Response of Blood Cells to Hemorrhagic Shock in the Dog

Shigeharu KUDO, Hirotsugu ONAI* and Ryo OGAWA**

Response of canine blood cells to hemorrhagic shock was examined using ultrastructural and ultracytochemical techniques. Blood platelets responded to hemorrhagic shock with a decrease of α-granules and the simultaneous development of the platelet canalicular system which opened to the extracellular environment, and platelets having no or few granules appeared at the end of the experimental period. Neutrophil leukocytes also responded to shock, with the decrease of both specific and azurophil granules. Eosinophil leukocytes were morphologically unchanged before and after shock but basophils were not found in the present observations. The effects of dexamethasone and phenoxybenzamine on inhibition of blood cell degeneration during hemorrhagic shock were examined. These drugs were considerably effective to inhibit degeneration of blood cells. (Key words: hemorrhagic shock, blood cells, granule release, lysosomal enzymes, dog)

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Experimental hemorrhagic shock causes an increase in serum lysosomal enzymes[1-3]. The major sources of these enzymes are thought to be the intestinal epithelium[3,4], liver[4-7], pancreas[8,9], spleen[10], or kidney[11]. The enzymes contribute directly to the pathophysiology of shock by exerting a splanchnic vasoconstrictor effect in the pancreas[12], or in the entire splanchnic region[12-15].

Blood platelets are known to be important in hemostasis, thrombosis and blood coagulation, and during this process or when appropriately stimulated they release biologically active substances[16,17]. Platelets contain lysosomes, α-granules and serotonin granules[18], and fusion with the canalicular system of the organelles results in extrusion of their contents into the canalicular system and thereby to the extracellular environment[17]. This process initially involves serotonin granules and then α-granules[19]. Neutrophil leukocytes migrate into damaged tissues within minutes[20]. They contain lysosomes and other granules[20-22] and release lysosomal enzymes in inflammation, tissue injury and in response to appropriate stimulation[20,23]. On the other hand, various cellular factors such as leukocytes, blood platelets, endothelial cells and macrophages have been discussed with regard to the genesis of a shock occurrence[24]. It is not clear in detail, however, whether or not platelets and leukocytes respond to hemorrhagic shock by the release of their granules containing lysosomal enzymes, thereby increasing the serum levels of these enzymes, resulting in mediation of irreversible tissue damage during hemorrhagic shock.

Corticosteriods have been shown to stabilize lysosomal membranes, thus providing cellular protection in various forms of shock[25,26]. Sakauchi et al.[27] stated that plasma β-glucuro-

*Departments of Anatomy, *Surgery and **Anesthesiology, Gunma University School of Medicine, Maebashi, Japan*

Address reprint requests to Dr. Kudo: Department of Anatomy, Gunma University School of Medicine, 3-29-22 Showa-machi, Maebashi, 371 Japan

nidase activity was markedly elevated in dogs when hemorrhagic shock was induced, but that the enzymatic activity was not elevated significantly when hemorrhagic shock was induced after pretreatment with phenoxybenzamine (POB). Thus, the effects of dexamethasone and POB on inhibition of blood cell degeneration during hemorrhagic shock were examined.

The present study was therefore undertaken in an attempt to obtain morphological evidence of response of blood cells to hemorrhagic shock and to facilitate a better understanding of the origin of lysosomal enzymes in blood serum during hemorrhagic shock.

Materials and Methods

Ten adult mongrel dogs of both sexes weighing 10–15 kg were anesthetized with sodium pentobarbital, 25 mg/kg intravenously, injected with succinylcholine (relaxin) 2 mg/kg, intubated with an endotracheal tube and ventilated with room air using a Harvard pump respirator at a constant volume and rate to achieve a PaO_2 of more than 90 mmHg and a $PaCO_2$ of 30–40 mmHg. Polyvinyl catheters were placed in the abdominal aorta and inferior vena cava for pressure measurement and sampling of blood. The carotid artery was then cannulated for rapid removal of blood with polyethylene tubing which was connected to a blood reservoir. The dogs were systemically heparinized, and following controlled measurements, hemorrhagic shock was rapidly produced by adjusting the height of the blood reservoir connected to the carotid arterial cannula. Systemic arterial blood pressure was maintained at 40 mmHg for 2–4 hr.

