Experimental Hemostasis in Normal Dogs and Dogs with Congenital Disorders of Blood Coagulation

By T. Hovic, H. C. Rowsell, W. J. Dodds, L. Jørgensen and J. F. Mustard

Investigations of hemostasis and thrombosis in the microcirculation of animals during the past century have provided considerable insight into the mechanisms involved. The vessel wall, platelets and blood coagulation are recognized to be important. Hughes considered that the speed with which platelets accumulate at the point of vessel injury is faster than can be explained on the basis of the known action of blood coagulation. He found that platelets adhere to mesenteric connective tissue, and Bounameaux observed that platelets adhere to subendothelial structures of the aorta in vitro. Studies by Zucker and Borrelli and by Hovic showed that platelets adhere to collagen and that this induces platelet aggregation. Hellem and Gaarder and associates had already found that adenosine diphosphate (ADP) from the red blood cells was able to cause platelet aggregation. Hovic and, subsequently, Spaet and Zucker demonstrated that collagen induced platelet aggregation through release of platelet ADP. It is also possible that ADP may be released from injured cells in the vessel wall. These observations provide a reasonable explanation for the mechanism by which platelets rapidly accumulate at a point of vascular injury.

The part played by coagulation may not be important in these early stages, but the well-known fact that subjects or animals with coagulation disorders (i.e., classic hemophilia) have abnormal hemostasis clearly indicates that coagulation is necessary for establishing an impermeable, stable, hemostatic plug. However, this is a complicated problem since subjects with one type of coagulation defect can have a more severe hemorrhagic disorder than those with another. In general, it appears that subjects with defects in the later stages of the intrinsic pathway of coagulation show a greater hemo-
EXPERIMENTAL HEMOSTASIS IN DOGS

static defect than those whose abnormality lies solely in the extrinsic pathway of coagulation,\textsuperscript{10,12} or in the first steps of the intrinsic pathway.\textsuperscript{53,56}

The basic problems are: which of the pathways of coagulation is important? What initiates coagulation in vivo? How does coagulation exert its effect on the platelet plug? The intrinsic clotting system is probably of greatest importance,\textsuperscript{12} but one cannot exclude a significant participation of the extrinsic system as well.\textsuperscript{36} It has been postulated that both coagulation pathways are triggered by tissue thromboplastin.\textsuperscript{61} Further, it has been suggested that factor XII is absorbed onto, and activated by, collagen.\textsuperscript{47} However, before one assumes that activation of Factor XII is important, it is necessary to explain why the lack of this factor has little, if any, effect on the hemostatic mechanism.\textsuperscript{8,53} Contact of platelets with collagen may make platelet factor 3 available,\textsuperscript{59} and it seems that the initial platelet mass serves as a focus for acceleration of clotting,\textsuperscript{53} perhaps even for its initiation. The formation of thrombin causes further platelet aggregation through the release of ADP\textsuperscript{22,27} and also potentiates coagulation through its effect on the activation of factor V\textsuperscript{62} and factor VIII\textsuperscript{49,52} and making available of platelet phospholipoprotein.\textsuperscript{25} Conceivably, these processes lead to the generation of sufficient thrombin to convert fibrinogen to fibrin at the contact area between the platelet mass and the extravasated blood,\textsuperscript{34,36} and thereby help to stabilize the plug.

The availability of dogs with congenital defects in factor VII, factor IX or factor VIII provided an opportunity to observe in vivo the effect of these disorders on hemostasis. Furthermore, the effect of drugs which influence coagulation (heparin and Dicumarol) or the platelet collagen reaction\textsuperscript{44} (pyrazole compounds) was explored. Normal dogs served as controls. Both direct observation of platelet plug formation and light and electron microscopy of the plugs were carried out. In addition, the reaction of the dog platelets to collagen, ADP, and thrombin was tested with a turbidimetric technic.

**Materials and Methods**

**In Vivo Studies**

**Dogs.** The dogs used in these studies came from the colony maintained at the Ontario Veterinary College, Guelph, Ontario. The dogs with congenital defects of factor VII and factor IX have been previously described.\textsuperscript{45,46} There are also dogs, which have not been described, with a congenital defect of factor VIII.\textsuperscript{48} These dogs have genetic characteristics and values for clotting tests similar to those found in man with corresponding defects. The factor VIII deficient dogs used in this study have the same characteristics as the hemophilic dogs described by Graham and associates.\textsuperscript{21} The factor IX and factor VIII deficient dogs have a hemorrhagic tendency, whereas the factor VII deficient dogs do not.

Altogether, 21 dogs with coagulation defects were studied: 7 with factor VII deficiency, 9 with factor IX deficiency, and 5 with factor VIII deficiency. In addition, 11 normal dogs were included as controls. The diagnosis of the coagulation defects was based on tests carried out as previously described.\textsuperscript{46,48} The results for the abnormal dogs are shown in Table 1.

**Drugs.** (1) Bishydroxycoumarin (Dicumarol, Abbott Laboratories, Chicago, Ill.) was administered by mouth to 3 factor IX deficient dogs (10 to 15 Kg. body weight) in the form of 25 mg. tablets, one tablet 3 times daily for 2 to 4 days. Coagulation studies, including the prothrombin time and Factor VII assay, were carried out prior to and during the experiments.
Table 1.—Coagulation Factor Assays

<table>
<thead>
<tr>
<th>Dogs</th>
<th>Factor VII Assay</th>
<th>Factor IX Assay</th>
<th>Factor VIII Assay</th>
<th>Platelet Count</th>
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* Normal means that the assay gave a value greater than 70 per cent.

(2) Heparin (Connaught Medical Research Laboratories, Toronto, Ont.) was given to 2 factor VII deficient dogs. To one of the dogs, 10 mg. heparin (1000 U.S.P. units) per Kg. body weight was administered intravenously 15 minutes prior to the experiment. The other dog received 20 mg. heparin (2000 U.S.P. units) per Kg. body weight subcutaneously one hour before the experiment. Clotting tests were done immediately prior to and during the experiments.

(3) Phenylbutazone (Butazolidin, Geigy, Montreal, Quebec) was given by mouth to 2 normal dogs and 2 factor IX deficient dogs (10 to 15 Kg. body weight) in the form of 100 mg. tablets. The dose used was 200 mg. 3 times daily for 2 to 3 days.

