Microangiopathic Hemolytic Anemia in Rats with Malignant Hypertension

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Microangiopathic hemolytic anemia in humans complicates a variety of vascular diseases. This hemolytic process is characterized by fragmentation of erythrocytes with the production of irregularly contracted erythrocytes (including "burr" cells, "helmet" cells, and schistocytes) and spherocytes. The condition may develop in thrombotic thrombocytopenic purpura, malignant hypertension, bilateral renal cortical necrosis, periarteritis nodosa, the hemolytic-uremic syndrome, acute glomerulonephritis, systemic lupus erythematosus, intracardiac plastic prostheses, and advanced cancer.1-9 Brain et al.1 have implicated arteriolar lesions as the basic factor in the pathogenesis of this anemia.

We have observed that induction of malignant hypertension in rats with desoxycorticosterone and salt load consistently results in this type of hemolytic anemia. Although this particular experimental preparation has been used by several investigators,10,11 hematologic observations have not been recorded previously. Acute forms of microangiopathic hemolytic anemia have been produced experimentally by induction of the generalized Shwartzman reaction12,13 and by injection of thrombin14,14 or snake venom.15,16 In these situations, hemolysis appears to coincide with intravascular coagulation. In the current experiments, hemolysis continues over a period of several weeks as the rats develop malignant hypertension, a phenomenon more nearly comparable to the forms of microangiopathic hemolytic anemia commonly recognized in man. With this experimental model we have studied the development of the hematologic disorder in order to gain insight into the cellular mechanisms responsible.

Materials and Methods

We studied 140 male and female white rats of the Charles River strain. Extensive hematologic studies were carried out on 25 hypertensive rats and 25 control rats with or without
unilateral nephrectomy. These rats were maintained on standard Purina® rat chow, and left nephrectomy was performed on each animal except for one half of the control group. Following recovery, the experimental group was given 1 per cent sodium chloride solution ad libitum for drinking water. In addition, the experimental group received 30 mg./Kg. of Percorten (desoxycorticosterone pivalate microcrystalline suspension) subcutaneously once a week. The control group was studied with the same procedures as the experimental group.

Each week the animals were weighed and the systolic blood pressure was measured. Arterial tension was determined using the tail and a microphone manometer.17 Urine was examined weekly for albumin and blood with Albustix® and Hemastix®. Fresh centrifuged urine was also studied microscopically for erythrocytes and the supernatant was tested for hemoglobin. Biweekly microhematocrits and blood films were obtained from the tail. Forty-eight hour fecal samples were collected prior to sacrifice for urobilinogen studies.

After the development of marked erythrocyte abnormalities in the blood smears or severe clinical illness, the animals were sacrificed. Rats dying in the course of the experiment were used only for histopathologic study. At sacrifice, heparinized blood was collected for blood urea nitrogen and creatinine determinations using an AutoAnalyser and blood anticoagulated with EDTA for hematologic measurements18 and phase-contrast studies of the erythrocytes. Erythrocytes and leukocytes were enumerated with the Coulter Counter, and platelets with the phase-contrast microscope. All blood smears were classified by two observers in a blind study for grading of the severity of the erythrocyte changes with remarkable agreement in evaluation.

Tissues were fixed in neutral formalin and Zenker's solution for light microscopy. In many randomly selected cases, tissues were fixed for electron microscopy by excision and fixation or by arterial-perfusion fixation. Fixation was carried out with buffered 4 per cent glutaraldehyde followed by 1 per cent osmic acid or with osmic acid alone. Tissues were embedded in Epon 812,19 cut with glass or diamond knives, stained with uranyl acetate and lead citrate,20 and were examined on bare copper grids in an RCA EMU3G electron microscope.

Light microscopic studies were carried out with hematoxylin and eosin, Weigert's elastic, trichrome, and Perls' iron stains. Wright stains of peripheral blood, and Wright stains and Prussian blue stains of bone marrow and splenic imprints were also studied.