Arterial blood samples (8 ml) were drawn just before hemorrhage and at 30 min, 1 hr, 2 hr and sometimes 3 and 4 hr after bleeding. The blood was collected in plastic tubes and centrifuged at 1,500 rpm for 15 min after which the plasma was decanted. For electron microscopy, leukocyte- and platelet-rich plasma was then pipetted into 5 vol. of 1.25% glutaraldehyde phosphate-buffered at pH 7.4 containing 1% sucrose. The fixation for 1 hr was followed by the alternate repeat of both washing with the same buffer and centrifugation, and then the cells were osmicated for 2 hr at 4°C. For cytochemistry for revealing peroxidase activity, the cells were incubated in a diaminobenzidine medium[28], followed by osmication. Cytochemistry for demonstrating serotonin granules in platelets was carried out according to the method of Wood[29]. Glutaraldehyde-fixed platelets were incubated for 24 hr at 4°C in a solution of 2.5% potassium dichromate and 1% sodium sulphate in a 0.2 M acetate buffer (pH 4.1). The platelets were then dehydrated in graded alcohols without postosmication. The cells for electron microscopy were embedded in Epon 812.

The drugs used, dexamethasone (5 mg/kg) and POB (2 mg/kg), were intravenously administered before induction of hemorrhagic shock. Pieces of hepatic tissue and blood samples were excised or drawn at various time intervals, and they were processed for electron microscopy.

The number of leukocyte and platelet granules before and after hemorrhagic shock was counted in electron micrographs, and represented as the mean in 300 μm^2 of cytoplasm in the former and in 3,500 μm^2 of cytoplasm in the latter in five dogs.

Results

Platelets before and after shock

Since the general ultrastructure of the platelet is similar with regard to cell organelles and other constituents which have been thoroughly reported in several species[17,18,20,29], only platelet granules and the canalicular system before and after hemorrhagic shock will be characterized in detail here in determing morphological alterations due to the response of platelets to hemorrhagic shock.

Before hemorrhagic shock, three kinds of granules, α-granules, serotonin and dense granules (possibly lysosomes), were present scattered among other organelles with a tendency to be gathered in the central part of the cell. The α-granules were spherical or oval in shape, measuring 190–380 nm in diameter, and were the most aboundant of the three kinds of granules (fig. 1). They were of moderate electron opacity and tightly surrounded by a limiting membrane. The serotonin granules were 190–320 nm in diameter with an eccentrically-located dense core surrounded by a clear halo (fig. 2). The dense granules measured

