. In either of these two ways mitosis could result in the relatively small endothelial openings observed in the present study.

The possibility that microhemorrhage is related to endothelial mitosis is further supported by the relatively large number of endothelial cell divisions encountered in the stimulated thyroid gland. The peak incidence of microhemorrhage—at about the fifth day of thiouracil treatment—correlates in general with the period of maximum mitotic activity for stromal (nonepithelial) cells in thiouracil-treated animals reported by Santler [11] and by Wollman *et al.*[4] In addition, microhemorrhage has been widely reported in a variety of tissues undergoing neovascularization,[12-14] in which growing vessels are characterized by fragile sprouts, reported to contain both proximal [9,15] and distal [16,17] populations of mitotic cells.

Site of RBC Extravasation

The four examples of apparent RBC extravasation noted in this study all involved endothelial openings of less than 1 μ. This raises the question of whether the sizeable numbers of extravasated RBCs observed could all have passed thru such small openings. In this regard, Inomata *et al.*[18] have shown the passage of quantities of RBCs through the endothelium lining the canal of Schlemm, where pores of 0.8 to 1.8 μ were identified. Similarly, Chen and Weiss [19,20] and Cho and De Bruyn [21] have found that RBCs, reticulocytes, and erythroblasts can routinely enter the splenic sinusoids by traversing endothelial pores of less than 1 μ diameter. Smelser [22] and Ohsaka *et al.*[23] have also illustrated the passage of RBCs through endothelial openings of this size in corneal and mesenteric capillaries, respectively. Although it thus seems possible that at least a modest number of RBCs could traverse such an opening, the size of this opening may be a factor in limiting the quantity of extravasated cells, so that a microhemorrhage rather than a macrohemorrhage results. At the present time it is not possible to determine whether all extravasating RBCs pass through these small openings, and it is certainly also possible that temporary larger openings in blood vessels occur and were simply not observed

in the present study, perhaps due to their low frequency or rapid closure. Such larger vascular openings might result in the formation of the more sizeable pockets of RBCs and/or pseudolumens sometimes observed.

Another consistent feature of the sites of apparent RBC extravasation observed in this study was the presence of dense microfilaments and granular material in the endothelial cytoplasm about the opening. A similar material has been reported around the endothelial pores through which RBCs enter splenic [20] or bone marrow [24] sinusoids, where it has been suggested that this material may play a role in the restriction of pore size. Possible considerations of the significance of such material under the conditions of the present study would include the following: a) If RBC extravasation occurs between endothelial cells, this material might represent the usual dense microfilaments associated with endothelial junctions. However, the quantity of microfilaments associated with endothelial openings appeared to exceed that generally associated with intercellular junctions in these cells. b) The dense material may represent microfilaments (sectioned at different orientations) involved in the healing or closure of the endothelial openings. Similar microfilaments have been associated with cellular wound closure in amphibian eggs [25-27] and amebas [28] where a contractile function for these structures has been proposed (see Pollard and Weihing [29] for review of contractile microfilaments). c) If RBC extravasation is related to sites of endothelial mitoses, the dense material may represent microfilaments associated with the mitotic cleavage furrow region or with cellular wound closure occurring in a mitotic fragile area.

Polarization of Microhemorrhage

The localization of the vast majority of microhemorrhage to epithelial rather than interstitial regions most probably results from some polarization in the route of RBC extravasation. Thus if extravasation is through locally injured portions of endothelial cells this would suggest a polarization of the sensitive zones of such cells toward the follicular epithelium. It does appear that the thin fenestrated regions of thyroid capillaries are primarily located adjacent to the epithelium.[30] Alternatively, if extravasation takes place mainly about mitosing endothelial cells, it would seem likely that either the midbody region, or a specific mitotic fragile zone, faces the follicular epithelium.