Anesthesia. The anesthesia was induced by intravenous injection of thiopental sodium (2.75 per cent). Following this, the animals were intubated and the anesthesia was maintained with halothane, using a semiclosed system with a Boyle’s anesthetic apparatus (Model 10).

Experimental Procedure. The microcirculation was studied in two areas: in the subcutaneous tissue and fasciae on the medial side of the thigh, and in the mesentery.

The subcutaneous and fascial vessels were exposed by a 1 to 3 cm. long incision and observed through a microscope (Zeiss Model—Standard WL microscope). A quartz rod with a curved tip coupled to a light source was inserted beneath the fascia in order to provide illumination of the area under examination. The vessels were then transected with a razor blade and hemostasis was observed directly. The wound was bathed in a continuously flowing Tyrode solution (30-37 C.) by means of a slow drip. In 3 experiments the wound was made in the thigh and then closed, so as to avoid the necessity of having to keep it moist with the Tyrode solution.

In order to study the mesenteric circulation, a small incision was made in the midline of
the abdomen. The peritoneal cavity was opened carefully and an intestinal loop gently pulled out and placed in an observation chamber mounted on a Zeiss microscope. The center part of the chamber was elevated, and transillumination was through a piece of glass inserted in the middle. The glass was surrounded by a rubber ring to which the mesentery was pinned. Again, vessels were transected with a razor blade. Both the mesentery and the intestinal loop were continuously bathed with the warm Tyrode solution. The blood was washed away from the area under observation by a drip of the same fluid.

Following completion of the experimental procedure the mesentery was replaced in the abdominal cavity and the peritoneum closed with catgut sutures and the abdominal wall with silk sutures. The animals with congenital defects in factor IX or factor VIII were then transfused with fresh frozen plasma at intervals for the next 24 to 48 hours. All of the animals survived the surgical procedure, although there was extensive bruising around the wounds in the factor IX and factor VIII deficient animals.

Cinematography. Motion pictures were taken of the hemostatic process in the mesenteric vessels. A Bolex 16 mm. movie camera was attached to the top of the Zeiss microscope, which had a light dividing device so that the area could be directly observed at the same time as the cinematographic recordings were made. The film used was Kodak Ektachrome 3500.

Bleeding Time. The length of time from the onset of bleeding to its arrest was recorded by direct observation of the transected vessels both in the thigh and in the mesentery. The vessels were observed for a period of up to 30 minutes after transection in order to record possible renewed bleeding from the transected vessels.

Tissue Preparation for Light and Electron Microscopy. The platelet plug and surrounding subcutaneous or mesenteric tissues were removed at various intervals from 10 min. to 30 min. following the transection.

Specimens for light microscopy were immediately placed in Helly’s solution (Zenker’s Formol) for 24 hours, rinsed in flowing tap water for another 24 hours, and dehydrated in increasing concentrations of ethanol and was treated in propylene oxide for one-half to one hour. The specimen was then placed in a mixture of equal parts of Epon 812 and propylene oxide for 8 to 12 hours and then was embedded in Epon 812. Half-micron thick sections were cut on a Porter-Blum microtome and stained with azure II for orientation by light microscopy. A suitable area was selected and ultrathin sections were cut and stained with lead hydroxide or with lead hydroxide and uranyl acetate. The sections were picked up on uncoated grids and examined in either a Philips 200 or RCA EMU-3F microscope. The primary magnifications varied from 1800 to 30,000. The conclusions are based on approximately 850 plates from 70 specimens.

In Vitro Studies

Blood Samples. Blood was withdrawn from the jugular vein or veins on the fore or hind legs of the dogs. Gauge 20 stainless steel needles were used, and the blood was collected in plastic or silicone-coated glass syringes. The blood was placed in silicone-coated glass tubes containing either no anticoagulant or 3.8 per cent trisodium citrate, nine parts of blood to one part anticoagulant solution. Platelet-rich plasma (PRP) was prepared by centrifuging at 120 g at 4 C. for 10 min.

Adenosine Diphosphate. Disodium adenosine diphosphate (ADP) (Sigma Chemical Co., St. Louis, Mo.) was dissolved in either imidazole buffered saline, as previously described, or in Ringer’s solution (pH 7.4). The concentrations of ADP were $2.0 \times 10^{-4}$ M and $2.0 \times 10^{-5}$ M.

Collagen. Collagen used in these experiments was prepared from either dog, pig, or rabbit, as previously described, or from commercial collagen devoid of tissue thromboplastin (Sigma Chemical Co., St. Louis, Mo.). The collagen was homogenized and
Fig. 1.—This is a movie frame sequence which demonstrates the series of events taking place after transection of a venule (V) and an arteriole (A) in the mesentery. (1): Thirty seconds after transection some platelets have started to accumulate, but the blood is streaming from the cut vessels. The blood flow from the other ends of the transected vessels is seen at the bottom of the picture. (2): Sixty seconds after the transection hemostatic plug formation is clearly evident at both ends of the cut vessels. Bleeding is through channels in the plugs. (3): Two minutes after transection there is still some bleeding through the plugs. (4): Three minutes after transection bleeding has stopped at both ends of the vessel. The blood stream has been reversed in the vessels at the bottom of the picture and the vessels seem to be almost empty of blood cells. There is stasis at the end of the venule in the upper part of the picture. Note the small hemostatic plug at the middle right.
Table 2.—Bleeding Times of Transected Mesenteric Vessels

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Observations</th>
<th>Initial Bleeding Time (Seconds)</th>
<th>Significance of Difference of Mean from Normal</th>
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<td></td>
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<td>Mean</td>
<td>S. D.</td>
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<tr>
<td>Normal</td>
<td>59</td>
<td>184.8</td>
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<td>35</td>
<td>242.9</td>
<td>105.5</td>
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<tr>
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<td>36</td>
<td>222.0</td>
<td>131.1</td>
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<tr>
<td>Factor VIII</td>
<td>33</td>
<td>&gt;450.1*</td>
<td>326.8</td>
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*In four of the observations there was no arrest of bleeding during the period of observation (20 to 30 min.)