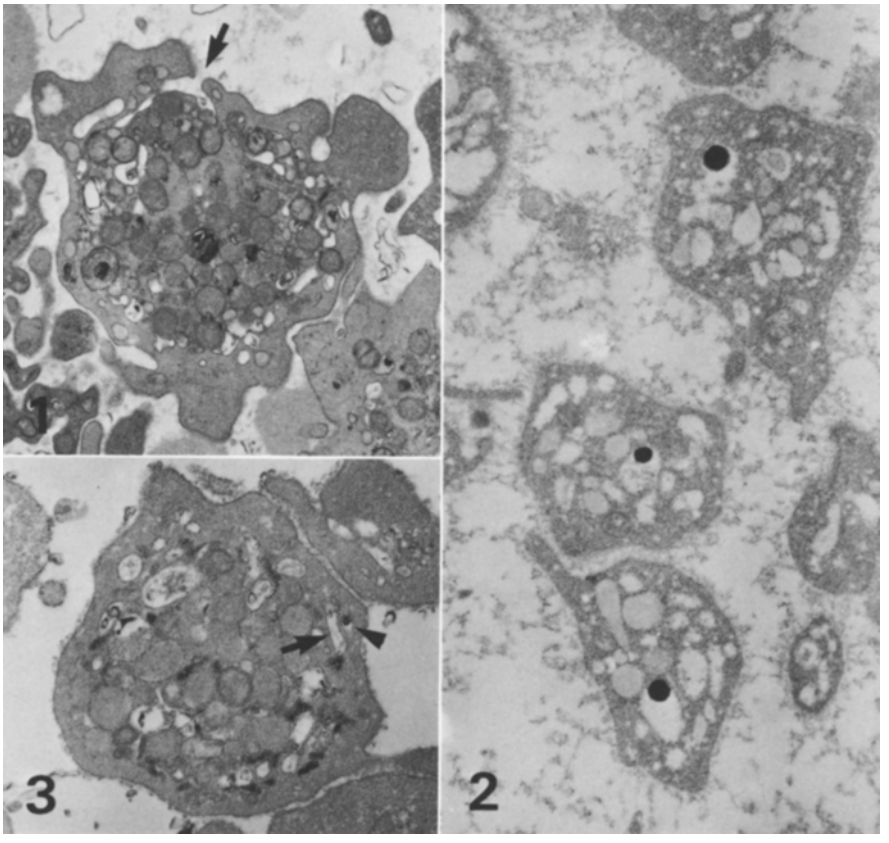

Fig. 1. Blood platelet before hemorrhagic shock. The α-granules are somewhat localized in the central region of the cell and the canalicular system is seen near the periphery, opening to the extracellular environment (arrow). × 11,900

Fig. 2. Serotonin granule in a platelet before hemorrhagic shock. The dense core is eccentrically located. The electron density of the granule has been produced by a technique for revealing the localization of catecholamines. × 18,700

Fig. 3. Localization of peroxidase activity in a platelet before hemorrhagic shock. Cisternae of the rough endoplasmic reticulum are positive for this activity but the canalicular system is not (arrow). The arrowhead shows a serotonin granule. × 18,700

160–190 nm. The number of both serotonin and dense granules was much less than that of α-granules, and only zero to three granules could be found in ultrathin-sectioned platelets. The platelet canalicular system ramified throughout the entire cytoplasm, varying quantitatively from cell to cell and frequently communicating with the surface membrane. These morphological characteristics were observed as fragmented channels of the system in ultrathin sections. Peroxidase activity was demonstrated in cisternae of the rough endoplasmic reticulum, to which ribosomal adhesion was very poor (fig. 3).

After hemorrhagic shock, the most striking change occurred in the canalicular system of the platelets. Development of the canaliculi became more marked with the passage of time, showing frequent openings to the extracellular environment and simultaneous decrease of α-granules (figs. 4 and 5), so that the channels of the canalicular system became dilated, showing a larger luminal diamter than in those before hemorrhagic shock (fig. 5). Their diameter was similar to or larger than the size of α-granules and their cavities were filled with a moderately electron-dense material similar in density and

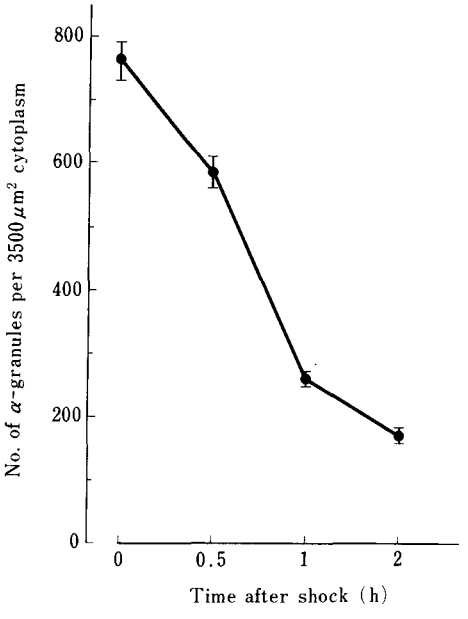

Fig. 4. The relationship between the number of α-granules in platelets and the passage of time after hemorrhagic shock

texture to that of α-granules and sometimes contained a spherical material similar to the core of serotonin granules or to dense granules. At 2 to 4 hr after the shock there were frequently some platelets which has no or few granules and a simultaneous lack of the canalicular system. Peroxidase activity in cisternae of the rough endoplasmic reticulum was unchanged, and serotonin granules were frequently found even at 4 hr after the shock (fig. 6). Such platelets containing serotonin granules had a few α-granules and a poorly-developed canalicular system, having suggested no release reaction.