Route of Entry of RBCs Into the Follicular Lumen

It is presumed that RBCs enter the follicular lumen by passing between epithelial cells and through the tight junction region. Alterations in this

tight junction region in association with thyroid hyperplasia have recently been reported by Tice *et al.*[31] using freeze-fracture techniques. These alterations included zones with very narrow tight junctions. Such zones might constitute weak spots through which RBC penetration could take place. It is also possible that RBC entry into the lumen occurs in the vicinity of mitosing epithelial cells where the follicular luminal seal may be temporarily weak.

Hemolysis of Extravasated RBCs

Apparent hemolytic processes were noted in the present study, involving both epithelium-embedded and intraluminal extravasated RBCs. This mode of destruction is not unexpected for RBCs which have been extravasated for a long time, and was noted most frequently in rats fed thiouracil for 7 or 10 days. The ultrastructure of the process differed in the two locations, possibly reflecting the influence of environment on the hemolytic process. Epithelium-embedded RBCs often appeared rounded in section when associated with hemolysis and may thus undergo the colloid osmotic swelling which appears to be a final common pathway in many varieties of hemolysis.[32] Intraluminal RBCs do not appear to undergo gross swelling prior to hemolysis, quite possibly owing to the high concentration of luminal protein.[33] The actual membrane lesions associated with hemolysis were not seen in epithelium-embedded RBCs, perhaps because of their resealing prior to tissue fixation.[32] Such lesions, however, were clearly visualized (by adjacent hemoglobin) in intraluminal RBCs undergoing hemolysis (see Klebanoff and Cook [34] for comparison), probably because of the slowness of hemoglobin diffusion in luminal colloid.

The precise etiology of hemolysis is not known for the present cases. Membrane alterations secondary to gradual metabolic exhaustion would appear to be one explanation. In the case of intraluminal hemolysis, another consideration would relate to RBC iodination which can produce hemolysis in other systems.[35] Iodination, however, seems unlikely under the conditions of the present study where thyroperoxidase is largely inhibited by thiouracil.

Clinical Correlation

Hemorrhage in the human thyroid gland has occasionally been observed in conjunction with various abnormalities and has sometimes been associated with intraluminal RBCs and or hemosiderin deposits within follicular epithelial cells.[36] Hemorrhage associated with thyroid hyperplasia is known to occur in cases of sporadic nontoxic goiter, sometimes associated with a nodule or cyst.[37] It is possible that the pathophysiologic

mechanisms discussed above play a role in such cases. The generally mild degree of hemorrhage and the specific time period during thyroid stimulation in which it was found to occur in the present studies in rats may explain why hemorrhagic phenomena are reported only infrequently in human hyperplastic thyroid disease.

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Legends for Figures

Figures 1–4—Light microscopic appearance of toluidine blue-stained hyperplastic thyroid tissue from rats fed thiouracil for 5 days. Prominent features in all figures are dilated perifollicular capillaries (*C*), columnar epithelial cells, and narrow follicular lumens (*L*). The majority of perifollicular capillaries have been well perfused and show no RBCs; however, occasional RBC-containing capillaries are evident. Extravasated RBCs are also visible in all figures and are uniformly associated with the epithelial rather than the interstitial face of capillaries.

Figure 1—This survey view shows several regions with basal microhemorrhage (*short arrows*) and microhemorrhage into a lumen (*long arrow*) (×120).

Figure 2—Columns of extravasated RBCs (*short arrows*) as well as isolated RBCs can be seen embedded within the basal region of epithelial cells on the left side of this follicle. Several larger pockets containing variable numbers of extravasated RBCs (*long arrows*) and a "pseudolumen" pocket (*PL*) (containing no RBCs) are apparent on the opposite side. (× 490)

Figure 3—Rather extensive basal microhemorrhage is present in these two follicles (*arrows*). The proximity of the involved regions to dilated capillaries (*C*) is apparent. (× 560)

Figure 4—RBCs within a follicular lumen (*L*) are depicted here and exhibit essentially normal morphology. Basal columns of extravasated RBCs may also be seen (*arrows*). (× 560)