Table 3.—Renewed Bleeding in Transected Mesenteric Vessels

<table>
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<tr>
<th>Group</th>
<th>Number of Observations</th>
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<tr>
<td></td>
<td></td>
<td>No.</td>
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<tr>
<td>Normal</td>
<td>59</td>
<td>52</td>
</tr>
<tr>
<td>Factor VII</td>
<td>35</td>
<td>33</td>
</tr>
<tr>
<td>Factor IX</td>
<td>36</td>
<td>9</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>33</td>
<td>10*</td>
</tr>
</tbody>
</table>

*In four of these observations the vessels bled during the entire period of observation (20 to 30 min.)
†In 11 of these observations there was no further arrest of bleeding during the period of observation (15 to 30 min.)
‡In three of these observations there was no arrest of bleeding at the time when the experiment was stopped (20 to 30 min.)

suspended in a Tyrode solution without magnesium and calcium. The suspension was centrifuged at 810 g for 15 minutes to remove large collagen fragments. In other studies large collagen fragments were wound around the stir-bars used in the turbidimetric device.

Thrombin. Crude bovine thrombin (Parke, Davis & Co., Detroit, Mich.) was dissolved in imidazole buffered saline to the desired concentration, usually 5 NIH units per ml.

Platelet Adherence and Aggregation. This was studied using a turbidimetric method which has been described in detail.38 These studies were carried out at 37 C. or at room temperature. In some experiments large collagen fragments were wound around the siliconized metal stir-bar and placed at the bottom of the silicone-coated glass tube so that the mass was kept beneath the light beam passing through the platelet-rich plasma. The plasma was stirred at 1100 r.p.m. for 5 to 60 minutes. Following the experiments the collagen fragments were prepared for light and electron microscopy.

Statistical Methods. The statistical methods used were the conventional tests. The mean and standard deviation of the bleeding times within each animal were calculated separately. Afterwards, the common mean and standard deviation for all animals within each group were determined. The values used for comparison between the groups were the common means and their standard deviations. If the bleeding time exceeded the observation period, the observation period was taken as the bleeding time, giving, therefore, a minimum estimate.

RESULTS

In Vivo Studies

The transected vessels on the thigh tended to be somewhat larger than the transected vessels in the mesentery; the thigh vessels ranged from ap-
Fig. 2.—This is a section from a normal hemostatic plug at the end of a transected vein as seen in the light microscope. The mass of platelets (PLT) extends across the cut end of the vessel and appears to be anchored to the connective tissue (CT) at the sides. Fibrin (FIB) is evident around the margins of the platelet mass. Red blood cells (RBC) can be seen in the arrested column of blood in which there is no evidence of coagulation. MSB × 490.

Approximately 50 to 200 microns in diameter, whereas those in the mesentery usually did not exceed 150 microns. Both in the thigh and in the mesentery the arteries and veins were usually located close together and, therefore, they were frequently transected at the same time. The findings in the two areas of observation did not differ. Immediately after the transection the vessels showed various degrees of transient constriction, the arteries more than the veins. There appeared to be no difference in the pattern of constriction between the normal dogs and the dogs with coagulation defects.

Normal Dogs. The platelets accumulated almost immediately on the lips of the transected vessels and the deposit gradually increased in size so as to encroach on the central stream of blood (Fig. 1). The bleeding was gradually reduced as the platelet mass formed across the mouth of the vessel. When the central stream of blood was nearly arrested, one or more new channels were usually formed in various directions through the plug. These channels gradually became closed, and by about 3 minutes the bleeding stopped (Table 2). In a few instances brief renewed bleeding occurred (Table 3).
Fig. 3.—This is an electron micrograph of the peripheral area of a hemostatic plug which is representative of hemostatic plugs from normal as well as factor VII deficient dogs. Note the marked pseudopod (PSD) formation of the platelets at the lower left. Many of the platelets (PLT) appear to be swollen and empty with reduced electron density of the cytoplasm. Some of the degranulated structures may be swollen pseudopods. Note strands of fibrin (FIB) and red blood cells (RBC) on the surface.

Light microscopy of section prepared from these plugs revealed a platelet mass covering the mouth of the transected vessels (Fig. 2). Usually the platelet aggregate did not extend into the lumen of the vessel. The plug was
Fig. 4.—This electron micrograph of the periphery of a hemostatic plug shows fibrin (FIB) in close association with the platelet (PLT) membrane. In some of the fibrin strands cross striations can be seen. There appear to be breaks in the limiting membranes of some platelets (unlabeled arrows). The platelets in this region have lost their internal structure.

attached to the connective tissue at the edge of the cut vessel. In the region of the connective tissue and around the periphery of the plug, material with the staining characteristics of fibrin was present. Occasionally, there were spaces in the platelet mass, presumably channels, often containing red cells and sometimes lined by what appeared to be fibrin. Most of the platelets in the plug seemed to be granular, but at the periphery many of them were swollen and empty.

Sections for electron microscopy were taken from the periphery of the plug, from the central regions of the mass, and from the side of the plug where it was adherent to the connective tissue. In sections from the periphery the presence of a network of fibrin was confirmed (Fig. 3). The fibrin was usually fibrillar in appearance and occasionally showed the characteristic cross striation of fibrin (Fig. 4). The strands of fibrin were lying between the
Fig. 5.—This is an electron micrograph of the central region of a hemostatic plug which is representative of hemostatic plugs from normal and factor VII deficient dogs. Note the close packing of the platelets (PLT) which have retained their limiting membranes (PM). There appears to be some loss of internal structure in some of the platelets but others contain their granules (GR), glycogen (GLY), and mitochondria (MIT).

platelets at and near the surface of the plug. The distance between the fibrin fibrils and the platelet surface varied; sometimes these structures seemed to merge (Fig. 4). The platelets in this region often had what appeared to be gaps in their membranes (Figs. 3 and 4). In general, the platelets near the surface were closely adherent to each other. They appeared to have lost their organelles (granules and mitochondria) and showed pseudopods of varying length. Some of the structures which appeared to be degranulated platelets were, in effect, long pseudopods with a swollen end extending from the center of the platelet mass.

