Infectious Canine Hepatitis: Animal Model for Viral-induced Disseminated Intravascular Coagulation

D. H. Wigton, G. J. Kociba, and E. A. Hoover

The objective of this study was to characterize the hemostatic defect in dogs with infectious canine hepatitis (ICH), a naturally occurring viral disease of dogs. Five littermate dogs were inoculated with $10^3$ TCID$_{50}$ of ICH virus intravenously. Two littermates were controls. The clinicopathologic manifestations of ICH were fever, depression, anorexia, hematemesis, melena, widespread mucocutaneous petechiae, prolonged bleeding from venipunctures, facial edema, leukopenia, and proteinuria. The hemostatic defect of ICH was characterized by thrombocytopenia, abnormal platelet function, prolonged one-stage prothrombin time and activated partial thromboplastin time, normal thrombin times, depressed factor VIII activity, and increased fibrin-fibrinogen degradation products. These findings suggested that the central pathologic mechanism of the abnormal hemostasis in ICH was disseminated intravascular coagulation (DIC). ICH is an example of DIC induced by viral infection. This disease is a suitable model for investigation of the detection, pathogenesis, and therapy of DIC.

INFECTIOUS CANINE HEPATITIS (ICH) as described originally by Rubarth in 1947 is an acute infectious disease of young dogs characterized by severe hepatitis, edema of the gall bladder, tonsillitis, multifocal vasculitis, and hemorrhage. ICH is caused by canine adenovirus type 1 (ICHV) which is a double-stranded DNA virus with intranuclear replication. ICHV has a tropism for endothelial, mesothelial, and hepatic parenchymal cells. Endothelial cell damage occurs primarily in the liver, spleen, and kidney, and less frequently in other tissues. Infected dogs usually show fever, pronounced leukopenia, elevated serum levels of liver enzymes, proteinuria, and impaired and unstable clot formation. Prolonged bleeding from venipunctures and widespread hemorrhages are reported consistently. Impaired synthesis of liver-derived clotting factors and concomitant massive heparin release as a sequela to extensive hepatic necrosis have been proposed as an explanation for the hemorrhage.

In the light of recent concepts concerning hemostatic aberrancies, disseminated intravascular coagulation (DIC) as a complication of diseases featuring diffuse vasculitis offers an alternative explanation for the hemorrhage in ICH. Since endothelial disruption with release of tissue thromboplastin and exposure of adventitial collagen are recognized effects of vascular degeneration, such as develops in ICH, generalized activation of the hemostatic system is a probable pathologic consequence of ICH.
Characterization of the hemostatic abnormality seen in ICH as it relates to DIC could offer a suitable animal model for in vivo studies of the pathogenesis, detection, and therapy of DIC. In the present study the hemostatic defect of dogs with infectious canine hepatitis was characterized and correlations were made between the nature and severity of the hemostatic defect and other clinicopathologic abnormalities.

MATERIALS AND METHODS

Experimental Animals

Seven large mixed-breed littermate dogs were raised from birth in isolation units. The dogs were treated for ascariid infestation at 2 wk of age, but they were not vaccinated for any diseases.

Inoculum and Inoculation

ICHV isolated by and obtained from J. Carmichael (Cornell University Veterinary Virus Research Institute, Ithaca, N. Y.) was used to prepare a viral stock in canine thyroid carcinoma cell culture (T-Ca). The virus stock was titrated in T-Ca cells using standard techniques. An inoculum of approximately 10 median tissue culture infectious doses (TCID50) of ICHV contained in 1.0 ml of cell culture fluid was administered slowly in the external jugular vein of five dogs. The inoculum was prepared by freezing infected T-Ca cultures at -90°C, thawing to 37°C, centrifuging at 1000 rpm for 10 min, and decanting the supernatant. This procedure was repeated twice, and the supernatant was used. Two control dogs received an inoculum of uninfected T-Ca cell culture prepared and administered in a similar manner. The dogs were 14 wk old at the time of inoculation.

Hematologic Evaluation of Inoculated Animals

Blood samples were collected at 12-hr intervals from 24 hr before inoculation to 226 hr post-inoculation (pi) patterned after trial inoculation of two dogs. Similar collections were made on all moribund dogs prior to euthanasia and necropsy. Blood samples were collected from the external jugular veins into silicone-coated glass vials.

