Cross-Reactivity of the Anti-Human D-dimer Monoclonal Antibody 1C9-6F10 to Canine Fibrin Degradation Products

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Disseminated intravascular coagulation (DIC) is a life-threatening condition characterized by abnormal systemic coagulation followed by secondary fibrinolysis. An elevated concentration of fibrin degradation markers indicates the activation of fibrinolysis and is thus one of the diagnostic criteria for DIC in humans and dogs [6, 12]. Fibrinogen comprises a central globular E region and two identical D regions that form a D-E-D structure. As such, the cleavage of fibrinogen produces four fragment patterns: D-E-D, D-E, D or E. Fibrin is a complex form of fibrinogen connecting each D region. Thus, fibrin degradation products (XDPs) comprise D-D/E-D/E-D-E-D (molecular weight, 1040 kDa), D-D/E-D/E-D/E-D (780 kDa), E-D/D-D/D/E (520 kDa) and D-D/E (260 kDa) fragments in blood and are visualized as E-D-D/E-D-D/E-D-D fragments by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) after cleavage of the simple disulfide bond [8, 11]. All XDPs include the D-D complex, which is called a D-dimer, while the fibrinogen degradation product does not include the D-D complex. In general, most laboratory “FDP” tests measure the degradation products from both cross-linked fibrin and fibrinogen itself, while the “D-dimer” test measures only XDPs. Thus, the “D-dimer” test is thought to be suitable for the evaluation of secondary fibrinolysis. The measurement of D-dimer concentrations in dogs is used to diagnose DIC and other fibrinolysis-related diseases [2–5, 7, 9, 10]. However, the specific cross-reactivity of the canine D-dimer antibodies used in these tests has not been investigated. Therefore, we investigated whether an antibody developed for the human D-dimer that is used in the Factor Auto D-dimer kit (Q-may, Oita, Japan) and is commercially available from Mitsubishi Chemical Medience Corporation can cross-react with canine XDPs. We then evaluated the potential utility of the latex agglutination assay using this antibody to measure canine D-dimer concentrations in plasma.

The fibrinogen was prepared from dog plasma, passed through lysine-Sepharose 4B (Pharmacia Biotech AB, Uppsala, Sweden) and gelatin-Sepharose 4B (American Bioscience AB, Uppsala, Sweden), centrifuged three times (12,900 × g for 20 min) and subjected to precipitation cycles with ammonium sulfate (1 M). The final precipitate was dissolved in buffer containing 0.05 M Tris-HCl (pH 7.5) with 0.9% NaCl. The cross-linked fibrin was prepared by incubation with thrombin (0.25 U/ml) and Fibrogammin P (0.11 U/ml) in the fibrinogen at 37°C for 1 hr. The cross-linked XDPs were produced by incubation with both urokinase (0.01 U/ml) and plasminogen (0.04 U/ml) at 37°C for 1 hr. The digestion was terminated by the addition of aprotinin (51.4 U/ml). Protein concentrations were determined using the Bradford assay with bovine serum albumin as a standard.

Four micrograms of fibrinogen (cFg), canine XDP (cXDP) and human XDP (hXDP) on 3% to 10% gradient SDS-PAGE were visualized and stained. The separated cFg (0.5 µg) cXDP (0.5 µg) and hXDP (0.2 µg) on 3% to 10% gradient SDS-PAGE were transferred to polyvinylidene difluoride membranes and reacted with the 1C9-6F10 antibody at a concentration of 1 µg/ml. The membrane was then reacted with horseradish peroxidase-conjugated sheep anti-mouse antibody (20,000 dilutions; GE Healthcare Biosciences, Chicago, IL, U.S.A.), and the signals were detected with an ECL plus chemiluminescence detection kit (GE Healthcare Biosciences,) using an LAS-1000 luminescent image analyzer (Fujifilm).
To investigate the specificity of the antibody to canine plasma, samples obtained from a dog clinically suspected to have DIC and a healthy dog were analyzed by similar immunoblotting techniques, except that the sample was reduced by 2-mercaptoethanol and 6% SDS-PAGE. We suspected that the dog had DIC, because of its clinical condition and platelet count; it showed an acute onset of depression after prolonged outdoor activity and a platelet count of 68,000/µl.

Next, the possible usefulness of the antibody using a latex agglutination assay kit (Factor Auto D-dimer kit; Q-may) to measure cXDPs with Accute (Toshiba Medical, Ohtawara, Japan) was evaluated, and the results were expressed as absorbance values. The plasma sample obtained from the dog without DIC, because of its clinical condition and a platelet count of 68,000/µl.

The SDS-PAGE results of the purified cFg, cXDP and hXDP are shown in Fig. 1A. A major broad band was observed at 300 to 350 kDa with other bands detectable at over 400 kDa, 80 and 70 kDa on the cFg column. The cXDP was observed as a major band at 200 kDa with minor bands at 260, 70 and 60 kDa. The hXDP was observed as two major bands at 260 and 200 kDa with multiple bands between 300 and 500 kDa, a single band at 50 kDa and a weak signal at 100 kDa. The corresponding immunoblotting results of the purified cFg, cXDP and hXDP are shown in Fig. 1B. The signal for canine fibrinogen was detected as a broad band at approximately 300 to 380 kDa. The signal for cXDP was detected at 200 kDa, corresponding to the size of the D-dimer (DD-fragment) detected by SDS-PAGE. For hXDP, strong signal bands were seen at 200 and 260 kDa with weak signals of various sizes. The immunoblotting results of plasma from the dog clinically suspected to have DIC and the dog without DIC with hXDP are shown in Fig. 1C. Sharp bands were detected at 260, 200, 90 and 80 kDa with a broad band at over 300 kDa in the dog with suspected DIC. The two bands at 260 and 200 kDa in the dog with suspected DIC corresponded to the bands in the hXDP samples, which used the D-dimer as a positive control. Only broad bands were detected in the dog without DIC.