In sections from the middle of the hemostatic plug, the platelets were lying close together; the distance between neighboring platelets was of the order of 100–300 Å (Fig. 5). Many platelets had retained most of their organelles but showed varying degrees of pseudopod formation. In some areas, platelets
Fig. 6.—This electron micrograph demonstrates fine filaments (FIL) in the cytoplasm of platelets in the central region of a normal hemostatic plug in which there has been only moderate structural change in the platelets. Dark zones (DZ) can be seen in some regions where the platelets are in contact with each other. In some areas the filaments appear to run into dark zones. Tubule-like (TUB) structures are also seen. Granules (GR) and mitochondria (MIT) are evident in the platelets.

seemed to have lost their internal structure and the cytoplasm showed reduced electron density (Fig. 5). At intervals dense zones between adjacent platelets were observed. Fine fibrils with a diameter of 50–100 Å could be seen in the platelet cytoplasm, particularly in the pseudopods. These fibrils were often
Fig. 7.—An electron micrograph from the central region of a hemostatic plug. Microtubules (TUB) are apparent. This was especially noticeable in the platelet pseudopods (PSD) in plugs from both normal dogs and dogs with coagulation defects. Glycogen granules (GLY) are also evident in this picture.

orientated towards the dark zones (Fig. 6). Tubule-like structures of 200 to 400 Å in diameter were also observed (Fig. 7). These structures, which tended to lie parallel to each other, had a variable structure, but a light inner zone with a dark membrane was often found in both the longitudinal and cross sections. In cross sections they appeared as bundles. They were frequently found
Fig. 8.—This electron micrograph shows the area of contact between the platelet mass and the collagen fibers (COL) at the end of the cut vessel. The platelets (PLT) in contact with the collagen are swollen and less electron dense than unaltered platelets. The platelets toward the center of the mass at the top right of the picture are better preserved. In some platelets glycogen (GLY), granules (GR), and mitochondria (MIT) can be seen. There is some fibrin (FIB) interspersed amongst the platelets. Red blood cells (RBC) are present.

in association with platelets that showed some loss of electron density, swelling and pseudopod formation.

Sections from the part of the plug in contact with the vessel wall and adjacent
connective tissue showed platelets closely adherent to collagen fibrils (Fig. 8). The gap between the platelet membrane and collagen was usually less than 30 Å (Fig. 9), and the platelets in contact with collagen often showed breaks in their membranes, particularly at the points where the collagen contacted the platelets (Fig. 9). Parts of the platelet membrane seemed to interlock with the dense part of the collagen striation. The platelets had lost their internal structure, were swollen and less electron dense (Figs. 8 and 9). Fibrin strands frequently occurred between the platelets and between the platelets and the collagen (Fig. 8). The plugs prepared from injured vessels which were not bathed with Tyrode solution were similar in appearance to those which had been so treated.

Both light and electron microscopic examination of the lumen of the vessel in the regions immediately adjacent to the plug failed to show evidence of coagulation in the static column of blood (Fig. 2).
Fig. 10.—This shows the appearance in the light microscope of a hemostatic plug from a factor IX deficient dog 30 minutes after transection of the vessels. Note the size of the plug and the numerous channels (CH) in it. The platelet masses (PLT) appear to be less tightly packed than in the normal plugs. One channel appears to be in direct contact with the lumen (L) of the transected vein, which contained red blood cells (RBC). The platelets are in contact with the connective tissue (CT) at the sides of the cut vessel. This vessel is considerably contracted. There is little evidence of fibrin in the plug. MSB × 70.

Factor VII Deficient Dogs. In the dogs with congenital deficiency of factor VII, the same sequence of arrest of bleeding from transected vessels was observed as in normal animals (Tables 2 and 3). Furthermore, in sections prepared for light and electron microscopy the plugs from these animals were indistinguishable from those of normal dogs.

Factor IX Deficient Dogs. In dogs with a congenital defect of factor IX, the platelet plugs formed at the ends of the transected vessels in the same manner as in normal dogs, and the initial arrest of bleeding occurred in about the same time as in the normal dogs (Table 2). However, seconds or minutes later bleeding recurred through the plug in most instances (Table 3). The duration of the renewed bleeding varied and was usually followed by cessation of bleeding during the period of observation. When this took place further episodes of renewal and arrest of bleeding often occurred. The rebleeding took place through channels running in various directions in the plugs. During each period of bleeding, new platelet material accumulated, leading to the
formation of a plug greater in size than in normal dogs. Fragments of the plug were occasionally observed to break off.

Sections examined by light microscopy showed large plugs containing what appeared to be many channels filled with platelets, and red and white blood cells (Fig. 10). The fibrin around the surface was less prominent than normally seen and was often lacking completely.

Sections for electron microscopy from the periphery of the plug showed less fibrin and change in platelet structure than in plugs from normal dogs. In several areas fibrin was lacking and the platelets were loosely packed, with little evidence of swelling (Fig. 11).

In the center of the plug the structure of the platelets did not differ from that of the normal plugs (Figs. 11 and 12). However, channels were frequently seen which were lined by swollen platelet pseudopods and some degranulated platelets (Fig. 12). Well-preserved single platelets and red and white blood cells were found within the channels. Fibrin lining of the channels could not be found in the central regions of the plug.
Fig. 12.—This is an electron micrograph showing the appearance of a channel through a 20-minute-old platelet plug from a factor IX deficient dog. The platelets close to the channel appear to be degranulated, but these structures may also represent swollen platelet pseudopods. Red blood cells (RBC) and polymorphonuclear leukocytes (PMN) can be seen within the channel. There is no morphologic evidence of fibrin. The granules in the channel (indicated by arrows) may have come from platelets or disintegrated white blood cells.

In sections from the part of the plug in contact with the vessel wall, the platelets were just as closely adherent to the collagen as in normal plugs (Fig. 13). Furthermore, platelets with complete loss of internal structure were observed. Figure 13 shows an endothelial cell at the point of transection and the adjacent part of the platelet plug. Some platelets are lying beside what appears to be the cut surface of the endothelial cell. The distance between the platelets and the cut surface varies from about 50 to 400 Å. These platelets do not appear to be as swollen and empty as those lying close to the collagen. Fibrin strands were found between the platelets near the collagen, although the amount of fibrin in this region was less than normal (Fig. 13).