Leukocytes before and after shock

Before hemorrhagic shock, the cytoplasm of neutrophil leukocytes is rather dark and contained a great number of granules which were spherical or ellipsoid, and sometimes dumbbell- or rod-shaped (fig. 7). They could be morphologically divided into two main types: one larger and denser, and the other smaller and less dense. The former had a tendency to be ellipsoid and frequently contained crystalloids. The spherical form frequently observed in the

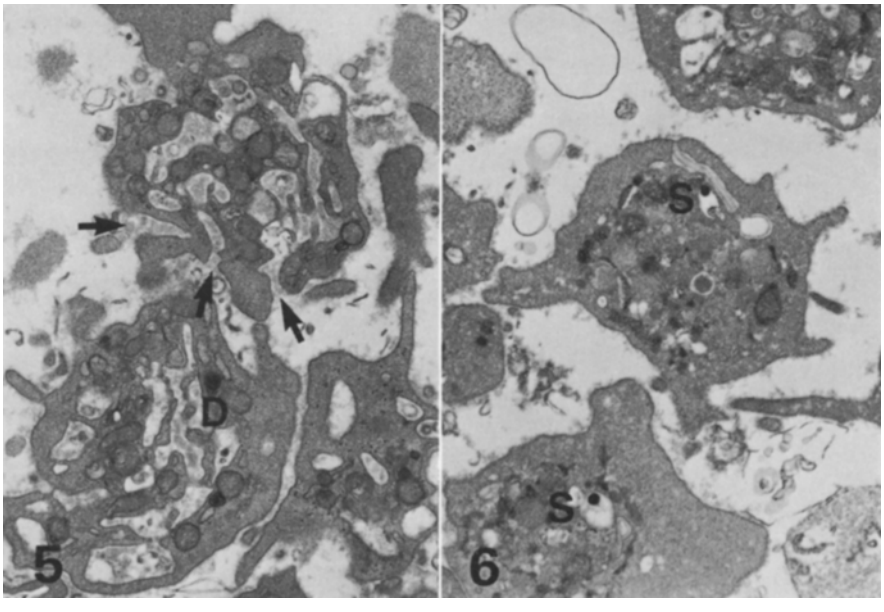

Fig. 5. A platelet at 2 hr after hemorrhagic shock, showing decrease of α-granules and the simultaneous development of the canalicular system. The arrow shows the opening of the canaliculi to the extracellular environment. D; dense granule. × 12,300

Fig. 6. Localization of peroxidase activity in platelets 4 hr after hemorrhagic shock. The localization is similar to that in the preshock period. S; serotonin granule. × 15,100

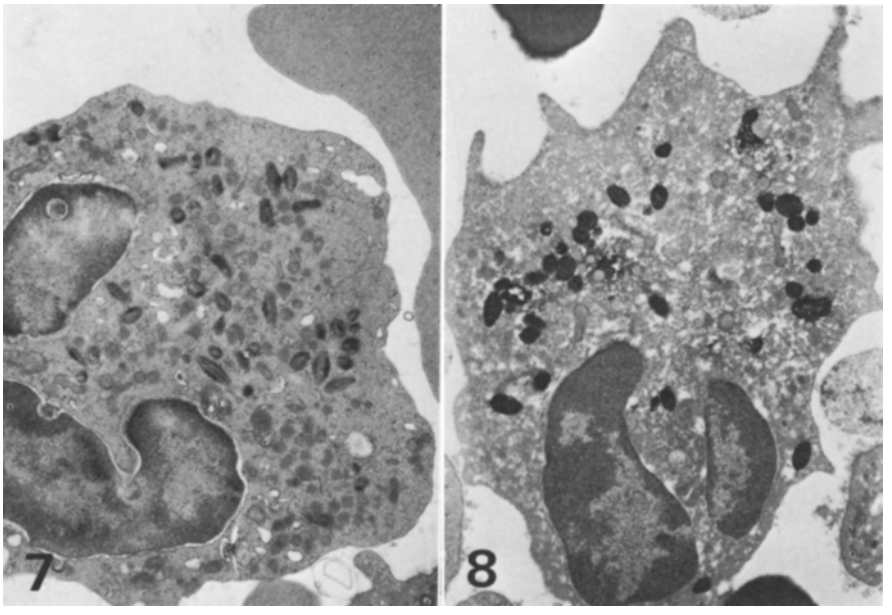

Fig. 7. A neutrophil leukocyte before hemorrhagic shock. A large number of granules are seen in the cytoplasm. × 8,900

Fig. 8. Peroxidase-positive azurophil granules in a neutrophil before hemorrhagic shock. About thirty granules can be counted in the cytoplasm. × 9,500

former may have been a cross-section caused by slicing through an ellipsoid. Since the former granules were reactive to peroxidase activity, they were identified as azurophil granules which have been reported to contain peroxidase and large amounts of four lysosomal hydrolases[21] (fig. 8). The other granules which were non-reactive to peroxidase activity were identified as specific granules.

