Persistent Hematomas in Japanese Black Cattle with Impaired Platelet Aggregation Function and Large Granule Eosinophils

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ABSTRACT. In Japanese black cattle with large and long-existing hematomas, platelets was impaired in collagen aggregation function in vitro. There was no statistically significant difference from control animals in the tests of PT (prothrombin time) and PTT (partial thromboplastin time) for extrinsic and intrinsic blood coagulation system. Aside from impaired collagen aggregation function, platelets in the hematoma cattle showed the similar aggregation patterns as the normal cattle, when ADP, serotonin (5-HT), thrombin, arachidonic acid, epinephrine and ristocetin were used as agents for inducing aggregation. Decreased aggregation function as well as impaired collagen-induced release response in platelets suggested the hematoma cattle to be of storage pool disease (SPD). The impaired platelet was postulated to be a main cause of the large and long-existing hematomas. All of the hematoma cattle with impaired platelet functions had the eosinophils in peripheral blood of which granules were fewer and larger than normal ones. These large eosinophil granules were peroxidase positive and periodic acid Schiff (PAS) staining negative as typical eosinophil granules.—KEY words: hematoma, large granule eosinophil, platelet aggregation deficiency.


A hematological investigation of Japanese black cattle with hematomas revealed the affected animals to be classified into 2 groups by the size of eosinophil granules. One had small typical eosinophil granules and the other had larger and fewer specific eosinophil granules than those of typical bovine eosinophils. Cattle with large and long-existing hematomas were usually found to have eosinophils with large specific granules and at the same time impaired platelet aggregation function in response to collagen in vitro [1]. In the present study, further investigation concerning the extrinsic and intrinsic coagulation and the platelet aggregation function was performed on the hematoma cattle with large eosinophil granules. Most of the recognized platelet abnormalities are known to be identified by means of aggregation tests in response to ADP, collagen, arachidonic acid and ristocetin [9]. In the present study, aggregation test was performed with the inducing agents described above as well as with serotonin (5-HT), thrombin, and epinephrine which were also known to induce platelet aggregation in vitro in man [9, 12]. Collagen-induced release response of platelets was also investigated in this study, because platelet aggregation induced by collagen was accompanied by a release response of platelets, which might be abnormal in cases of impaired platelet aggregation [9].

The hematoma cattle resembles Chediak-Higashi syndrome cattle [3, 5, 13, 19] in regard to impaired platelet aggregation as well as large granules contained in eosinophils which are peroxidase-positive [5]. The decreased number of eosinophil granules is also seen in a certain human anomaly in which peroxidase staining of eosinophil granules is negative [16]. PAS (periodic acid Schiff) staining of large granules in eosinophils are positive in many human cases [15, 18]. There were no references in PAS staining on large granules in Chediak-Higashi syndrome in cattle. In the study, staining of eosinophil granules on peroxidase and PAS were performed to compare with these anomalies.

MATERIALS AND METHODS

Twenty eight matured Japanese black cattle with large and long-existing hematomas on 26 different farms were studied. In all of the hematoma cattle, eosinophils in peripheral blood included small numbered and large sized specific granules. The control consisted of 28 clinically healthy Japanese black cattle with small typical eosinophil granules and without a history of hematomas. There were 2 familial cases with large eosinophil granules among
the cattle studied, one of full-sibling sister cows and the other of 2 dams and their heifer calves (Fig. 1). In these families, hematomas occurred only in the cattle with large eosinophil granules.

Blood samples were obtained with a plastic syringe by jugular venipuncture. One part of blood was anticoagulated with EDTA 2K (1 mg/ml blood) for the determination of platelet count by the counter (PL-100; Toa Medical Electronics Co., Ltd.). The other part was mixed with 3.2% sodium citrate in physiological saline by a ratio of 9:1 in polycarbonate tubes, and centrifuged for 10 min at 100 × g to obtain PRP (platelet rich plasma). After the PRP was obtained, the residue was centrifuged again for 15 min at 1,700 × g to obtain PPP (platelet poor plasma). PT (prothrombin time) and PTT (partial thromboplastin time) were determined with PPP using reagents Simplastin and Platelin (Orga-non Teknika Co., Ltd.) respectively and a blood coagulation analyzer (CA-100, Toa Medical Electronics Co., Ltd.). Platelet count, platelet aggregation with collagen (final concentration; 90.9 μg/ml) and ADP (final concentration; 18.0 μM, PT and PTT were investigated using 20 hematoma cattle and 19 control cattle.