Figure 5—A RBC is shown in apparent transit through a capillary wall. This RBC has a rounded appearance with a marked central constriction (*long arrow*) at the level of the endothelial cell. It has not penetrated the follicular basal lamina (*BL*) in this section. Endothelial fenestrations (*short arrows*) may be seen. *C* = capillary lumen. (× 9900)

Figure 6—This higher magnification of a portion of Figure 5 shows dense microfilamentous (*F*) and granular (*G*) material in the cytoplasm on both sides of the 0.4-μ opening in the capillary wall. (× 24,900)

Figure 7—This nonconsecutive serial section of the RBC shown in Figure 5 passes through endothelial cytoplasm just adjacent to the RBC neck. Numbers of microfilaments (*arrows*) are evident in this region, but no evidence of an intercellular junction is seen. The closest endothelial junction observed in this plane was several microns away from the RBC neck region and was located in very similar position to that seen in the plane of Figure 5. (× 40,000)

Figure 8—Extravasated RBCs are shown embedded between the basal portions of two follicular epithelial cells (E_I, E_{II}). No reaction to these RBCs is noted in the adjacent epithelial cytoplasm (*long arrows*). (× 6400)

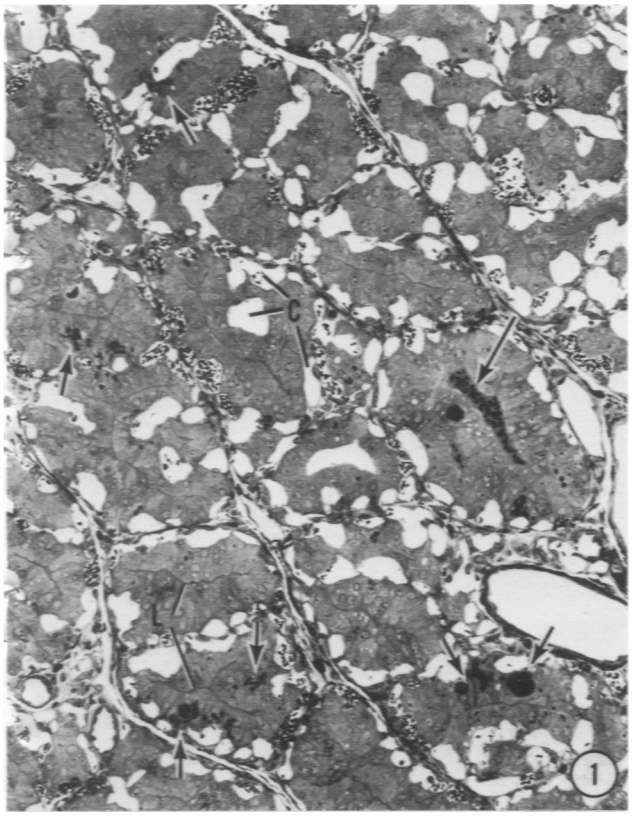
Figure 9—This higher magnification of a portion of Figure 8 shows dense microfilaments (*F*) and granular (*G*) material in the cytoplasm on both sides of the 0.3-μ opening in the capillary wall. (× 31,900)

Figure 10—Part of a pocket of tightly packed extravasated RBCs located at the base of the follicular epithelium (similar to that in Figure 2) is shown. No reaction to these cells is apparent in the adjacent epithelial cytoplasm (*long arrows*). The epithelial cells are attenuated where the RBCs approach the follicular lumen (*L*) and have complex cell-to-cell boundaries (*short arrows*). Microhemorrhage into the lumen was also seen in this follicle, accounting for the presumed RBC phagosomes (*PH*) shown. (× 10,000)

Figure 11—RBCs are shown within a follicular lumen. Although normal RBC morphology is present in some regions, an apparent hemolytic process is evident. This is characterized by raised focal eruptions (*short arrows*) of flocculent material similar in density to the matrix of intact RBCs. Such material can also be seen apparently free within the lumen (*long arrows*). Portions of two pseudopods (*P*), engaged in erythrophagocytosis, are also visible. (× 9100)