Factor VIII Deficient Dogs. In dogs with congenital defect of factor VIII, the observations in vivo were similar in most respects to those made in factor
Fig. 13.—This electron micrograph demonstrates part of an endothelial cell (END) and the vessel wall with platelets attached, just at the point where the vessel is transected. The endothelial cell is vacuolated and appears to be damaged. Note the close contact between the collagen fibrils (COL) of the vessel wall and the platelets. A few platelets (PLT) are swollen, with a marked reduction of electron density of the cytoplasm. Fibrin (FIB) can be seen between some of the platelets. The platelets remote from the collagen do not show these marked structural changes. The nucleus (NUC) of the endothelial cell is shown. This is from a factor IX deficient dog.

IX deficient dogs. However, the initial arrest of bleeding was usually delayed (Table 2). During the period of prolonged bleeding, the blood stream through the plug was often temporarily reduced; then suddenly the flow increased again through the same or a new channel. This could repeat itself several times, giving rise to waxing and waning of the bleeding. Renewed bleeding occurred frequently and the plugs became large (Table 3). The structure of the plugs did not differ from that of the plugs of factor IX deficient dogs. The platelets were loosely aggregated in many regions, particularly toward the surface of the plug. In these regions the structure of the platelets was well preserved; the pseudopod formation was not marked and there were no dense zones between platelets. The amount of fibrin was even less than in the plugs of factor IX deficient dogs, and often it was completely lacking, even
Fig. 14.—This is an electron micrograph of platelets (PLT) in contact with collagen (COL) at the exposed end of the transected vessel wall in a plug from a factor VIII deficient dog 30 minutes after transection. Swelling of the platelets with almost complete loss of internal organelles and cytoplasm is evident. Gaps in the limiting membrane of the platelets are indicated by the arrows. Note the close contact between the platelets and the collagen.

in the areas of contact with the vessel wall (Fig. 14). Platelets in contact with collagen showed the usual structural changes. The characteristic gaps in the limiting membranes of platelets in contact with collagen were evident. In some areas there was marked evidence of tubule formation in the platelets near the collagen (Fig. 15).

The Effect of Dicumarol, Heparin and Phenylbutazone on Hemostasis

The administration of Dicumarol to factor IX deficient dogs, in doses sufficient to reduce factor VII levels to 2 per cent or lower (Table 4), caused a slight prolongation of the bleeding time and did not increase the frequency of renewed bleeding (Table 5). The main difference in plugs from untreated factor IX deficient dogs was that the amount of fibrin appeared to be less.

Administration of heparin to factor VII deficient dogs impaired hemostasis.
The clotting time values in glass and silicone-coated glass tubes were prolonged to more than 60 minutes. While platelets seemed to accumulate at the normal rate in arteries, the formation of plugs in veins was much slower. The plugs which formed seemed small, loose and friable, and portions often broke off. The prolonged initial bleeding waxed and waned (Table 5). In addition, in the experiments in which there was an initial arrest of bleeding it almost invariably restarted (Table 5). Electron microscopy of the plugs revealed various degrees of platelet packing. In the periphery the platelets were loosely aggregated and there was no fibrin. Even in the region where the platelets came in contact with the collagen and had undergone swelling and loss of organelles (Fig. 16), there were areas of loosely packed platelets. Fibrin was absent from this region.

Normal dogs given phenylbutazone did not show any difference from untreated dogs in the arrest of bleeding (Table 5) and structure of the plugs. However, in factor IX deficient dogs administration of phenylbutazone further

Fig. 15.—This is an electron micrograph of another area from the zone of platelet contact with collagen (COL) in a factor VIII deficient dog. The platelets in this picture show less loss of electron density. There is marked evidence of tubules (TUB) in these platelets, particularly in the pseudopods. A mitochondrion (MIT) can be seen.
Table 4.—Coagulation Data in Two Factor IX Deficient Dogs Given Dicumarol

<table>
<thead>
<tr>
<th>Dogs</th>
<th>Whole Blood Clotting Time (Minutes)</th>
<th>Prothrombin Time (Seconds)</th>
<th>Factor VII Assay (Per cent of normal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K-63</td>
<td>60</td>
<td>&gt;60</td>
<td>77</td>
</tr>
<tr>
<td>K-73</td>
<td>&gt;25</td>
<td>&gt;60</td>
<td>55</td>
</tr>
</tbody>
</table>

Impaired hemostatic plug formation. In some instances the building up of the plug was slower than that found in untreated factor IX deficient dogs. Furthermore, fragments of the plug were broken off and washed away. Either the bleeding did not stop during the observation period or the initial arrest of the bleeding was greatly delayed (Table 5).

Structurally, the plugs were smaller than in untreated Factor IX deficient dogs, and they were crossed by wide channels. The platelets were loosely packed and, apart from those in the contact zone with the perivascular tissue, most of them had retained their internal structures (Fig. 17). As could be expected in a factor IX deficient dog, very little fibrin was present. Many of the platelets had an oval shape, indicating that they had probably preserved their intravascular disc form. The platelets in contact with the collagen showed swelling and loss of organelles.