For platelet counts 1 ml of blood was added to 0.02 ml 7.5% potassium ethylenediamine tetraacetate (K3 EDTA) solution. For platelet retention 5 ml of blood with no anticoagulant were tested immediately after collection using the glass bead method of Salzman as modified by Bowie. All platelet counts were performed immediately after collection using an electronic cell counter (Coulter Model B). Platelet retention was done on all samples with platelet counts over 50,000/cu mm.

For clotting factor assays, one-stage prothrombin time (OSPT), activated partial thromboplastin time (APTT), thrombin time, and serial dilution of protamine sulfate fibrin-fibrinogen degradation product (FDP) test, 9 ml of blood were added to 1 ml of 0.13 M sodium citrate. The blood was centrifuged at 2000 g for 20 min, and the plasma was preserved in polyethylene containers. Plasma for clotting factor assays and thrombin time was stored at -70°C.

Clotting times were determined by the method of Lee and White, and OSPT by Quick's one-stage technique. Fibrinogen was determined by the technique of Kaneko and Smith. The techniques used to measure APTT and to determine relative activities in plasma of PTA (factor XI), Christmas factor (factor IX, PTC), and antihemophilic factor (factor VIII) were those described by Forman et al. using canine plasma substrate. The technique to determine relative activity in plasma of Stuart factor (factor X) was that of Dodds et al. using human plasma substrate. The control for clotting factor assays was canine plasma pooled from six normal adult dogs and stored at -70°C. Plasma activity for experimental and control dogs was expressed as a percentage of normal adult activity. Plasma thrombin time was determined by the technique of Jim as modified by Bowie. The serial dilution of protamine sulfate (SDPS) was determined by the technique of Gurewich and Hutchinson.

For latex agglutination FDP determinations 10,000 kallikrein inactivator units (Trasylol, Baychem, Leverkusen, Germany) were added to 1 ml blood to inhibit in vitro fibrinolysis. FDP was determined semiquantitatively using the latex agglutination test.
For the complete blood count (CBC), which included hemoglobin determination, packed cell volume (PCV), total protein, total leukocyte count, and differential leukocyte count, 1 ml of blood was added to 0.02 ml of 7.5% K3 EDTA solution. Blood films were made immediately upon sample collection.

Ancillary clinicopathologic determinations included blood urea nitrogen, serum glutamic pyruvic transaminase (SGPT), serum alkaline phosphatase (SAP), lactic dehydrogenase (LDH), and serum glucose. Urine samples were collected once daily by catheterization using sterile precautions, and a complete urinalysis was performed. Quantitative urine protein determinations were done using a turbidometric technique.26

Necropsy Evaluation of Experimental and Control Dogs

Complete necropsies were performed on all experimental dogs that died or became moribund during the experimental period. One surviving dog and control dogs were electrocuted and necropsied at 226 hr pi.

Tissue sections from liver, gall bladder, kidney, spleen, mesenteric and mediastinal lymph nodes, tonsils, lung, brain, bone marrow, intestine, stomach, urinary bladder, ovarian arteries in females, adrenal gland, and eye were preserved in phosphate-buffered formalin. Microscopic evaluation of histologic sections was performed on representative tissues.

RESULTS

Clinical Signs

Initial signs detected at 36 hr pi in all virus-infected dogs were fever, depression, partial anorexia, mild conjunctivitis, and tonsillitis. By 48 hr pi infected dogs had rectal temperatures ranging from 40°–41.1°C. At 60 hr pi, all infected dogs were severely depressed, and vomiting and dehydration were evident. Enlargement of the tonsils was pronounced and peripheral lymph nodes were palpably swollen and tender.

At 72 hr pi additional signs of bilateral photophobia and aqueous flare were seen in two dogs. Two dogs developed edema of the head, lower cervical area, and ventral body midline. At 84 hr pi, generalized petechial hemorrhages of mucosal surfaces and prolonged bleeding from venipunctures were constant features in infected dogs.

Petechiae were observed in the skin of the pinna, oral and genital mucosa, and inguinal skin at 96 hr pi in all infected dogs. Hematomas developed after venipuncture and areas of hemorrhage were found in the interdigital spaces of some dogs. Mild icterus was present in two dogs at 96 hr pi and persisted until death.