Platelet aggregation function with the following inducing agents was investigated using 4 hematoma cattle and 5 control cattle. For the determination, PRP was adjusted with autologous PPP to a platelet count ranging from 3 to 5 × 10^5 /μl counted by the platelet counter.

Aggregation-inducing agents (25 μl) were added to PRP (250 μl) in the platelet aggregation analyzer (AA-100, Toa Medical Electronics Co., Ltd.). Final concentrations of the inducing agents in PRP were 90.9 μg/ml in collagen (Hormon-Chemie, München, GMBH), 4.5, 9.0 and 18.0 μM in ADP (Sigma diagnostics, St Louis, U.S.A.), 10, 50 and 250 μM in 5-HT (Sigma), 0.22, 0.45 and 0.90 U/ml in thrombin (Sigma), 1.1, 4.5 and 9.0 mM in arachidonic acid (Sigma), and 280 and 560 μM in epinephrine (Sigma). In ristocetin (Sigma), final concentrations were 1.5 and 3.0 mg/ml in PRP. For the reaction in the final concentration of 3.0 mg/ml, plasma in the PRP was diluted to 1:1 with physiological saline without a change in platelet count.

Collagen-induced release response of platelets was determined using 4 hematoma cattle and 4 control cattle as the method shown in Fig. 2. Each 250 μl of PRP from the hematoma and the control cattle respectively was incubated at 37°C with 25 μl of 0.1% collagen (90.9 μg/ml in the final concentration) for 20 min in a test tube of the platelet aggregation analyzer. Supernatant was obtained by centrifuging a test tube at 1,000 × g for 10 min. Thereafter, each of the supernatant (100 μl) was mixed with PRP (150 μl) from the hematoma cattle and was reacted with 0.1% collagen (25 μl) to
determine platelet aggregation.

Blood smears were made just after venipuncture and stained with May-Giemsa technique, periodic acid Schiff (PAS) reagent and peroxidase using 3,3-diaminobenzidine as a substrate.

Data were analyzed by means of Duncan’s multiple range test and differences with p<0.05 were considered to be significant.

RESULTS

Clinical signs: Hematomas were present subcutaneously or intramuscularly on the hip, thigh, paralumbar fossa, chest and/or shoulder (Fig. 3). There were relative differences in severity and time required for recovery in hematomas. There were some cattle which had histories of prolonged bleeding after dehorning and easy bleeding from nasal septum at a nasal ring when pulled strongly by the nose ring. These bleedings were not so massive but prolonged for a few days, and in some cases bleeding continued until the nose ring was taken off. Young cattle which had one or more large and long-existing hematomas were usually emaciated and delayed in growth. In some cows a large intrapelvic hematoma developed following parturition. A few cattle had more than 20 liters of blood in the hematoma and failed to resolve for the period of a year or more. No sex and age related occurrence of hematomas was observed in the cattle.

Laboratory findings: There was statistically significant decrease in collagen-induced aggregation (p<0.05), with no statistically significant difference in platelet count. ADP-induced aggregation, PT and PTT between 20 hematoma cattle with large granule eosinophils and 19 control cattle (Table 1).

The platelet aggregation was examined with various agents for inducing aggregation in the 4 hematoma and the 5 control cattle. Platelet aggregation in the hematoma cattle was impaired in the test with collagen, but similar to that in the control cattle when tested with ADP, serotonin (5-HT), thrombin, and arachidonic acid. The aggregation with epinephrine was weak in all the cattle examined. While no mass was apparent in a test tube in the low concentrations of thrombin, aggregates were observed in the high concentration (0.90 U/ml). Constant strong response was not obtained with ristocetin. However, when PRP was diluted by half in plasma concentration with physiological saline without a change of platelet count, the similar rate of strong aggregations with ristocetin was seen both in the hematoma cattle and in the control cattle (Table 2).