In Vitro Studies

Addition of ADP (final concentrations of $2.0 \times 10^{-6}$ M and $2.0 \times 10^{-6}$ M) to native or citrated PRP from normal dogs induced platelet aggregation (Fig. 18). The same reaction was observed when ADP was added to native or citrated PRP from dogs with congenital defects of factor VII, factor IX, or factor VIII. ADP-induced platelet aggregation in normal native PRP shortened the clotting time, in agreement with earlier observations. When ADP was added to native PRP from the dogs with factor IX or factor VIII deficiency (Fig. 19), the time until the onset of clotting was longer than with PRP from normal factor VII deficient dogs, even though platelet aggregation was normal. Studies on the effect of collagen on platelet aggregation in PRP from the dogs proved difficult. In contrast to observations with human, pig and rabbit PRP, we could not induce routinely platelet aggregation in canine PRP with the standard collagen suspensions. However, by winding a collagen fragment around the stir-bar used in the test tube, platelet aggregation could be induced (Figs. 19 and 20). The collagen-induced platelet aggregation in factor IX deficient and factor VIII deficient dogs was normal but it did not cause any clotting during the 1-hour period of observation. The results of a study with plasma from a factor VIII deficient dog are shown in Figure 19. Light and electron microscopy of the collagen fragments which had been exposed to native PRP from normal dogs showed platelet aggregates in contact with the collagen. Swollen and degranulated platelets were observed, together with fibrin in and around the periphery of the platelet aggregates, similar to the effects seen in the vivo studies. In contrast, there was no evidence of fibrin in association with the aggregates formed in a
Fig. 16.—This is an electron micrograph of platelets attached to the vessel wall in a plug from a factor VII deficient dog given 10 mg. of heparin per Kg. body weight, intravenously, 15 minutes prior to the experiment. Note that some of the platelets (PLT) show marked structural changes with gaps (indicated by arrows) in the limiting membranes. No morphologic evidence of fibrin is seen. Note again the close contact between the platelets and collagen (COL).

similar manner in native PRP from factor VIII or factor IX deficient dogs.

Thrombin added to citrated PRP from dogs with congenital coagulation defects produced platelet aggregation similar to that in citrated PRP from normal dogs (Fig. 21).

DISCUSSION

The observations in vivo of hemostasis in normal dogs are in agreement with those made in rabbits and rats by Apitz,1 Chen and Tsai,15 Zucker,66 and Hugues.30 The appearance of sections from normal plugs examined by light microscopy corresponds with that earlier reported in other species by Apitz,1,2 Zucker,66 and Jørgensen and Borchgrevink.34 The plugs were attached to the perivascular connective tissue. Most of the platelets in the central part of the plug appeared to be granular, whereas the platelets in the peripheral part were
Table 5.—The Effect of Dicumarol, Heparin, and Phenylbutazone on Hemostasis

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Observations</th>
<th>Bleeding Time (Seconds)</th>
<th>Frequency of Renewed Bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>S.D.</td>
</tr>
<tr>
<td>Factor IX</td>
<td>36</td>
<td>222</td>
<td>131.1</td>
</tr>
<tr>
<td>Factor IX and Dicumarol</td>
<td>13</td>
<td>279.5</td>
<td>135.8</td>
</tr>
<tr>
<td>Factor IX and Phenylbutazone</td>
<td>10</td>
<td>&gt;874.7</td>
<td>—</td>
</tr>
<tr>
<td>Factor VII</td>
<td>35</td>
<td>242.9</td>
<td>105.5</td>
</tr>
<tr>
<td>Factor VII and Heparin</td>
<td>6</td>
<td>&gt;656.7</td>
<td>—</td>
</tr>
<tr>
<td>Normal</td>
<td>59</td>
<td>184.8</td>
<td>87.2</td>
</tr>
<tr>
<td>Normal and Phenylbutazone</td>
<td>15</td>
<td>193.1</td>
<td>54.5</td>
</tr>
</tbody>
</table>

* In one observation the animal bled continuously after the first renewed bleeding occurred (>25 min.).
† In five of the observations the bleeding did not stop during the period of observation (10 to 20 min.).
‡ Three of the four animals the bleeding did not stop during the period of observation (10 to 20 min.).
§ In four of the five animals the bleeding did not stop during the period of study (8 to 15 min.).
‖ In five of the five observations the bleeding did not stop during the period of study (8 to 15 min.).
* See Table 3.
EXPERIMENTAL HEMOSTASIS IN DOGS

Fig. 17.—This is an electron micrograph showing loosely packed platelets in a hemostatic plug. This plug was taken 30 minutes after transection of a vessel from a factor IX deficient dog treated with phenylbutazone, 600 mg. daily for 3 days. There is good preservation of the platelet structure and no evidence of fibrin formation. The platelets are disc-like in appearance, and some show pseudopods.

swollen and empty. Jørgensen and Borchgrevink found more extensive “balloonning” of the platelets throughout the plugs from normal human beings. The findings with electron microscopy are in keeping with those made by Kjaerheim and Hovig and French and associates. The presence of fibrin in the contact zone between the platelet mass and the perivascular connective tissue was not reported in previous studies with the electron microscope. All the above differences may be due to species variation, vessel size, the interval between transection and removal of plugs, and the use of washing fluid. However, no evidence was found in the present study that the use of the washing fluid changed the appearance of the plugs. It is important to point out that, in contrast to what is thought, we could not find evidence of coagulation in the static column of blood adjacent to these plugs.

Fine filamentous structures were observed in the platelets, particularly in the pseudopods. These structures sometimes seemed to continue across the limiting
Fig. 18.—This is a copy of the tracings obtained with a turbidimetric device upon the addition of ADP to citrated platelet-rich plasma from a normal dog and from dogs with coagulation defects. Upward deflection indicates increased light transmission occurring as the platelets aggregate. With a final concentration of ADP of 10 μg per ml. (2 × 10⁻⁵ M), no difference in platelet aggregation among the various plasma samples was observed. (The platelet counts of the various plasma were adjusted to 300,000 per cu. mm.) ADP was added at the points indicated by the arrows.

Fig. 19.—These are tracings obtained during platelet aggregation in vitro in stirred native platelet-rich plasma from a factor VIII deficient dog. In (a) only Tyrode's solution was added to the plasma. Aggregation occurred as indicated by the rise in the curve, but no coagulation occurred during the observation period. In (b) ADP to a final concentration of 10 μg per ml. (2 × 10⁻⁵ M) was added at the time indicated by the arrow. Aggregation occurred but no coagulation. In (c) a collagen fragment was added to the plasma at the point indicated by the arrow. Aggregation occurred in a normal manner, but there was no evidence of clotting. (d) Addition of thrombin (5 units per ml.) induced clotting, as indicated by the downward and then upward deflection of the tracing without oscillations.
membranes of adjacent platelets at some points, occasionally giving the appearance of desmosome-like structures. The filamentous material may be related to the actomyosin-like material referred to as thrombosthenin. The dark zones may represent points of tight binding between the platelets. The possibility exists that they may represent early formation of fibrin.