The first death occurred at 96 hr pi, and by 158 hr pi four dogs had died or were moribund. Subnormal temperatures and dark tarry stools were noted in all fatally infected dogs during the 12 hr prior to death. Each fatally infected dog vomited a sanguineous fluid within 5 hr of death.

Necropsy and Histopathologic Results

The most prominent feature on postmortem examination of infected dogs was widespread vascular degeneration and hemorrhage. Fibrin thrombi were observed in lung, liver, kidney, thymus, and spleen. The stomach consistently contained dark sanguineous intraluminal fluid, and the gastric mucosa had severe congestion and numerous petechiae. Diffuse mucosal petechiae and widespread subserosal hemorrhage were present in the intestinal tract.
Mesenteric lymph nodes were enlarged and dark red. Histopathologic examination revealed necrosis and depletion of lymphocytes, vascular degeneration, edema, and hemorrhage. Similar changes were seen in bronchial, mandibular, axillary, and popliteal lymph nodes. Atrophy, edema, and hemorrhage of the thymus were present in all infected dogs. The tonsils were grossly enlarged, and intranuclear inclusion bodies appeared in tonsillar epithelial cells. Although the spleen appeared grossly normal, fibrinoid degeneration was observed in muscular arteries with large basophilic intranuclear inclusion bodies in endothelial cells.

The liver appeared enlarged and dark red. Histopathologic examination demonstrated marked congestion and multifocal coagulation necrosis with large basophilic intranuclear inclusion bodies in hepatocytes. Edema of the gall bladder was present in all but the one surviving infected dog.

Large multifocal hemorrhages were seen throughout the lungs of all infected dogs. Numerous petechiae were observed on the parietal pleura chiefly in the ventral thorax.

Petechiae of the urinary bladder mucosa and subserosa were consistently seen associated with vascular degeneration. Renal lesions varied from isolated large basophilic inclusion bodies in glomerular tuft endothelium in rapidly fatal infections to interstitial nephritis featuring chiefly lymphocytes and plasmacytes in the surviving infected dog.

Bone marrow examination of all fatally infected dogs revealed myeloid, erythroid, and megakaryocytic hypoplasia and erythrophagocytosis. The surviving dog’s bone marrow was hyperplastic.

**Hematologic Changes**

The platelet count, platelet retention, OSPT, APTT, thrombin times, and individual factor activity of control dogs are representative for normal dogs tested in our laboratory. The platelet count of the infected dogs was significantly decreased (analysis of variance for repeated measures, $p < 0.05$) by 60 hr pi and declined to below 50,000/cu mm by 84 hr pi (Fig. 1). The counts

![Fig. 1. Total platelet numbers in five dogs infected with infectious canine hepatitis (ICH) and two littermate controls. Brackets = 1 SD.](image)
of the fatally infected dogs remained less than 50,000/cu mm until death. The survivor's platelet count did not recover to 50,000/cu mm until 192 hr pi. Evaluation of peripheral blood smears revealed many megaplatelets detected initially at 60 hr pi and persisting until death.

In infected dogs platelet adhesiveness as demonstrated by the glass bead column retention technique progressively declined from a preinoculation average of 80% to 24% at 72 hr pi. The absolute number of platelets not retained remained constant. Preinoculation retention for control dogs was 77% in contrast to 66% at 72 hr pi.

The changes in OSPT were not pronounced but were statistically significant. The OSPT became significantly prolonged at 72 hr pi and remained so thereafter except for the 120 hr pi sampling (Fig. 2). The maximum OSPT was approximately 15 sec at 96 hr pi.

The APTT became significantly prolonged at 36 hr pi and reached a maximum average of over 90 sec at 84 hr pi (Fig. 3). The APTT of the infected dogs remained significantly longer than that of the control dogs throughout the experiment.

Thrombin times of infected dogs remained statistically identical with control values except in one dog at 72 hr pi only.

Factor VIII plasma activity decreased from preinoculation levels of 109% of normal adult activity to less than 6% activity at 48 hr pi (Table 1) and remained markedly reduced. Factor IX plasma activity was significantly decreased only at 96 hr pi. At all other sampling periods infected and control values were similar (Table 1). Factor XI plasma activity became reduced at 96 hr pi in the infected group, with 25% activity compared to 64% activity in the controls. A moderate decrease persisted (Table 1). Fibrinogen levels decreased significantly ($p < 0.05$) at variable intervals prior to death in each infected dog. Significant changes in factor X were not observed.

