ビーグル犬における出血の仮の指標としての糞便および尿中の炭酸脱水酵素アイソエンザイム-I（CA-I）濃度

Carbonic anhydrase isoenzyme I (CA-I) concentration in faeces and urine as a temporary marker of occult blood in beagle dogs（Exp. Anim. 56(1), 43-49, 2007掲載）

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要 目 ヘモグロビンに代わる潜血の仮の指標として糞便および尿中の炭酸脱水酵素アイソエンザイム-I（CA-I）濃度を酵素免疫測定法（ELISA）により評価した。健康な各種年齢の実験用ビーグル犬113頭（雄50頭，雌63頭）における糞便中のCA-I濃度は，4.3から16.7 ng/g便（平均；7.0±2.9 ng/g便）であった。3頭の健康なビーグル犬から採取した血液1ml中に含まれるCA-Iは，それぞれ1.047, 1.062および1.150 μgであった。自己血（10ml）を胃内へ注入したイヌの糞便CA-I濃度は大幅低かった。しかし，自己血（5ml）を上行結腸部へ注入したイヌの糞便CA-I濃度は大変高かった。糞便中のCA-Iの検出は大腸からの出血があるイヌを見分けるのに有用であろう。健康な55頭のビーグル犬から採取した尿を化学的検査で調べた結果，44頭が陰性であったが，これら尿中のCA-I濃度はELISAで1.8から12.6 ng/ml（平均；6.9±5.4 ng/ml）であった。また，尿の潜血検査の結果が陽性であった11頭のイヌのCA-I濃度は，ELISAで41.2から525.0 ng/mlであった。CA-Iは赤血球の特異的な指標ではないが，Hbに対する抗体を用いた特異的な免疫学的検査キットが開発されるまで，CA-Iはイヌの糞便および尿の潜血の検出に用いられるであろう。

1. Purpose

This study was undertaken to investigate whether the concentration of carbonic anhydrase isoenzyme I (CA-I) in canine faeces and urine is useful as a temporary marker of occult blood. Concentrations of CA-I were measured by enzyme-linked immunosorbent assay (ELISA).

2. Materials and Methods

Dogs, blood, faeces and urine

Eleven healthy beagle dogs (5 males and 6 females; age, 12–13 months) were kept in the experimental animal facilities of the Research Institute of Biosciences at Azabu University (temperature, 20 ± 2°C; relative humidity, 50 ± 10%; 12/12 light/dark cycle (6:00–18:00); air exchange occurred 13 times per hour) and were used in this study. Beagle dogs were fed Clea CD-5 dog feed (Clea Japan, Inc., Tokyo, Japan), and were allowed free access to water. Heparinized blood for isolation and quantification of CA-I and faeces for determination of CA-I were collected from these dogs. For isolation of CA-I from erythrocytes, heparinized blood was washed seven times in physiological saline by centrifugation at 1,600 × g for 10 min. Sedimented erythrocytes were then lysed in
distilled water and an equal volume of 0.02 M phosphate buffered saline (PBS; pH 7.2). Erythrocyte lysate containing CA-I was obtained by centrifugation at 1,600 × g for 10 min. This lysate was mixed with 0.1% sodium azide and was stored at −80°C until use.

For immunological quantification of faecal CA-I by ELISA, faeces samples were treated and stored within 6 h of collection. One g of faeces was mixed with 1 ml of distilled water, after which 1 ml of 0.02 M PBS containing 0.2% sodium azide was added. The supernatant obtained by centrifugation (1600 × g for 10 min) was stored immediately at −80°C until use.

In order to quantify the amount of CA-I in faeces from healthy beagle dogs of various ages, faeces were collected from 113 beagle dogs (50 males and 63 females; age, 3–24 months), kept in isolators at the Breeding Division of Hongo Beagle Farm, Kitayama Labes Co., Ltd. (Yamaguchi, Japan) at a temperature of 23 ± 2°C, a relative humidity of 55 ± 10%, and a 12/12 light/dark cycle (6:00–18:00) with air exchange occurring at least 12 times per hour. Dogs were fed a DS-E diet (Oriental Yeast Co., Ltd., Tokyo, Japan), and were allowed free access to water. For immunological quantification of CA-I, faeces samples were treated and stored as described above.

In order to quantify the amount of CA-I in urine from healthy beagles dogs, urine was collected by catheter from 55 beagle dogs (28 males and 27 females; age, 6–24 months) kept in isolators at the Breeding Company, CSK Co., Ltd., (Nagano, Japan) (44 dogs) and in the experimental animal facilities at Azabu University (11 dogs). Urine was used for both quantification of CA-I by ELISA and detection of haemoglobin by chemical testing. Urine was also mixed with 0.1% sodium azide and stored at −80°C until use.