Structures similar to the microtubules which have been reported in platelets were found in many of the sections in these osmic acid-fixed tissues. It has been suggested that glutaraldehyde fixation is necessary to demonstrate these structures. The results in the present study, however, show that this is not the case. We found the tubular-like structures to be mainly evident in platelets which already had undergone some structural changes, especially pseudopod formation. Since they are not evident in unaltered platelets fixed in osmic acid but can be shown in areas in which platelets have been exposed to stimuli such as collagen and thrombin, this indicates that some alterations of the platelets are necessary before the tubules can be demonstrated with osmic acid fixation.

A striking feature of platelets in contact with collagen was the presence of gaps, probably representing holes in the limiting membrane of the platelet. These were most evident at the points where the platelets were in closest contact with the collagen. This could mean that the release of some platelet constituents by collagen is in part due to formation of gaps in the platelet membrane. It is important to note that these changes were found not only in normal dogs, but also in those with defects in coagulation.

Three factors are recognized to play a part in the establishment of a hemostatic plug: collagen, ADP, and thrombin. The in vitro studies of platelet aggregation in plasma prepared from the dogs with congenital coagulation defects showed that there was no abnormality of platelet aggregation induced by these stimuli. The morphologic observations from the in vivo studies in-
Fig. 21.—These tracings illustrate that the addition of thrombin to citrated platelet-rich plasma from a normal dog and from dogs with coagulation defects induced platelet aggregation in a similar manner. Platelet aggregation occurred before clotting.

dicated that there was less thrombin formation in these dogs than in normal dogs. Therefore, it is apparent, on the basis of the in vivo and in vitro observations, that the primary abnormality of hemostasis in the hemophilic dogs is attributable to insufficient thrombin formation due to impaired coagulation.

The frequency of renewed bleeding and the waxing and waning of bleeding in the hemophilic dogs probably reflect the same phenomenon—the opening and closing of channels through the plug. Before hemostasis can be effective, the consolidation of the plug must be sufficient to resist the intravascular pressure. Conceivably, the strength of the plug is dependent on (1) the fastening of the platelet mass to the vessel and the surrounding tissue, (2) the mutual cohesion of the platelets, and (3) the presence of fibrin. During the observation period, in hemophilic dogs only small portions of the plug broke away and this was never from the point of attachment to the vessel wall. This indicates a firm binding to the connective tissue despite the coagulation defects. This assumption is supported by the evidence of a close contact between the platelets and collagen in the electron micrographs. The presence of channels through the platelet mass suggests a regional weak binding between the platelets. The frequent finding of such channels in hemophilic dog plugs suggests that coagulation is particularly important for maintaining a firm platelet mass. This could be related to the action of thrombin in platelet binding, formation of fibrin, or both. The presence of loosely packed platelets with little pseudopod formation in the periphery of many hemophilic plugs and the absence of desmosome-like structures in this area could mean that thrombin is important in
producing tight binding of the platelets in this region. The peripheral fibrin net was incomplete even when hemostasis did occur in the hemophilic dogs. This suggests that a complete fibrin net is not essential for the provisional arrest of bleeding, which is in accordance with earlier observations.\textsuperscript{18,35,39,66}

In vitro there was no fibrin in association with the platelet aggregates produced by collagen in hemophilic native PRP, in contrast to normal native PRP. This suggests that the delayed fibrin formation in hemophilia is not corrected in spite of the potential stimulus of collagen and platelet aggregates. However, the occurrence of some fibrin in the plugs from even the most severe cases of hemophilia shows that there must exist additional coagulation stimuli, not present in our in vitro system. The loss of internal structure of the platelets along some channels and in some regions at the surface of the plug may also indicate that traces of thrombin were formed. Thrombin formation could be caused by the intrinsic pathway of coagulation, but in view of the in vitro experiments a more reasonable explanation is that the extrinsic pathway is stimulated by tissue thromboplastin released from injured tissues, presumably not present in the in vitro experiments. Obviously, this coagulation stimulus was not sufficient in the present experiments to form an impermeable plug at the normal rate. On the other hand, a defective extrinsic pathway, as manifested by factor VII deficiency, did not influence hemostasis, a finding in accordance with that made by Jørgensen and Borchgrevink\textsuperscript{36} in the case of “primary bleeding” in man. Although this evidence could be interpreted as indicating that the intrinsic pathway of coagulation is the more important one in hemostasis, the problem of comparing the severity of the defects precludes any conclusion on this point.

In our experiments factor VIII deficiency created a more severe defect in hemostasis than factor IX deficiency, judged both by functional and morphologic criteria. It would seem that factor VIII is more important than factor IX in formation of the hemostatic plug. This may be related to the observation that thrombin activates factor VIII and thereby potentiates the intrinsic pathway.\textsuperscript{49,52} This activation is still possible in factor IX deficiency.

In order to create combined defects in both coagulation pathways, factor IX deficient dogs were treated with Dicumarol and factor VII deficient dogs were given heparin. The suppression of factor VII to 2 per cent or less in factor IX deficient dogs produced only a slight effect on hemostasis. These observations further support the evidence that the extrinsic pathway, as manifested by this degree of factor VII deficiency in dogs, is not important in hemostasis.

Heparin treatment of factor VII deficient dogs had a pronounced effect on hemostasis. Examination of electron micrographs of these plugs showed a complete absence of fibrin in all regions. This is probably due to the well-known antithrombin effect of heparin, preventing fibrin formation although other actions of heparin on clotting may have played a part. The swelling and loss of internal structure in platelets in contact with collagen in these dogs further emphasizes the point that the platelet interaction with collagen is independent of blood coagulation. This probably explains why a hemostatic plug was formed even though thrombin formation was inhibited. These findings are in agreement with other studies showing that heparin does not prevent the
formation of a platelet mass at the point of injury. The delay in the primary arrest of bleeding in the heparin-treated factor VII deficient dogs may have been due to an additional effect of heparin, such as its reported action on “platelet adhesiveness.”