Figure 12—A pocket of RBCs in between several epithelial cells (E_I, E_{II}, E_{III}) is depicted. Small extracellular accumulations of dense material (*arrows*) are apparent about the extravasated RBCs. (× 11,200)

Figure 13—A pocket of microhemorrhage similar to that in Figure 12 is shown. In this area, however, a homogeneous material (*long arrows*) similar in density to the matrix of intact RBCs surrounds the RBCs and is present about some of the lateral cellular interdigitations (*long arrows*). A thin pale zone, just beneath the RBC membrane, is also visible in these cells. Small accumulations of dense material (*short arrows*) are again noted. (× 10,300)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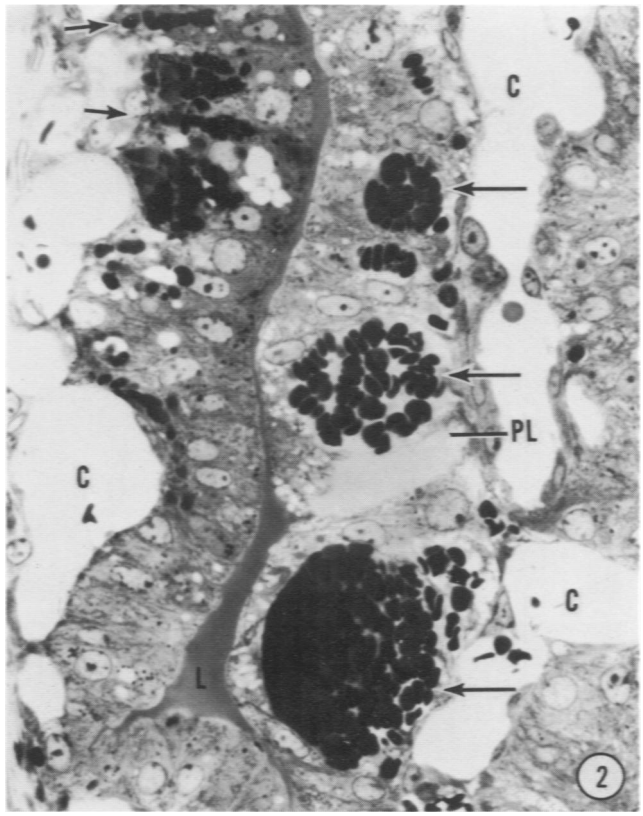
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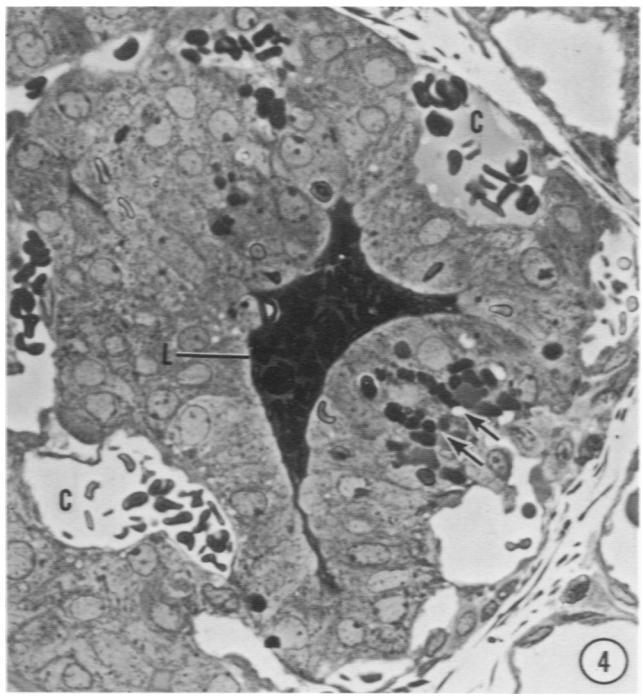
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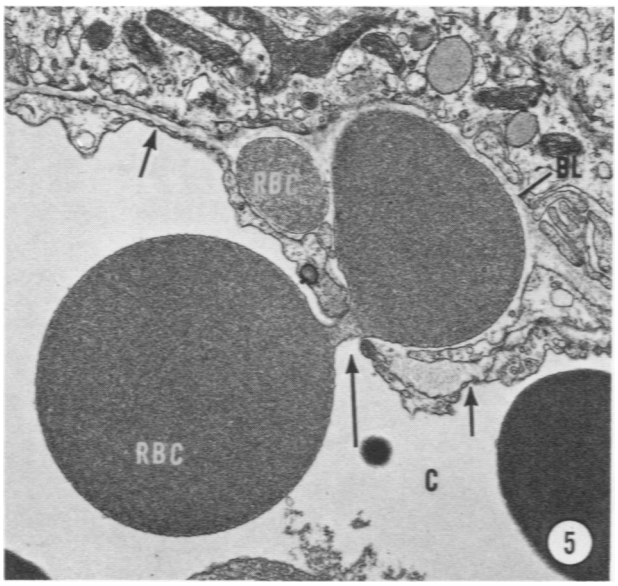
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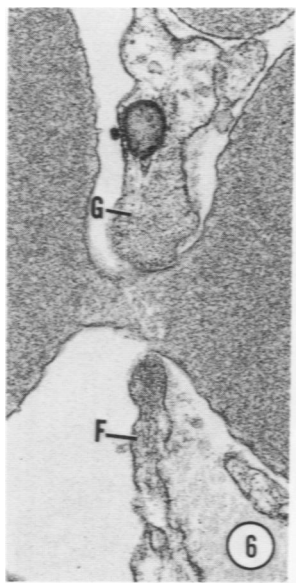