Increased FDP (any positive dilution) were detected by 72 hr pi using the serial dilution of protamine sulfate test. Elevated levels were observed thereafter (Table 2). The more sensitive latex agglutination test for FDP revealed low levels in control dogs and in preinoculation samples. Elevations (> 1:10) were
first observed at 48 hr pi and remained throughout the study (mean dilution 1:50). Positive results were seen in dilutions of 1:100 in individual dogs.

The leukocyte count dropped sharply at 48 hr pi and progressively declined until death (Fig. 4). Initially the leukopenia was accountable chiefly to a pronounced lymphopenia which was most severe at 48 hr pi. A marked neutropenia ensued, first detected at 84 hr pi, and progressively worsened until death. Band neutrophil numbers increased sharply from a preinoculation average of

Table 1. Clotting Factor Activity in Five Dogs Infected With ICH and Two Littermate Controls*

<table>
<thead>
<tr>
<th>Postinoculation Day</th>
<th>VIII Clotting Factor Activity (%)</th>
<th>IX Clotting Factor Activity (%)</th>
<th>XI Clotting Factor Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ICH C</td>
<td>ICH C</td>
<td>ICH C</td>
</tr>
<tr>
<td>0</td>
<td>105 109</td>
<td>82 70</td>
<td>42 35</td>
</tr>
<tr>
<td>2</td>
<td>6 200</td>
<td>60 70</td>
<td>45 52</td>
</tr>
<tr>
<td>4</td>
<td>10 111</td>
<td>37 82</td>
<td>25 64</td>
</tr>
<tr>
<td>6</td>
<td>42 99</td>
<td>87 90</td>
<td>17.5 42</td>
</tr>
</tbody>
</table>

*Measurements are mean percentage of normal adult activity.

Table 2. Serial Dilution of Protamine Sulfate Results in Five Dogs Infected With ICH and Two Littermate Control Dogs

<table>
<thead>
<tr>
<th>Day Postinoculation</th>
<th>ICH</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>1/40</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1/40</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
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<tr>
<td>7</td>
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<td>0</td>
</tr>
<tr>
<td>8</td>
<td>1/40</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are highest positive dilution containing gel or fibrin strands. Highest dilution tested was 1/40.
164 cells/cu mm to over 450 cells/cu mm at 36 hr pi. At 60 hr pi, examination of peripheral blood films revealed a change in lymphocyte morphology characterized by increased size, deeply basophilic cytoplasm, and azurophilic cytoplasmic granules. These lymphocytes persisted as predominant forms until death of fatally infected dogs.

Nucleated erythrocytes were observed in peripheral blood smears at 36 hr pi and increased until death. Anemia (PCV < 30%) was not seen in infected dogs before 96 hr pi. Mild anemia (PCV = 29%) was seen solely in terminal samples of three dogs dying between 96 and 120 hr pi. The dog dying at 150 hr pi and the surviving dog both developed severe anemia (PCV < 20%), initially detected at 108 hr pi. Reticulocytosis was not observed in infected dogs. The total plasma protein of infected dogs was not statistically different from control values.

Elevations in SGPT were detected at 72 hr pi and rapidly increased to peak values of over 1600 I.U./liter at 96 hr pi (mean control = 50 I.U./liter). Increases in LDH were first seen at 72 hr pi. A sharp increase to over 3700 I.U./liter occurred at 84 hr pi, representing maximum elevation (mean control = 300 I.U./liter). Significant increases in SAP were first observed at 84 hr pi, with a subsequent increase until death.

Mild proteinuria (60 mg/100 ml) was first observed at 60 hr pi and increased to an average of 418 mg/dl at the termination of the experiment. Variable numbers of hyaline and finely granular casts were seen at 60 hr pi and increased until death. Variable numbers of erythrocytes were seen in the urine, first detected at 72 hr pi and increased thereafter.

**DISCUSSION**

In this study the hemostatic defect of ICHV infected dogs has been characterized by thrombocytopenia, abnormal platelet function, prolonged OSPT and APTT, depressed factor VIII plasma activity, normal thrombin times, and increased circulating FDP levels. These findings suggest that the central pathologic mechanism of the abnormal hemostasis in ICH is disseminated intravas-
cular coagulation.\textsuperscript{12,13} ICH is, therefore, a suitable model of DIC related to viral infection and probably to virus-induced vascular lesions. The experimental disease reproduces the naturally occurring disease in which the same hemostatic abnormality occurs. ICH should prove a useful model to investigate the abnormality in platelet function present in virus-induced DIC and the genesis and in vivo anticoagulant effects of FDP. Also, ICH offers an in vivo system for the evaluation of various therapeutic regimes in DIC.