**Observations**

Following the institution of Percorten and sodium chloride therapy, the rats developed a progressive rise in systolic blood pressure starting in 2 to 3 weeks. By 6 to 7 weeks, a systolic pressure of 190–270 mm. Hg. was reached with an average of 218 mm. Hg. Within the first week, polydipsia and polyuria were noted and were probably due to the salt load. After the onset of severe arterial hypertension, with or without renal failure, the initial weight gain ceased and weight loss was common. The average duration of illness terminated by sacrifice or death was 3.7 months. Criteria for sacrifice were moribund state, marked hematuria or hemoglobinuria, or marked erythrocyte abnormalities in the peripheral blood films. Male rats met these criteria in an average of 2.3 months, while female rats met them at 4.3 months.

**Clinical Laboratory Findings During Life**

Repeated urinalyses during the experiments revealed that 77 per cent of the animals developed severe proteinuria. Significant amounts of blood or hemoglobin were present in the urine in 75 per cent of rats.

In the twenty-five animals of both sexes in which a complete series of serial studies was obtained, 77 per cent of the animals developed severe proteinuria. Significant amounts of blood or hemoglobin were present in the urine in 75 per cent of rats.

†The Percorten used in this experiment was generously provided by Robert Gaunt, Director of Biological Research of the CIBA Pharmaceutical Company.
blood smear was available, the average time of onset of significant erythrocyte abnormalities was sixty days from the onset of the experiment. Significant red blood cell changes consisted of anisocytosis, poikilocytosis, microspherocytosis, and increased polychromasia. These changes, in general, became progressively more severe until death or sacrifice. Estimates of platelet numbers were performed on all blood smears; in none was a significant decrease noted. Progressive development of anemia was confirmed by biweekly tail microhematocrits. This anemia was absent in control rats tested concurrently. Stool was collected for 48 hours prior to sacrifice in 9 experimental rats and 6 control rats for urobilinogen excretion. The control average urobilinogen value was 0.56 mg./24 hr. (range 0.4 to 0.77 mg.), while the experimental average was 1.25 mg./24 hr. (range 0.45 to 2.4 mg.). This indicates an increase in red blood cell destruction in the experimental rats.

Clinical Laboratory Findings at Sacrifice

Hematologic Findings (Table 1). All but three of the experimental animals were anemic. Hemoglobins averaged 10.3 Gm./100 ml., hematocrits 30.6 per cent, and erythrocyte counts 4.61 \times 10^{12}/cu. mm. The anemia was normocytic and normochromic. On air-dried films and in wet preparations, a significant proportion of the erythrocytes was usually abnormal (Fig. 1). Irregularly contracted erythrocytes included "burr" cells and schistocytes (fragments). In contrast to crenated cells, the spicules of these erythrocytes varied in size and shape and had rounded ends. Spherocytes were common but variable in number, and frequently had spicules. In some severely anemic rats, a small number of normoblasts were seen in the blood films. Polychromasia and reticulocytosis were prominent in most anemic animals; reticulocytes ranged from 1.5 to 51.3 per cent. Though a mild leukocytosis was occasionally seen, the leukocyte counts were usually normal, and differential counts were not significantly different from normal. Platelet counts did not differ from controls in 4 animals tested from each group; range in experimental rats was 0.88 to 1.47 million/cu. mm., control range 0.86 to 1.24 million/cu. mm.

Osmotic fragility studies (Fig. 2) revealed, in test rats, a small population
MICROANGIOPATHIC HEMOLYTIC ANEMIA

Fig. 1.—Air-dried film of peripheral blood. Upper left: Note three irregularly contracted cells with increased density, three schistocytes (erythrocyte fragments) and several platelets. Upper right: Dense microspherocytes and schistocytes are present. The larger unevenly staining cells in the center are polychromatic erythrocytes. Lower left: Several irregularly contracted cells, two neutrophils, and numerous platelets can be seen. Lower right: Irregularly contracted erythrocytes and a target cell are noted.

In these illustrations, one can see how loss of spicules from the irregularly contracted cells leads to the formation of small, dense spherocytes. Wright stain; 1,000 x.

of osmotically fragile erythrocytes. This roughly corresponded to the proportion of morphologically abnormal erythrocytes in the blood films.

The bone marrow was generally hypercellular, though the M:E ratio was approximately normal. Iron was only occasionally slightly increased. Splenic imprints were not significantly different from those of control animals, with regard to cellular composition and stainable iron.