For collagen-induced release response of platelets, supernatant of PRP from the hematoma and the control cattle were mixed respectively with PRP from the hematoma cattle. When these mixtures were reacted with collagen, the platelets aggregated in the mixture with the supernatant from the control cattle PRP, but not in the mixture with the supernatant from the hematoma cattle PRP (Table 3).

Specific granules in eosinophil leukocytes in the hematoma cattle were larger and fewer (Fig. 4) than those in the control cattle (Fig. 5). These eosinophil granules in the hematoma cattle were PAS staining negative and peroxidase positive, which were similar to that of the control cattle. PAS and peroxidase staining in other leukocytes in the hematoma cattle were also similar to those in the control cattle. Morphologically abnormal platelet in the hematoma cattle was not apparent.

DISCUSSION

Hemorrhagic poisoning due to bracken fern,
Table 2. Aggregation response of PRP from the hematoma and the control cattle

<table>
<thead>
<tr>
<th>Aggregating agents</th>
<th>Final concentrations</th>
<th>Maximal aggregation rate (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hematoma Mean±SD (N=4)</td>
</tr>
<tr>
<td>Collagen 90.9 μg/ml</td>
<td>13.5±4.4a</td>
<td>91.8±3.8</td>
</tr>
<tr>
<td>ADP 4.5 μM</td>
<td>65.8±13.7</td>
<td>56.2±11.3</td>
</tr>
<tr>
<td>9.0</td>
<td>90.8±9.0</td>
<td>87.2±9.4</td>
</tr>
<tr>
<td>18.0</td>
<td>94.0±7.1</td>
<td>91.4±4.0</td>
</tr>
<tr>
<td>5-HT 10 μM</td>
<td>18.5±2.9</td>
<td>18.2±1.9</td>
</tr>
<tr>
<td>50</td>
<td>23.0±4.3</td>
<td>23.8±2.2</td>
</tr>
<tr>
<td>250</td>
<td>14.8±3.3</td>
<td>16.2±1.6</td>
</tr>
<tr>
<td>Thrombin 0.22 U/ml</td>
<td>26.3±3.5</td>
<td>25.4±3.9</td>
</tr>
<tr>
<td>0.45</td>
<td>56.0±14.6</td>
<td>57.6±17.5</td>
</tr>
<tr>
<td>0.90</td>
<td>89.5±17.1</td>
<td>97.2±3.6</td>
</tr>
<tr>
<td>Arachidonic acid 1.1 mM</td>
<td>3.5±2.1</td>
<td>2.8±1.6</td>
</tr>
<tr>
<td>4.5</td>
<td>58.3±20.9</td>
<td>63.4±26.8</td>
</tr>
<tr>
<td>9.0</td>
<td>79.0±6.1</td>
<td>81.6±8.6</td>
</tr>
<tr>
<td>Epinephrine 280 μM</td>
<td>8.5±1.3</td>
<td>9.8±1.5</td>
</tr>
<tr>
<td>560</td>
<td>9.8±2.8</td>
<td>11.0±2.9</td>
</tr>
<tr>
<td>Ristocetin 1.5 mg/ml</td>
<td>3.5±2.4</td>
<td>36.8±40.4</td>
</tr>
<tr>
<td>3.0</td>
<td>6.8±2.6</td>
<td>43.2±44.9</td>
</tr>
<tr>
<td>Dil. pl30 3.0 mg/ml</td>
<td>94.5±6.1</td>
<td>87.0±14.6</td>
</tr>
</tbody>
</table>

a) P<0.05
b) Ristocetin was reacted with both PRP and PRP diluted by half in plasma concentration with physiological saline without change in platelet count.