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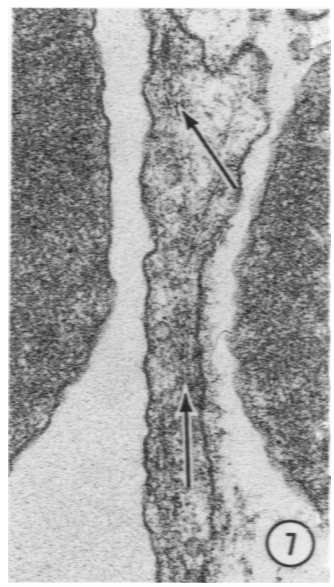
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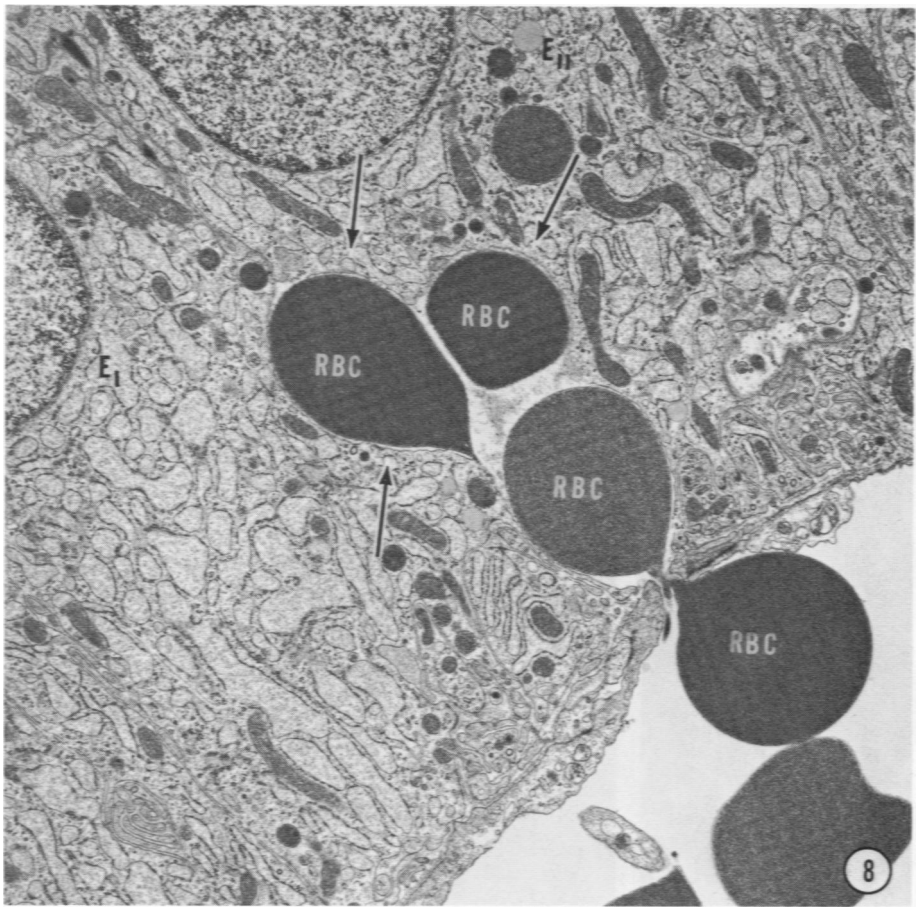
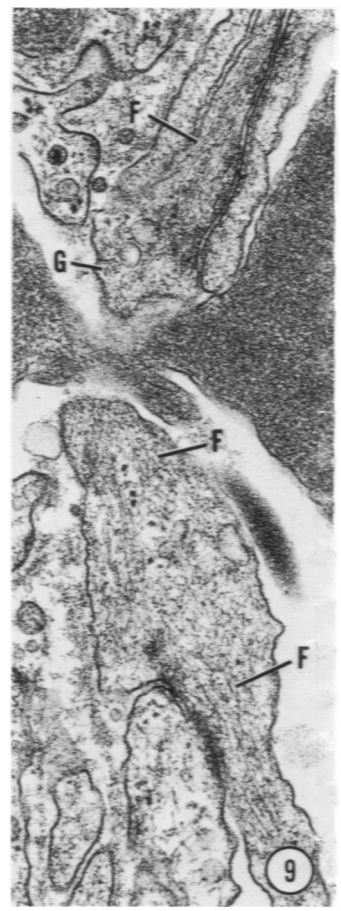
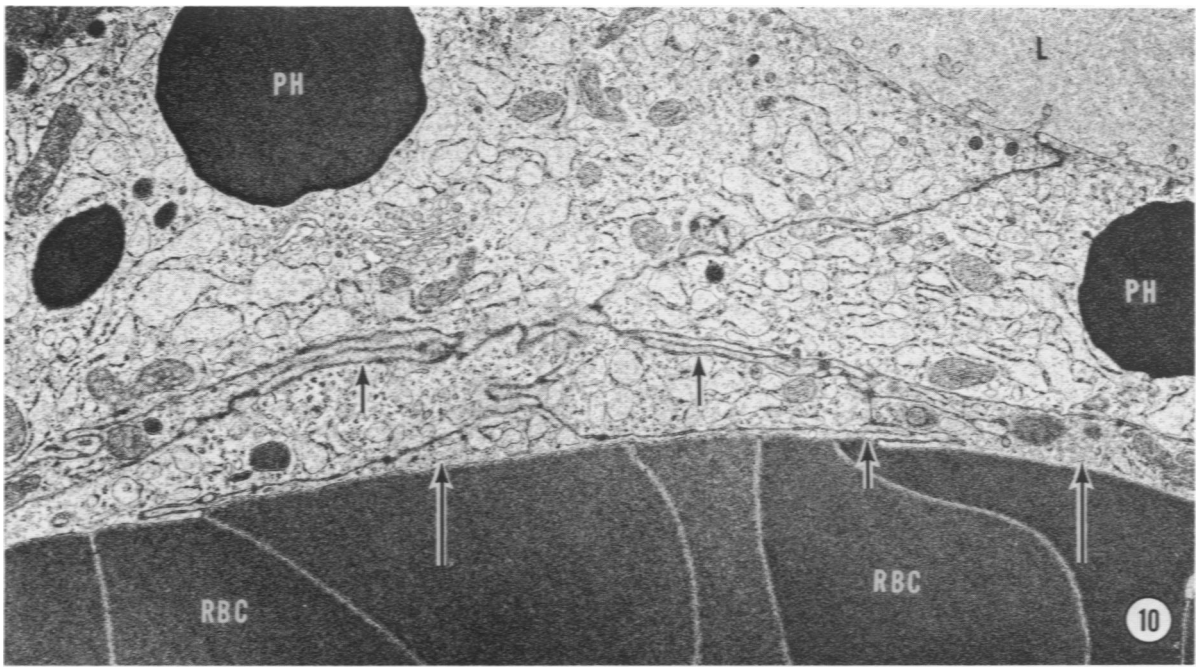
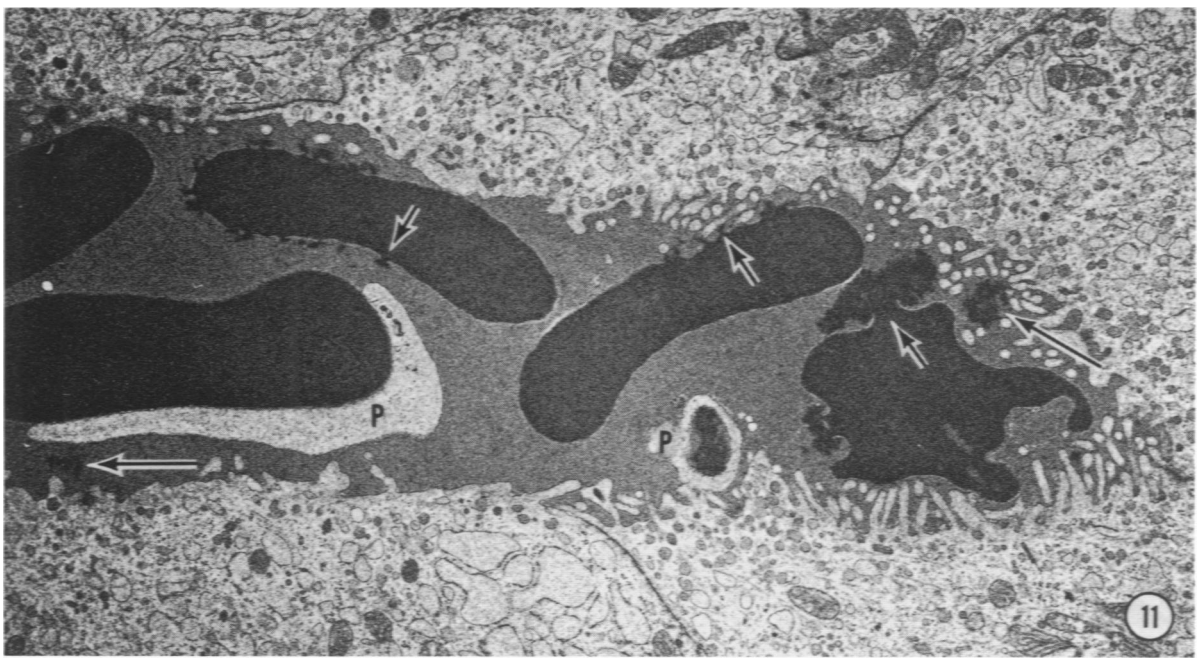