After hemorrhagic shock, both azurophil and specific granules decreased in number with the passage of time (figs. 9 and 10). Thirty minutes after hemorrhagic shock, both granules had a tendency to decrease in the periphery of the cells. After 60 min, both granules were scattered through the whole cytoplasm or located in small groups in the periphery of leukocytes whose cytoplasm became clearer than in that before induction of the shock and sometimes contained a few deformed vaculoes. Exocytosis of the specific granules was sometimes found (fig. 11), but no evidence could be obtained for exocytosis of peroxidase-positive azurophil granules, while the positively-reacting granule contents which had been discharged were very frequently found

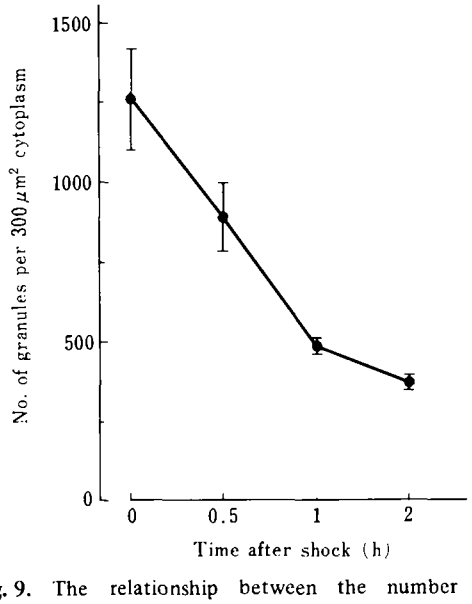

Fig. 9. The relationship between the number of specific and azurophil granules in neutrophils and the passage of time after hemorrhagic shock

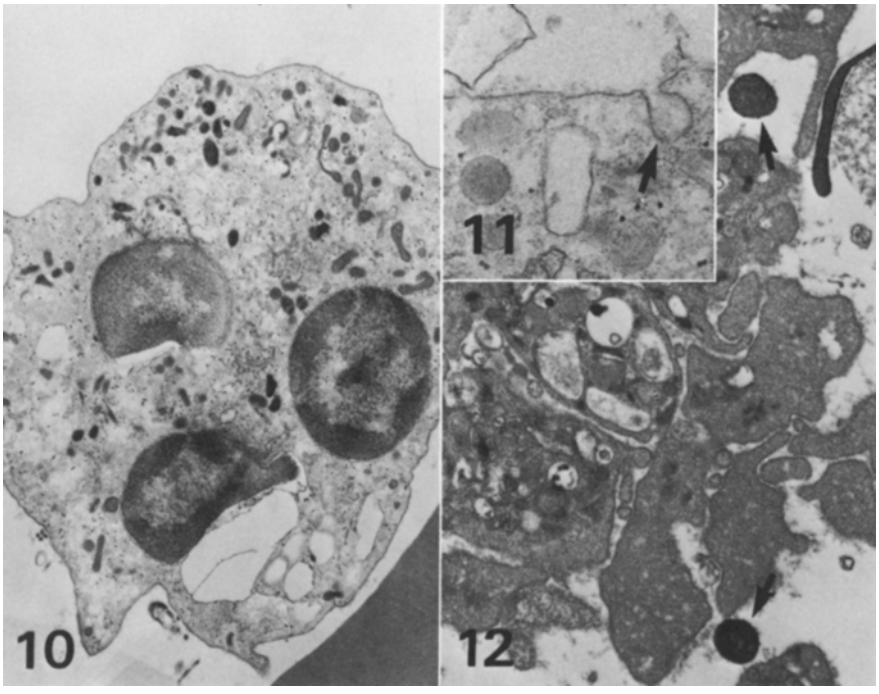

Fig. 10. A neutrophil 2 hr after hemorrhagic shock, showing a remarkable decrease of the granules compared with those in the preshock period (fig. 7). ×7,700

Fig. 11. Exocytosis of a specific granule (arrow) from a neutrophil 3 hr after hemorrhagic shock. × 26,900

Fig. 12. Peroxidase-positive azurophil granules (arrows) which have been discharged to the extracellular environment 2 hr after hemorrhagic shock. × 16,600

in the extracellular environment (fig. 12). Eosinophil leukocytes remained unchanged in morphology during hemorrhagic shock and there was an apparent absence of basophils in the present experiments.