All experiments conformed to the Japanese regulations on animal care and use, based on the Guidelines for Animal Experimentation (Japanese Association for Laboratory Animal Science, JALAS, 1987), and were approved by the Institutional Animal Care and Use Committees of CSK Co., Ltd.

Isolation of canine CA-I

Canine CA-I was isolated using the method of Nishita et al. [16]. Briefly, the CA fraction was obtained from lysate of washed canine erythrocytes using the chloroform-ethanol denaturation method, which is used for the preparation of CA in a number of other mammals. Haemoglobin was removed as a precipitate and the supernatant was then collected and dialysed against 10 mM Tris-HCl buffer (pH 8.0). CA-I was isolated from the supernatant by liquid chromatography, as described by Funakoshi and Deutsch [6]. For fast liquid chromatography (FPLC; Amersham Bioscience, Uppsala, Sweden), a HiLoad 16/10 Q sepharose column (Amersham Bioscience) was used for ion-exchange chromatography, a Mono P HR5/20 column (Amersham Bioscience) was used for chromatofocusing and a Superdex 200 16/60 column (Amersham Bioscience) was used for gel filtration. CA enzyme activity and haemoglobin content of each fraction on FPLC were detected by p-nitrophenyl acetate [4] and 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Zymed Laboratories, South San Francisco, CA, USA), respectively.

Anti canine CA-I antibody

A New Zealand rabbit was initially immunized subcutaneously with 1 ml of CA-I emulsion prepared with Freund’s complete adjuvant. The rabbit subsequently received ten further injections of CA-I solution alone. Serum was collected at 7 days after the final immunization and was stored at −80°C until needed.

IgG antibody was obtained from this antiserum by affinity chromatography on a Protein A column (Amersham Biosciences) [8]. Subsequently, specific IgG antibody against canine CA-I was isolated by affinity chromatography using a HiTrap NHS-activated HP gel coupled with canine CA-I. The specific IgG antibody to canine CA-I was used for ELISA. Peroxidase (Sigma Chemical Co., St Louis, Mo, USA)-conjugated IgG antibody was prepared by the method of Nakane and Kawai [15]. This peroxidase-conjugated IgG antibody was used for ELISA.
Quantification of proteins

Isolated CA-I was quantified by the method of Bradford [3] using Coomassie brilliant blue G-250. Rabbit anti canine CA-I IgG antibody was quantified using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

SDS-PAGE and Western blotting were performed using the methods of Laemmli [13] and Towbin et al. [23], respectively. After SDS-PAGE for isolated CA-I, gels were stained with Coomassie brilliant blue R-250.

The specificity of the rabbit anti canine CA-I IgG antibody was confirmed by Western blotting analysis followed by SDS-PAGE.

ELISA for CA-I

ELISA for CA-I was carried out using the sandwich method described by Yamamoto et al. [26]. Rabbit anti canine CA-I IgG antibody was adjusted with 0.05 M sodium hydrogen carbonate buffer (pH 9.6) to 10 µg/ml for use as the coating antibody. The coating antibody (100 µl/well) was incubated at 37°C for 1 h in an ELISA plate (Nalge Nunc International, Rochester, NY, USA). Next, 1% bovine serum albumin (BSA; Wako Chemical Co., Ltd., Osaka, Japan) in 0.05 M sodium hydrogen carbonate buffer (pH 9.6) was added to all the wells at 300 µl/well to block any unadsorbed binding sites (4°C, overnight). After washing, a known concentration of CA-I or samples were added at 100 µl/well and the plates were incubated at 37°C for 1 h. The peroxidase-conjugated rabbit anti canine CA-I IgG antibody, adjusted with PBS to 2.5 µg/ml, was added to all the wells at 100 µl/well and was allowed to react at 37°C for 1 h. Substrate at 0.05% in 0.05 M citrate buffer (pH 4.2) was added at 100 µl/well. The results were determined by measuring the absorbance at 415 nm with an immunoplate reader.

Experimental models for detection of CA-I in faeces

Three healthy beagle dogs each received 5 or 10 ml of autologous blood infused from a syringe via a catheter into the stomach. After 1 week, the same 3 healthy dogs each received 1 or 5 ml of autologous blood similarly infused into the ascending colon. Samples were collected from the first to the fourth spontaneous defecations thereafter for detection of CA-I. Faeces obtained from these dogs prior to infusion of autologous blood were used as controls. Faeces (1 g) were treated as described above.

In order to assess changes in CA-I antigenicity in faeces, 1 g of faeces from 2 healthy dogs was mixed with 100 µl of blood and was allowed to stand at room temperature for 12 h. CA-I was then extracted as above, and its antigenicity was assessed by ELISA.