E II
RBC
RBC
RBC
RBC
E I
8


F
G
F
F
9


PH
L
PH
RBC
RBC
10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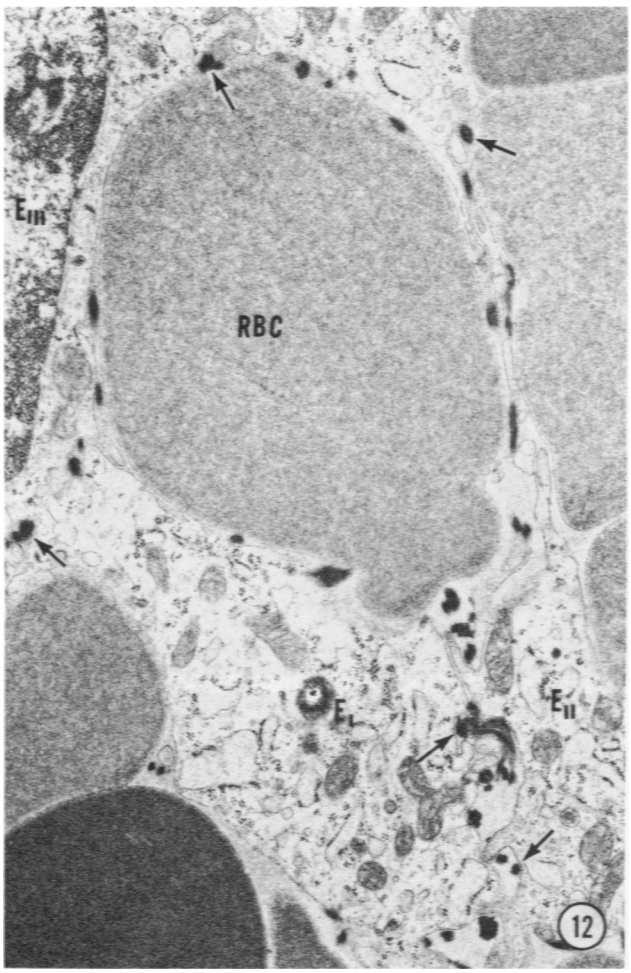
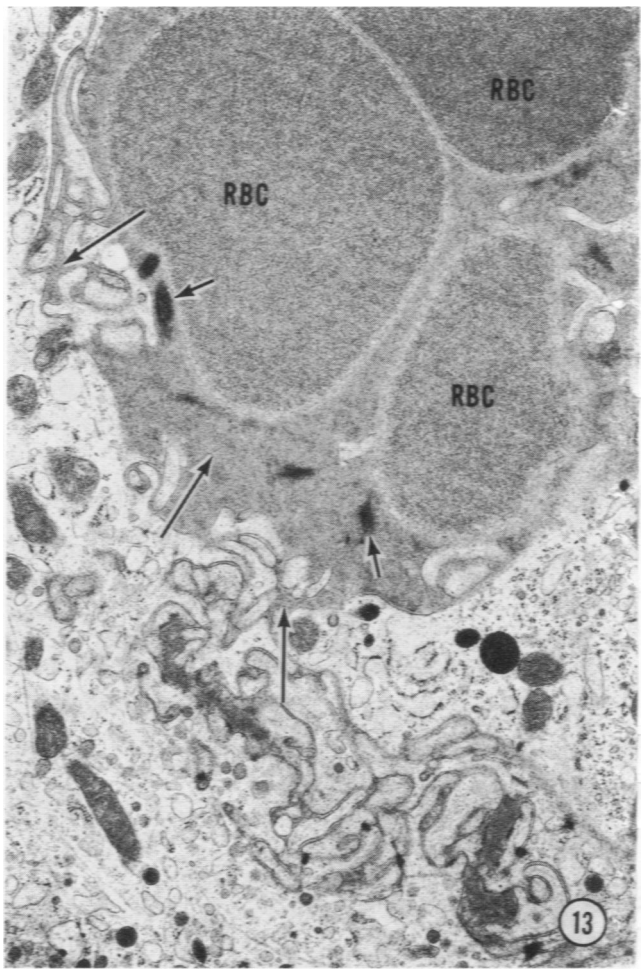

E_{III}
RBC
E_I
E_{II}
12


RBC
RBC
RBC
13