Degeneration of blood cells and Kupffer stellate cells

Blood cells phagocytosed by Kupffer stellate cells in hepatic sinusoids were also examined by electron microscopy. Thirty minutes after induction of shock, a considerable number of erythrocytes and platelets were seen to be phagocytosed by the stellate cells (fig. 13). The phagocytosed platelets contained few granules, suggesting that blood platelets begin to discharge their granules during the course of circulation within 30 min, and that considerable numbers of them are in a state of degeneration after granule release. At 1 hr after shock, the numbers of neutrophil leukocytes phagocytosed by Kupffer stellate cells were considerable (fig. 14), but eosinophil and basophil leukocytes were not phagocytosed. The phagocytosed leukocytes contained much fewer granules than those present before the shock. At 2 hr after shock, phagocytosed neutrophils showed a clear cytoplasmic matrix and cytoplasmic degeneration in addition to degranulation. At 4 hr, Kupffer stellate cells were hypertrophic (Fig. 15), having vigorously phagocytosed platelets and degenerated neutrophils. Degradation of degenerated neutrophils themselves was frequently observed without subsequent phagocytosis by Kupffer stellate cells.

Effect of drugs on inhibition of blood cell degeneration

Intravenous preadministration of dexamethasone or POB almost completely inhibited morphological alterations in platelets and leukocytes even at 1 hr after induction of