Evidence for DIC in viral diseases of humans includes exanthematous viruses, i.e., varicella, variola, rubella, rubeola, and vaccinia, and arboviruses that cause hemorrhagic fevers in the Philippines, Bolivia, and Argentina.\textsuperscript{27} Viral diseases of animals associated with DIC include fowl plague, hog cholera, and probably blue tongue and epizootic hemorrhagic disease of deer.\textsuperscript{27-29}

The most probable mechanism for the genesis of DIC in dogs infected with ICHV is endothelial disruption, which is thought to allow release of tissue thromboplastin into the blood stream, platelet adherence at the site of injury, and activation of the intrinsic clotting mechanism as well as enhancing the activation of plasminogen to plasmin.\textsuperscript{12,13} The thrombocytopenia observed initially at 48 hr pi may be the result of an increased consumption of platelets as an effect of diffuse vascular degeneration seen on histopathologic examination\textsuperscript{12,13} or may reflect direct damage of platelets by the virus such as occurs in hog cholera,\textsuperscript{28} or both. The appearance of megaplatelets at 60 hr pi is considered to reflect an increased platelet turnover rate.\textsuperscript{30} The severe thrombocytopenia that persisted until death may reflect the additional complication of decreased production of platelets due to bone marrow degeneration from virus infection.\textsuperscript{9,10}

The abnormal platelet retention of 23\% in infected dogs at 72 hr pi may be an effect of elevated circulating FDP,\textsuperscript{12,13} or may reflect abnormal platelets produced by diseased megakaryocytes. FDP increases were initially significant at 48 hr pi, which corresponded with the onset of abnormal platelet retention.

The APTT became prolonged to 6–7 times control values, while the OSPT was prolonged only to 1–2 times control values and thrombin times remained essentially unaltered. The dramatic prolongation of the APTT appeared to be chiefly due to markedly depressed factor VIII plasma activity. Depressed factor VIII plasma activity has been reported in DIC as an effect of consumption.\textsuperscript{12,13} Elevated FDP also contributes to prolonging the APTT and OSPT by competitive inhibition of thrombin activity and formation of nonclottable complexes with fibrin monomers.\textsuperscript{12,13} Prolongation of the APTT with depressed factor VIII activity precedes detection of elevated FDP by 12 hr, suggesting that consumption of factor VIII leads to the initial change in APTT.

The leukopenia observed at 48 hr pi reflected a pronounced lymphopenia which is reported to be a manifestation of viral proliferation within lymphoid tissue.\textsuperscript{12,25,6} The subsequent neutropenia observed first at 84 hr pi is reported to be an effect of viral proliferation within bone marrow.\textsuperscript{9} The changes in lymphocyte morphology observed at 60 hr pi are consistent with observations by Schalm and are thought to represent immature lymphocyte release or proliferation.\textsuperscript{31}

The appearance of nucleated erythrocytes in the peripheral circulation of nonanemic dogs may be associated with virus proliferation in endothelial cells...
within the bone marrow. The distortion of normal labyrinth architecture may allow premature release of nucleated erythrocytes. The increased numbers of band neutrophils at 36 hr pi may reflect a similar pathogenesis or may reflect a response to tissue inflammation, or both.

The degree of anemia corresponds with the duration of virus infection. Necropsy examination suggests that large volumes of blood may be lost via the gastrointestinal tract. The long life span of erythrocytes (99–110 days in dogs) may delay the recognition of depressed erythropoiesis. Changes in liver enzyme levels and chemical and microscopic changes in urine are similar to those of earlier studies. The onset of proteinuria at 60 hr pi may reflect altered glomerular permeability from viral replication in glomerular endothelium, or may be associated with microthrombosis of glomeruli as an effect of DIC, or both.

This system represents an easily reproduced naturally occurring disease of dogs which is a suitable model of DIC related to viral infection, and probably virus-induced vascular lesions.

REFERENCES

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Infectious canine hepatitis: animal model for viral-induced disseminated intravascular coagulation

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