The results of the latex agglutination assay are shown in Fig. 2. The relative absorbance values in the assay were not altered by additional canine fibrinogen from 13.3 to 40 µg/ml (Fig. 2A). In contrast, the absorbance values were increased in a dose-dependent manner following addition of cXDPs (Fig. 2B).

We then evaluated the cross-reactivity of the 1C9-6F10 antibody developed for the human D-dimer and its potential usefulness for the measurement of canine D-dimer using a latex agglutination assay kit.

To elucidate the cross-reactivity of the 1C9-6F10 antibody, we prepared the cFg, cXDP and hXDP and visualized the results using SDS-PAGE. We speculated that the major band at 300 to 350 kDa was canine fibrinogen, because the...
The molecular weight of fibrinogen is 340 kDa (Fig. 1A). The larger bands (>400 kDa) may have been multiple forms of fibrinogen, and the small bands (70–80 kDa) may have been degraded fibrinogen or co-purified contaminants. For hXDP, a positive control for canine XDP production was visualized as two major bands at 260 and 200 kDa with multiple other bands that indicated the hXDP containing the undigested fibrinogen multimore or fibrin. The 260- and 200-kDa bands in canine XDP were identical to those in hXDP and appeared to correspond in size with the D-D-E complex and D-D fragments, respectively. These results indicate that the canine D-dimer was successfully produced.

In the immunoblotting, the signal was detected as a broad band at approximately 300 to 380 kDa in the cFg column. We speculated that these bands were fibrinogen, because of the corresponding size of canine fibrinogen. Some anti D-dimer antibodies can reportedly react to solid-phase fibrinogen, because of the conformational structural changes that occur during the denaturing process in the SDS [1]. These antibodies, however, may recognize an epitope that is part of the D-fragment that exists in fibrinogen, although the epitope is not fully exposed in native fibrinogen. In cXDP, the signal was detected at 200 kDa, corresponding to the size of the D-dimer (DD-fragment) detected by SDS-PAGE. The 1C9-6F10 antibody detected strong-signal bands at 200 and 260 kDa with weak signals of various sizes in hXDP, despite the fact that lower quantities of hXDP protein (0.2 µg) were applied relative to cXDP (0.5 µg). This result may indicate that the 1C9-6F10 antibody has a higher affinity to hXDP than to cXDP.

The canine plasma immunoblotting results (Fig. 1C) indicate that the 1C9-6F10 antibody can detect various XDPs in canine plasma, such as the D-D-E complex (260 kDa), DD-fragment (200 kDa) and single D-fragments (90 and 80 kDa) [8, 11]. In contrast, a broad band at over 300 kDa was detected in both plasma samples, indicating that the antibody also reacted to denatured canine fibrinogen, despite the fact that the canine plasma did not undergo a purification process. Although two XDP bands were detected in the plasma from the dog suspected to have DIC, the 1C9-6F10 antibody detected only one band at 200 kDa corresponding to the synthesized cXDP, despite the fact that there were two bands in the chemically produced XDP by SDS-PAGE. It is unclear why the 1C9-6F10 antibody did not react to the chemically produced XDP at 260 kDa, but this may simply reflect the breakdown of the conformational structure of XDPs during the chemical production process. The bands at 80 and 90 kDa detected in the plasma of the dog with suspected DIC indicate that the 1C9-6F10 antibody detected single D-fragments derived from fibrinogen and/or fibrin degradation. This finding suggests that the epitope recognized by the 1C9-6F10 antibody exists on a portion of the D-fragment.

Finally, the potential usefulness of the antibody using a latex agglutination assay kit to measure cXDPs was evaluated. Because the 1C9-6F10 antibody reacted with solid-phase canine fibrinogen, the presence of fibrinogen may interfere with the utility of the assay kit to quantify the D-dimer concentration in dog plasma. The results indicate that fibrinogen does not affect the XDP concentration values measured by the Factor Auto D-dimer assay. In contrast, the assay can measure canine D-dimers in a liquid phase in the presence of endogenous fibrinogen.

In conclusion, the anti-human D-dimer antibody 1C9-6F10 cross-reacted to the canine D-fragment with high specificity, but lower affinity compared with the human D-dimer. The latex agglutination assay using the 1C9-6F10 antibody was used to measure the canine plasma D-dimer concentration. However, the major limitation of the present study is that we were not able to definitively diagnose DIC in the dog used for the validation of cross-reactivity, despite the fact that the clinical and biological findings were suggestive of DIC. Further assays of dogs definitively diagnosed with...
DIC are needed to establish reference values and determine the sensitivity and specificity of this test for the diagnosis of DIC in dogs.

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