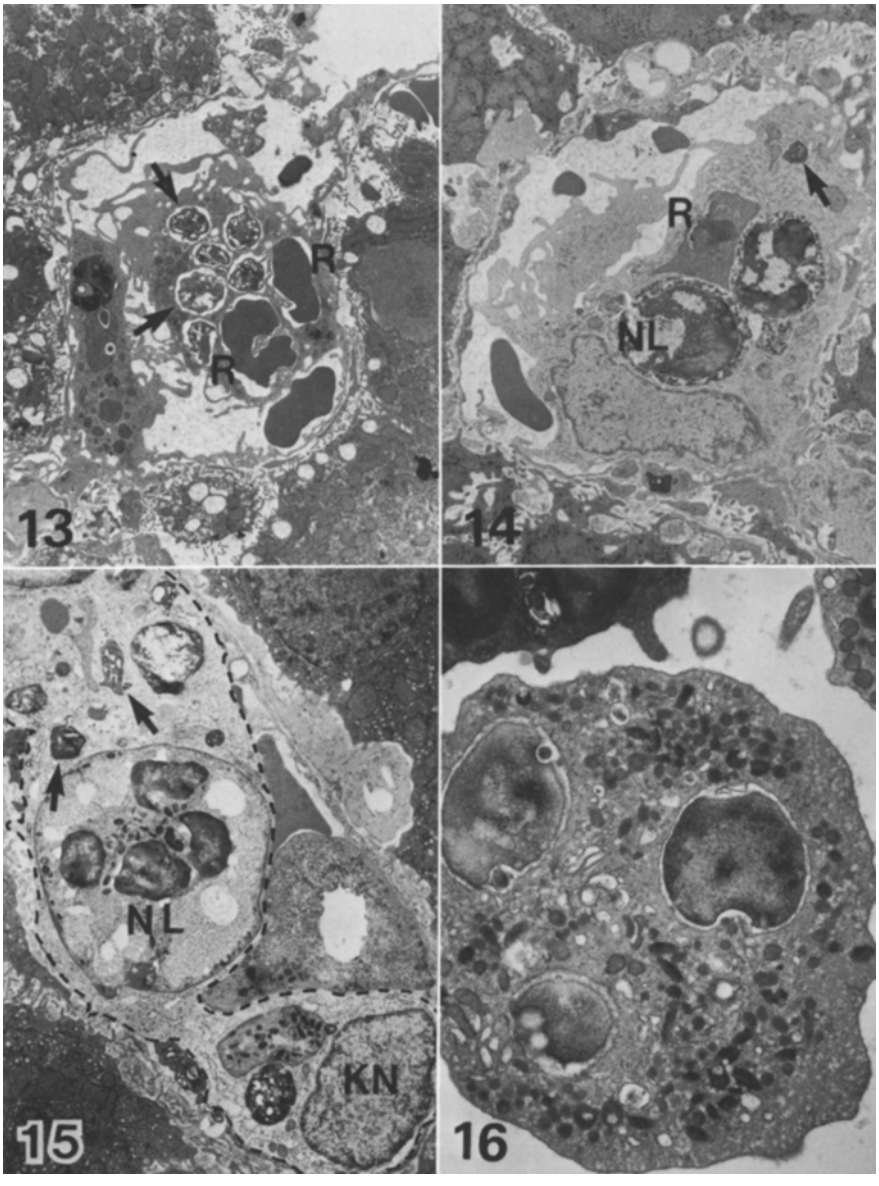

Fig. 13. Blood platelets (arrow) and erythrocytes (R) phagocytosed by a Kupffer stellate cell 30 min after induction of hemorrhagic shock. × 2,500

Fig. 14. Neutrophil leukocytes (NL) and an erythrocyte (R) phagocytosed by a Kupffer stellate cell at 1 hr after induction of hemorrhagic shock. The arrow indicates a platelet which has probably been digested. × 3,000

Fig. 15. Hypertrophy of a Kupffer stellate cell. The outline of the cell is indicated by a broken line, but the whole periphery is not covered by this frame. Neutrophil leukocytes (NL) and platelets (arrow) phagocytosed by the stellate cell are seen. KN: the nucleus of a Kupffer stellate cell. × 3,400

Fig. 16. A neutrophil leukocyte, 2 hr after hemorrhagic shock following preadministration of POB. The morphology is almost similar to that observed before induction of hemorrhagic shock (compare with fig. 7). × 8,900

hemorrhagic shock, although the canalicular system of several platelets had developed somewhat. Two hours after shock, the development of canaliculi in the platelets, which were not very numerous, was accompanied by various degrees of decrease in α-granules, but many platelets and neutrophils had not altered morphologically (fig. 16). No phagocytosis of these cells by Kupffer stellate cells was observed, except for erythrocytes. At 3.5 hr, a striking development of the canaliculi in platelets was accompanied by a marked decrease in α-granules, and the granules of neutrophils were also decreased without any signs of cytoplasmic degeneration. Phagocytosis of both types of cell by Kupffer stellate cells had begun by this stage. Development of the canaliculi in platelets became more striking than at the previous stage 4 hr after hemorrhagic shock, being attended by a marked decrease in α-granules or sometimes by a lack of granules. In neutrophils also, the decrease in granules was more marked. A high frequency of platelets and neutrophils phagocytosed by Kupffer stellate cells was observed. The extent of morphological alterations in the blood cells varied to a lesser degree with preadministration of POB than that occurring with preadministration of dexamethasone, when cell damage for the same period after hemorrhagic shock was compared.

Discussion

The results of this study indicate that platelets and neutrophil leukocytes in the dog release their granules into the surrounding medium (plasma) upon hemorrhagic shock, and may thus contribute to the rise in serum lysosomal enzymes.

Serum lysosomal enzymes have been shown to increase during hemorrhagic shock[1,2,4,8,30] The sources responsible for the rise of serum lysosomal enzymes have been thought to lie in the splanchnic region, as referred to in the Introduction. On the other hand, human neutrophil leukocytes indeed contain peroxidase- and AcPase-positive granules exhibiting similar properties to azurophil granules which have been shown to be true lysosomes and specific granules which contain lysozyme[21,31]. Further, lysosomal enzymes are released from human polymorphonuclear (neutrophil) leukocytes during phagocytosis or as a result of stimulation by agents such as cytochalasin B[32]. In the present study, canine neutrophil leukocytes were also seen to contain granules positive for peroxidase activity, which is a characteristic of azurophil granules. The granules decreased in number during hemorrhagic shock, and spherical material also exhibiting peroxidase activity and with contents similar in morphology to those of azurophil granules appeared in the extracellular environment, suggesting that they had been discharged from the cells. Blood platelets, including those of dogs, have been demonstrated to contain three kinds of granules; α-granules, serotonin granules and lysosomes[18]. When blood platelets are damaged in vivo by various pathologic processes, large amounts of lysosomal enzymes are released from platelet granules into the plasma[3]. These facts suggest that the granules observed to be released from platelets and leukocytes in the present experiments may have strongly contributed to the rise in serum lysosomal enzymes.