Blood Chemistry Findings (Table 1). Blood urea nitrogen (BUN) varied from 20 to 133 mg./100 ml. Seven test animals showed normal levels of BUN, but nevertheless had significant anemia and marked red blood cell changes. Serum creatinine and uric acid levels showed a mild increase over the control group; however, the number of determinations performed was insufficient to assess the significance.
Fig. 2.—Erythrocyte osmotic fragility curves, 27 normal and 27 experimental rats. The shaded area represents the range in control rats. In most experimental rats, a triangular tail to the left of the control range indicates that a small proportion of the erythrocytes are fragile.

Gross Anatomic Findings at Death or Sacrifice

In general, the experimental animals had enlarged hearts (mean, 1.49 Gm.; control mean, 1.15 Gm.) and kidneys (4.0 Gm.; control mean 2.2 Gm.). The livers (12.8 Gm.; control mean 11.3 Gm.) and spleens (0.98 Gm.; control mean 0.70 Gm.) were not significantly enlarged. Anasarca was seen occasionally. Seventeen animals showed grossly visible mesenteric artery aneurysms. Three of these animals died of intraperitoneal exsanguination from rupture of an aneurysm. A few animals showed severe bronchiectasis or pneumonitis and were excluded from the experiment. Severe wasting was seen in many of the severely ill animals.

Light and Electron Microscopic Observations

The heart characteristically showed hypertrophy and focal myocardial necrosis. A variety of severe arterial and arteriolar lesions were present in the kidney, pancreas, mesentery, and to a lesser extent in most viscera except the lungs, which were free of vascular lesions. The aorta showed predominantly

*Significance determined at the 5 per cent level with the Student t test.
adventitial fibrosis. Medium-sized muscular arteries showed subendothelial fibrin deposits, smooth muscle necrosis, marked smooth muscle hypertrophy and proliferation, adventitial fibrosis, and granulation tissue in the wall (Fig. 3). In lesions where fibrin deposition and smooth muscle necrosis were prominent, a moderate to severe degree of neutrophilic and histiocytic infiltration was seen in the adventitia and media (so-called hypertensive periarteritis). In spite of intimal and medial smooth muscle proliferation and hypertrophy plus adventitial fibrosis, the arteries underwent progressive aneurysmal dilatation. Thrombosis of these aneurysms was common. Marked extravasation of erythrocytes into the aneurysmal wall was often seen. Histiocytes sometimes showed erythrophagocytosis and were laden with ferritin-filled inclusions. Arterioles tended to develop smooth muscle necrosis with massive deposition of fibrin beneath the endothelium and within the necrotic muscle cells (Figs. 4 and 5). In severe small arterial and arteriolar lesions, endothelial damage was present as shown by chromatin clumping of nuclei, hydropic cytoplasmic swelling, damaged cytoplasmic organelles, or by shrunken darkly staining cytoplasm (Fig. 6).

The kidneys showed severe arterial and arteriolar lesions, patchy tubular atrophy and dilatation, some medullary calcinosis, and a variety of destructive glomerular lesions. Mild glomerular lesions consisted of mesangial cell vacuolar

Fig. 3.—Mesenteric artery showing smooth muscle necrosis (N) and massive accumulations of subendothelial fibrin (F). Lesions such as this precede the formation of aneurysms. Osmium fixation, uranyl acetate and lead citrate stain; 3,100×.
Fig. 4.—Afferent arteriole, kidney. Note the smooth muscle necrosis (N), swollen endothelial cytoplasm (E) which projects inward between erythrocytes, swollen endothelial basement membrane (BM), fibrin (F), platelets (P), and erythrocyte fragments or sections of erythrocytes apparently trapped in altered basement membrane and endothelium (arrows). Erythrocytes are closely applied to endothelial cells. Osmium perfusion fixation, uranyl acetate and lead citrate stain; 4,000×.

degeneration and hyaline droplets in podocytes. Severe acute lesions were characterized by swollen glomeruli with capillary lumens plugged by endothelial and mesangial cytoplasmic swelling, and by fibrin and other protein deposits both in the lumen and the interstices of the mesangium (Fig. 7). The damaged mesangial cells often showed prominent accumulation of lipid droplets. In older glomerular lesions, advanced capillary collapse and scarring were seen.