Chemical test for urinary occult blood

Occult blood in urine was detected using Multistix SG-L (Distributed by Sankyo Co., Ltd., Tokyo, Japan).

3. Results

Anti canine CA-I antibody

Figure 1A shows the Western blotting patterns of the antiserum to canine CA-I. This antiserum formed a single band with isolated canine CA-I and with a 30-kDa protein in lysates of canine erythrocytes.

Isolation of CA-I

CA-I was isolated from the lysates of canine erythrocytes by liquid chromatography using FPLC (Fig. 1B).

Concentrations of CA-I in blood

As shown in Table 1, CA-I concentrations in blood from 3 apparently healthy beagle dogs ranged from 1.047 to 1.150 µg/ml blood.

Antigenicity of CA-I

The antigenicity of canine CA-I stored in faeces at room temperature for 12 h did not change (Table 2).

Concentrations of CA-I in faeces from healthy dogs

The faecal CA-I concentrations in 113 healthy beagle dogs of various ages are shown in Table 3. Faecal CA-I concentrations ranged from 4.3 to 16.7 ng/g faeces (mean;
Detection of CA-I in faeces in experimental models of digestive tract bleeding

The faecal CA-I concentrations of dogs that received intragastric infusion of autologous blood were very low (Table 4). However, the faecal CA-I concentrations of the dogs that received infusion of autologous blood (5 ml) into the ascending colon were very high, as shown Table 4.

Concentrations of CA-I in urine

Urinary samples from 44 of 55 dogs were negative by chemical testing (detectable limit 150 ng haemoglobin/ml). As shown in Table 5, CA-I

<table>
<thead>
<tr>
<th>Time after mixing</th>
<th>Feces</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immediately</td>
<td>1,535</td>
</tr>
<tr>
<td>4 h</td>
<td>1,730</td>
</tr>
<tr>
<td>8 h</td>
<td>1,225</td>
</tr>
<tr>
<td>12 h</td>
<td>1,620</td>
</tr>
</tbody>
</table>

Table 2. In vitro changes in antigenicity of canine CA-I (ng/g) in faeces

Faecal materials (1 g) were mixed with blood (0.1 ml) and allowed to stand at room temperature for 12 h.

<table>
<thead>
<tr>
<th>Dogs</th>
<th>Concentration of CA-I (µg/ml)</th>
<th>Erythrocyte counts (µl)</th>
<th>Hematocrit value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,150</td>
<td>7,500,000</td>
<td>52</td>
</tr>
<tr>
<td>2</td>
<td>1,047</td>
<td>7,900,000</td>
<td>49</td>
</tr>
<tr>
<td>3</td>
<td>1,062</td>
<td>8,210,000</td>
<td>53</td>
</tr>
</tbody>
</table>

Table 1. Concentrations of CA-I in blood from healthy experimental beagle dogs by ELISA

<table>
<thead>
<tr>
<th>Months - old</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dogs</td>
<td>113</td>
</tr>
<tr>
<td>Means</td>
<td>7.0</td>
</tr>
<tr>
<td>Maximum</td>
<td>16.7</td>
</tr>
<tr>
<td>Minimum</td>
<td>4.3</td>
</tr>
<tr>
<td>SD</td>
<td>2.9</td>
</tr>
</tbody>
</table>
Table 4. Concentrations of CA-I (ng/g) in canine feces by ELISA after infusion of autologous blood

<table>
<thead>
<tr>
<th>Feces samplea</th>
<th>5 ml (blood)</th>
<th>10 ml (blood)</th>
<th>1 ml (blood)</th>
<th>5 ml (blood)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controlb</td>
<td>9</td>
<td>7</td>
<td>12</td>
<td>19</td>
</tr>
<tr>
<td>1c</td>
<td>11</td>
<td>8</td>
<td>30</td>
<td>2,950</td>
</tr>
<tr>
<td>2</td>
<td>21</td>
<td>49</td>
<td>44</td>
<td>1,450</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>28</td>
<td>12</td>
<td>2,150</td>
</tr>
</tbody>
</table>

aFeces (1g) was dissolved in distilled water (1ml), mixed with a two-fold concentration of saline (1ml) and centrifuged. Supernatant was used for ELISA
bFecal samples collected prior to infusion of blood into stomach or ascending colon were used as controls
cNumber of defecation after infusion of blood into stomach or ascending colon

Table 5. The relationship between chemical tests for occult blood and CA-I concentrations (ng/ml) of urine in experimental beagle dogs