Phenylbutazone in the dosage used did not affect hemostasis in normal dogs, whereas in factor IX deficient dogs it greatly aggravated the hemostatic defect. The pharmacologic effect of phenylbutazone is complex. One important action is its effect on cell membranes; in addition, it and its analogs have been found to inhibit platelet aggregation induced by collagen and to influence platelet economy in vivo. In the phenylbutazone-treated factor IX deficient dogs the plugs were fragile, and the sections revealed loosely packed platelets with slight morphologic changes. Since platelets from phenylbutazone-treated animals respond normally to ADP, this may mean that the amount of ADP released was diminished, and therefore the binding between platelets was weak. Furthermore, the slow formation of the plugs in some experiments is compatible with the observation that phenylbutazone depresses collagen-platelet interaction, particularly the release of ADP. In the factor IX deficient dogs it appears that platelets under the influence of phenylbutazone do not react adequately to the plug-forming stimuli. However, the results from normal dogs show that the degree of suppression of the platelet-collagen reaction produced by the doses of phenylbutazone used did not cause impaired hemostasis in the presence of a normal coagulation mechanism. The possibility exists that with further suppression of the platelet-collagen reaction, using higher doses of phenylbutazone, the compensatory effect of coagulation would be ineffective.

These studies show that in the presence of a marked congenital coagulation defect in the intrinsic pathway, platelets accumulated at the end of a transected vessel in the initial stages in a normal manner. This was also true for situations in which both the extrinsic and intrinsic pathway of coagulation were defective. The initial platelet interaction with the vessel wall and surrounding tissue was not dependent upon coagulation. Since partial suppression of this reaction did not produce abnormal hemostasis in normal dogs, but further impaired hemostasis in factor IX deficient dogs, this may mean that there is a combined action between the surface stimulus and the coagulation stimulus in the formation of the hemostatic plug. The intrinsic pathway of coagulation is important for the formation of a stable nonpermeable hemostatic plug.

SUMMARY

Hemostasis was examined after transection of vessels, 50–200 microns in diameter, both in normal dogs and in dogs with congenital defects of either factor VII, factor IX, or factor VIII. The formation of the hemostatic platelet plugs was observed by direct microscopy in vivo, and sections of the plugs were prepared for both light and electron microscopy 10 to 30 minutes after transection. Furthermore, the reaction of platelets from normal and abnormal dogs with adenosine diphosphate, collagen and thrombin was tested in vitro by a turbidimetric technic.

In normal and factor VII deficient dogs the initial arrest of bleeding took place about 3 minutes after transection, and rebleeding was infrequently ob-
served. Their platelet plugs were composed of densely packed platelets, anchored to the vascular and perivascular tissue and surrounded by a cap of fibrin.

In factor IX deficient dogs there was no definite prolongation of the initial bleeding time, but rebleedings were frequent. In factor VIII deficient dogs the initial bleeding time was prolonged and the intensity of the bleeding had a wave-like characteristic. The plugs in the hemophilic dogs were larger than normal, were rich in channels, and had areas of loosely packed platelets and an incomplete fibrin cap.

Treatment of factor IX deficient dogs with Dicumarol did not further impair hemostatic plug formation in the doses used. Treatment of factor VII deficient dogs with heparin, or factor IX deficient dogs with phenylbutazone, prolonged the bleeding time markedly, delayed the building up of the plug, and gave fragile, loosely packed plugs. Treatment of normal dogs with phenylbutazone did not alter the hemostatic process at the dosage used.

In the in vitro studies, platelets from the dogs with congenital coagulation defects reacted normally with the aggregating stimuli.

It is concluded that the initial platelet interaction with the vessel wall and surrounding tissue is not dependent upon blood coagulation. An intact intrinsic pathway of coagulation is necessary for the stabilization of the hemostatic plug after it is formed.

SUMMARIO IN INTERLINGUA

Esseva examinate le hemostase—tanto in canes normal como etiam in canes con congenite defectos de Factor VII, de Factor IX, o de Factor VIII—post le transsection de vasos de diametres de 50 a 200 micron. Le formation del hemostatic tappos de plachettas esseva observate in vivo per microscopia directe, e sectiones del tappos esseva preparate pro microscopia optic e electronic inter 10 e 30 minutus post le transsection. In plus, un technica turbidimetric esseva utilisate pro testar le reaction de plachettas (ab canes normal e anormal) a diphosphato de adenosina, collageno, e thrombina in vitro.

In canes normal e in canes a deficientia de Factor VII, le arresto del sanguination initial occurriva circa 3 minutus post le transsection. Resanguination esseva un observation infrequente. In iste casos le tappos de plachettas esseva componite de densemente paccate plachettas, ancorate al tissu vascular e perivascular e inoveloppte de un cappello de fibrina.

In canes a deficientia de Factor IX, nulle definite prolongation esseva notate in le tempore del sanguination initial, sed resanguination esseva frequente. In canes con deficientia de Factor VIII, le tempore del sanguination initial esseva prolongate, e le intensitate del sanguination sequava un curso undulatori. Le tappos in canes con hemophilia esseva de dimensiones plus que normal. Illos esseva rie in ductos e habeva areas de laxe pacce cellular, e le cappello de fibrina esseva incomplete.

Le tractamento con Dicumarol (al nivelllos de dosage utilisate) non damnificava addicionalmente le formation del tappos in canes a deficientia de factor IX. Le administration de heparina a canes deficiente in Factor VII o de phenylbutazona a canes deficiente in Factor IX prolongava marcatemente le tempore de sanguination, retardava le formation del tappos, e resultava in fragile tappos a laxe pacce. Le tractamento de canes normal con phenylbutazona (al nivelllos de dosage utilisate) non alterava le processo hemostatic.

In le studios effectuate in vitro, le plachettas ab canes con congenite defectos de coagulation reageva normalmente con le stimulus aggregatori.

Es concludite que le interaction initial inter plachettas e le parietes vascular (e le tissu adjacent) non depende del coagulation del sanguine. Un intacte circuito de coagulation es necessari pro le stabilisation del tappo hemostatic post su formation.
ACKNOWLEDGMENT

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REFERENCES

EXPERIMENTAL HEMOSTASIS IN DOGS


Experimental Hemostasis in Normal Dogs and Dogs with Congenital Disorders of Blood Coagulation

T. HOVIG, H. C. ROWSELL, W. J. DODDS, L. JØRGENSEN and J. F. MUSTARD