Relationship of Erythrocytes to Damaged Endothelium

In severely involved small arteries, arterioles, and glomeruli, a remarkable juxtaposition and molding of erythrocytes by damaged endothelium were often seen (Figs. 3, 4, and 8). Occasionally, platelets appeared to participate in this phenomenon, molded against both endothelial plasma membrane and red blood cells (Figs. 4 and 5). With fixation by perfusion, these close relationships were noted in experimental animals but not in controls; thus, the changes did not appear to be shrinkage artifacts such as can be seen in immersion fixation. Whole erythrocytes or cell fragments were sometimes enclosed complete-
Fig. 5.—Necrotic renal arteriole. A platelet (P) and two reticulocytes (R) are adjacent to damaged endothelium (E). Fibrin showing characteristic periodicity is beneath the endothelium. Osmium perfusion fixation, uranyl acetate, and lead citrate stain; 30,000×.

Morphologic Evidences of Increased Erythrocyte Destruction

The livers of diseased rats consistently showed a moderate accumulation of iron pigment in Kupffer cells and rarely in hepatocytes (Fig. 9A). Electron microscopy of the liver showed occasional Kupffer cells with phagocytized erythrocytes; almost every Kupffer cell had accumulations of ferritin in membrane-lined cytosomes. Control livers were uniformly negative with iron stains. Iron stains of spleens showed moderate iron content in both diseased animals and controls. Bone marrow iron was slightly increased in only part of the anemic group. In the kidney, moderate to marked deposition of iron was seen in tubular epithelial cells and, to a lesser extent, in the interstitium (Fig. 9B). A minimal deposit of ferritin was seen in vacuoles in mesangial cells. Control kidneys showed no iron or small traces of iron.

Anatomic-Hematologic Correlations

For comparison with blood hemoglobin levels at the time of sacrifice, the
Fig. 6.—Pancreatic arteriole. Note damaged endothelium (E), and a small early thrombus (T) composed of platelets and fibrin. Erythrocytes are juxtaposed to both the thrombus and the damaged endothelium. A smooth muscle cell (S) is relatively undamaged. Osmium perfusion fixation, uranyl acetate and lead citrate stain; 4,500×.

severity of the arterial, arteriolar, and glomerular lesions was graded from zero to three plus, based roughly on the number and size of lesions. The type of lesions, as described above, tended to be similar in affected animals regardless of severity. Animals with more severe vascular disease had a greater degree of anemia (Fig. 10); they also tended to have more striking red cell abnormalities and higher reticulocyte counts. No correlation was noted between blood urea nitrogen and either anemia or red cell morphology.

DISCUSSION

Significant anemia associated with striking red cell abnormalities was a regular finding in rats with malignant hypertension. A hemolytic process was confirmed by increased osmotic fragility, reticulocytosis, circulating normoblasts, and increased fecal urobilinogen. The increased osmotic fragility was limited to a small population of erythrocytes which probably represented the deformed cells seen in the blood smears.

The presence of hemoglobin in the supernatant of centrifuged urine and iron in renal tubules indicated that many erythrocytes underwent intravascular hemolysis. Kupffer cells showing erythrophagocytosis and siderosis indicated that many damaged red cells were removed from the circulation by the liver. Both intravascular and extravascular hemolysis were therefore present. The
Fig. 7.—Renal glomerulus. This badly damaged glomerular lobule shows three sections of residual capillaries containing erythrocytes, most of which appear to be in close approximation to endothelial cytoplasm. Collapsed capillaries, mesangial swelling, proliferation of mesangial cells, and protein deposits may also be noted. Osmium perfusion fixation, uranyl acetate and lead stain; 1,500×.

amount of iron in normal rat spleens made it impossible to evaluate a mild increase in iron deposition in this organ. In normal rats, the majority of erythrocytes is removed by the spleen.21 The marrows in the experimental rats only occasionally showed a minimal increase in iron. Regarding the extravascular hemolysis, it appeared that the liver was responsible for removal of most of the damaged red cells.

Mechanism of Erythrocyte Fragmentation

In accord with the observations of Schwartz and Motto22 and of Brain et al.4 we found no correlation between the degree of anemia and the level of the blood urea nitrogen. About one third of our animals with significant anemia and deformed erythrocytes had normal blood urea nitrogen levels.