<table>
<thead>
<tr>
<th>Chemical test (samples)</th>
<th>CA-I concentrations (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative (44)</td>
<td>1.8 ~ 12.6 (mean 6.9 ± 5.4)</td>
</tr>
<tr>
<td>Positive (11)</td>
<td></td>
</tr>
<tr>
<td>2+ positive (6)</td>
<td>41.2 ~ 98.0 (mean 69.4 ± 26.5)</td>
</tr>
<tr>
<td>3+ positive (5)</td>
<td>173.0 ~ 525.0 (mean 335.0 ± 132.7)</td>
</tr>
</tbody>
</table>

Concentrations in the negative urinary samples ranged from 1.8 to 12.6 ng/ml (mean; 6.9 ± 5.4 ng/ml) by ELISA. On the other hand, urinary samples from the other 11 dogs were positive by chemical testing. The CA-I concentrations of 6 urinary samples that were 2+ on chemical testing were 41.2 ~ 98.0 ng/ml, while the CA-I concentrations of 5 samples that were 3+ on chemical testing were 173.0 ~ 525.0 ng/ml by ELISA (Table 5).

4. Discussion

In human medicine, chemical tests for faecal occult blood have largely been replaced by immunological methods [19]. However, the development of useful methods that allow specific detection of internal bleeding in dogs and another animals remains at an early stage.

Although we have previously reported the usefulness of immunological tests using specific anti-canine Hb antibodies for occult blood in dogs [10, 11], the preparation of anti-canine Hb antibody is currently very difficult. Therefore, nonspecific chemical tests [12, 25] are generally employed to detect faecal occult blood in dogs, and these techniques are still used in for a variety of purposes, such as drug safety evaluation, or in veterinary clinics.

CA-I is not a specific marker of erythrocytes, as it is present in the tissues of the intestinal and urinary organs [20] and it may be secreted from these organs into their respective tracts. Unfortunately, it is impossible to determine the origin of CA-I. However, CA-I may be used to detect occult blood in canine faeces until a specific immunological test kit using antibodies for Hb is developed. This is because faecal CA-I concentrations in healthy dogs are very low, while faecal CA-I concentrations in dogs that received infusion of autologous blood into the ascending colon were found to be very high. When 3 dogs were infused 1 ml of autologous blood, CA-I was not detected in faeces, probably because faeces containing infused blood was not collected in test samples. Detection of faecal CA-I would thus be useful to identify dogs with hemorrhaging of the large intestine.

The antigenicity of human Hb changes as a result of digestive enzymes or faecal bacteria [5]. However, when faecal samples were mixed with blood and allowed to
stand at room temperature for 12 h, the reactivity of canine CA-I did not change. Consequently, CA-I in faeces may be used as an antigen for immunological tests.

Physiological bleeding into the intestine is seen even in healthy humans [2] and dogs [11]. Such internal bleeding should be carefully considered in the clinical application of immunological tests for faecal occult blood in dogs [10]. Eleven of 58 urinary samples from apparently healthy beagle dogs were positive by chemical testing and showed markedly increased CA-I by ELISA. This is thought to be due to scratching of the ureter mucuous membrane when the tube was inserted to collect urine.

Although sensitive methods, such as ELISA, are required in the quantification of CA-I in dogs, CA-I concentrations are a useful temporary marker for occult blood. In the future, it is necessary to develop quantitative methods for occult blood using Hb as a specific marker.

5. Summary

This study was undertaken to investigate whether the concentration of carbonic anhydrase isoenzyme I (CA-I) in canine faeces and urine is useful as a temporary marker of occult blood. Concentrations of CA-I were measured by enzyme-linked immunosorbent assay (ELISA). The reactivity of canine CA-I did not change for 12 h at room temperature. Faecal CA-I concentrations in 113 healthy beagle dogs (50 male and 63 female) of various ages ranged from 4.3 to 16.7 ng/g faeces (mean: 7.0 ± 2.9 ng/g faeces). One ml of blood from 3 healthy beagle dogs were found to contain 1,047, 1,062 and 1,150 µg CA-I. The faecal CA-I concentrations of dogs receiving intragastric infusion of autologous blood (10 ml) were very low. However, the faecal CA-I concentrations of dogs receiving infusion of autologous blood (5 ml) into the ascending colon were very high. Of 55 urinary samples collected from healthy beagle dogs by catheter, chemical tests for occult blood were negative in 44, but CA-I concentrations ranged from 1.8 to 12.6 ng/ml (mean; 6.9 ± 5.4 ng/ml) by ELISA. The CA-I concentrations of the other 11 samples, which tested positive for occult blood on chemical testing, ranged from 41.2 to 525.0 ng/ml by ELISA.

References
during the assembly of the head of bacteriophage T4.  

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