Clermont et al.[4] have already reported that while serum AcPase content rises to 311% of the control value by 2 hr after hemorrhagic shock in intact dogs, the average serum AcPase in dogs subject to enterectomy increases to 209% of the control value at 2 hr. This has been interpreted to mean that the rise in serum AcPase during the hemorrhagic shock period is due to the outpouring of lysosomal enzymes not only from the interstinal cells but also from other organs or tissues. It has been shown that subjecting the liver to ischemia for 30–60 min causes an increase in autophagosome formation, but that there are no signs of leakage of lysosomal enzymes as determined by cytosol assay[33]. This suggests that the liver may not be the major source of the rise in lysosomal enzymes in the blood plasma, at least in the early periods of hemorrhagic shock. In the pancreas, hypoperfusion has been thought to be an important link in the pathogenesis of circulatory shock, thereby establishing hypoxia, ischemia and acidosis, which constitute an effective stimulus for the release of pancreatic lysosomal hydrolases[11]. Hemorrhagic shock leads to a fall in the blood pH[4,34]. Since acidosis damages

lysosomes, an outpouring of lysosomal enzymes occurs in shock[10, 22, 25, 35, 36]. The lysosomal enzymes, in turn, damage capillary endothelia, cell membranes and mitochondria[10]. Such damage to capillary endothelia might have induced the granule release from blood platelets observed in the present experiments, and since some of the granular substances derived from blood platelets themselves induce the release of platelet granules[29], the increased release of such granules during hemorrhagic shock may allow a causal relationship to be hypothesized between the granule release of platelets and hemorrhagic shock in the present dogs. The liberation of endogenous mediators in the platelets might have an injurious effect on the participating cells. However, the platelet granule-releasing factor, which triggers the earliest granule discharge in hemorrhagic shock, remains unknown.

There is evidence that a variety of substances are actively released by leukocytes in vitro[3, 23]. While substances found in lysosomes of leukocytes are capable of degrading the materials relevant to tissue injury[6], granulocytes produce oxygen radicals and release proteolytic enzymes[37-39]. The factor(s) stimulating the release of granule contents from neutrophil leukocytes during hemorrhagic shock is unclear, but the process is possibly activated by a variety of stimuli. However, as demonstrated by considerable experimental evidence, hypoxia, ischemia and acidosis, which are characteristic of hemorrhagic shock, all cause tissue damage so that the materials emanating from the damaged tissues and cells may be released into the plasma, and may in turn trigger the release of granules from neutrophils. Further, since substances from leukocytes include lysosomal enzymes and the other materials[21], the release of these lysosomal enzymes into the plasma may mediate, in part, acute inflammatory reaction and irreversible tissue damage.

Our unpublished data on ultrastructural changes in the small intestine, liver and pancreas in dogs after hemorrhagic shock have shown that the liver is the most rapidly vulnerable of the three organs. Necrobiosis of a few hepatocytes appeared 1 hr after the shock, in contrast to the normal ultrastructure of the small intestine and pancreas. On the other hand, phagocytosis of platelets by Kupffer cells in the liver began within 30 min after hemorrhagic shock, and the phagocytosed platelets could be morphologically distinguished from those in the preshock period by having no or few granules. This suggests a very rapid "platelet release reaction" to the stimulation of hemorrhagic shock and a rapid vulnerability of the platelet plasma membrane. These data would be correlated with the release of granules from platelets in the early period of the present study, which might, at least, be associated with the earliest elevation in the circulating levels of AcPase and β-glucuronidase. (The assay for serum AcPase and β-glucuronidase activities was carried out before and after induction of hemorrhagic shock during the present experiments, and we obtained the results similar to the previous many data which have shown the rise in serum level of both enzymes during hemorrhagic shock.)

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