In the short-term experiments of Brain and his co-workers,12-16 hemolysis (hemoglobinemia) coincided in time with intravascular fibrin deposition and persisted only when fibrinolysis was prevented. Bull et al.23 also demonstrated in vitro fragmentation by forcing red cells through fibrin clots and felt that this might be a mechanism of fragmentation in vivo. Although our experimental model is different, this proposed mechanism could play a role, since we occasionally observed erythrocytes enmeshed in arteriolar thrombi.

In addition, however, we were impressed by the frequent close juxtaposition
of erythrocytes to endothelial cells, apparent molding of projections of endothelial cells around the erythrocytes, and occasional trapping of parts of erythrocytes by endothelium. Although it is impossible to demonstrate actual adherence with the technics employed, the following points suggest that these observations may be significant:

1. These findings were consistent in the kidneys of experimental rats perfused by osmium or glutaraldehyde fixatives, but not in control animals treated in the same manner.

2. Brain et al. also observed “red cells lying against the endothelium in palisade form, rather than free in the lumen.”

3. Although there is an apparent 250 to 500 A gap between plasma membranes of the erythrocytes and endothelium, experiments in progress using colloidal iron (which reacts with acid mucopolysaccharides) show that it is present in this space, suggesting that the space is occupied by the exterior glycoprotein coats of the cell membranes.

Sialic acid-containing glycoproteins which coat erythrocytes and endothelial cells are largely responsible for their negative charge and mutual repulsion. This negative charge may be reduced, and adhesiveness increased, by basic proteins including fibrinogen. Together with our observations, these considerations suggest that red cells may become adherent to endothelium, perhaps mediated in some manner by the clotting process. Under these conditions, the force of arterial blood flow could result in a shearing stress sufficient to break...
the red cells into two or more fragments. Rand\textsuperscript{27} has shown that after a limit of deformation is reached, an erythrocyte can lose successive fragments without gross lysis. In inflammation, Allison et al.\textsuperscript{28} have observed red cells trapped in endothelial defects breaking into two parts as a result of sudden changes in intravascular pressure. We postulate that a similar mechanism may be operating in this form of hemolytic anemia, possibly in addition to both fragmentation and intravascular hemolysis resulting from direct interaction of the erythrocytes and intravascular thrombi as proposed by Brain and his co-workers.

This experimental model is suitable for detailed study of the pathogenesis of the microangiopathic hemolytic anemia found in malignant hypertension.

**Summary**

Unilaterally nephrectomized rats given desoxycorticosterone and salt load developed malignant hypertension with widespread vascular lesions and, coincidentally, a hemolytic anemia. The anemia was characterized by red cell fragmentation, increased erythrocyte osmotic fragility, and evidence of both intravascular and extravascular hemolysis. Thrombocytopenia was not observed. Male rats were more severely affected than female animals. The degree of anemia correlated well with the severity of the vascular lesions, but not with the level of blood urea nitrogen.

Based upon electron microscopic observations of the relationship of red cells to damaged endothelial cells and thrombi, possible mechanisms for the red cell fragmentation are discussed.
Fig. 10.—Hemoglobin concentration of the blood at the time of death plotted against the severity of vascular lesions graded from one to three plus. In general, the greater the degree of vascular damage, the more severe the anemia.

SUMMARIO IN INTERLINGUA

Rattos subjicite a nephrectomia unilateral disveloppava—post le administration de desoxycorticosterona e cargation a sal—hypertension maligne con extense lesions vascular e, in coincidentia, anemia hemolytic. Le anemia esseva characterisate per fragmentation erythrocytic, un augmentate fragilitate osmotic del erythrocytos, e evidentia de hemolyse tanto intra-como etiam extravascular. Thrombocytopenia esseva absente. Animales mascule esseva afficite plus severmente que animales feminin. Le grado de anemia esseva ben correlationate con le severitate del lesions vascular sed non con le nivello del nitrogeno de urea in le sanguine.

A base de observationes a microscopia electronic relative al relation inter le erythrocytos e lesionate cellulas endothelial e thrombos, le possibile mechanismos del fragmentation erythrocytic es commentate.

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