Table 3. Difference of the maximal aggregation rate in PRP from the hematoma cattle mixed with supernatant of PRP reacted with collagen for 20 min

<table>
<thead>
<tr>
<th>Mixtures of supernatant and PRP</th>
<th>Maximal aggregation rate(% Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-sup30 100 μl+H-PRP 150 μl</td>
<td>61.0±8.7</td>
</tr>
<tr>
<td>H-sup30 100 μl+H-PRP 150 μl</td>
<td>13.4±5.8</td>
</tr>
</tbody>
</table>

a) Supernatant from the normal cattle (N=4).
b) Supernatant from the hematoma cattle (N=4).

sweet clover or coumarin was unlikely, as hematomas tended to develop in only one cattle in a pen [11].

Though large and long-existing hematomas were observed, there was no hematoma cattle which died of massive hemorrhage or infectious disease both in heifers and in adults. No defects of extrinsic or intrinsic blood coagulation factors seemed to be present, because statistically significant difference was not detected in PT and PTT between the cattle with hematoma and the control [6] and there was no cattle which had an episode of large hemorrhage suggesting hemostatic disorders. The values of PT as well as PTT in this study were longer than reported values [10], which suggested the necessity of further investigation for the methods and reagents employed in the study of Japanese black cattle.

Increase of platelet count in the hematoma cattle might indicate the normal response to a large hemorrhage or inflammation [6]. Hematomas were postulated to be caused by a decreased collagen aggregation function of platelets [2, 8] and to lead to severe anemia in the hematoma cattle. Differences in severity and the time required for recovery from hematomas suggested a possibility of concurrent hemostatic disorders.

Impaired platelet aggregation function in response to collagen in vitro in the hematoma cattle was similar to clinical signs of Chediak-Higashi syndrome. Ranges of platelet aggregation rates were significantly different between Chediak-Higashi syndrome and normal ones in Hereford cattle [3], Aleutian minks [4], human patients [20] as well as

Fig. 4. An eosinophil leukocyte with large granules.

Fig. 5. An eosinophil leukocyte with typical small granules.
the cases in the present study. It is necessary to consider the differences in methods (e.g. reagents, apparatuses, animal species) for the comparison of data. Ristocetin aggregated bovine platelets when plasma was diluted [7]. While ristocetin induced poor aggregation with intact platelets in the study, a strong reaction was obtained in washed platelets even with low concentration of ristocetin in a preliminary study.

The hematoma cattle was assumed to suffer from the storage pool disease because of the impaired aggregation with diminished collagen-induced release response in platelets and, on the other hand, the normal reaction in the other inducing agents such as ADP, 5-HT, ristocetin, thrombin, arachidonic acid and epinephrine [9]. Platelets from the hematoma cattle aggregated when mixed with substances released by collagen from platelets of normal cattle in vitro, which suggested the patient platelets had an ability to aggregate if they obtained substances from normal platelets. This finding was also thought to indicate the hematoma cattle to be of the storage pool disease. Deficiency of platelet storage pool substances, which substantiates the storage pool disease [3], is now investigated in our laboratory, and the level of 5-HT in platelets is lower in the hematoma cattle than in clinically healthy cattle with eosinophils containing typical small granules.

In the hematoma cattle with impaired platelet function, circulating eosinophils contained large sized granules, which were thought to be a marker for detecting a disposition of easy development of the large and long-existing hematomas. The cases in the present study resembles Chediak-Higashi syndrome in cattle, in regard to the impaired collagen aggregation function of platelets [3] and the presence of few and large granules in leukocytes [5, 14, 19] which are peroxidase positive [5]. As to the peroxidase staining of eosinophil granules, negative in a human anomaly [16] and positive in the hematoma cattle suggested the human anomaly might be different from that of hematoma cattle. The large eosinophil granules in the hematoma cattle were PAS negative and peroxidase positive, which are similar to those of typical eosinophils [17]. Further research is necessary for the PAS staining of large eosinophil granules, since most of the abnormal granules of leukocytes in Chediak-Higashi syndrome in human patients were positive but inconsistent [15, 18]. In the hematoma cattle, there was no conspicuous clinical characteristics observed in a young Japanese black cattle with Chediak-Higashi Syndrome such as partial cutaneous albinism and marked susceptibility to infection [19].

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REFERENCES


