C-Reactive Protein and α1-Acid Glycoprotein Levels in Dogs Infected with *Ehrlichia canis*

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To elucidate whether acute-phase protein responses occur in dogs infected with *Ehrlichia canis*, C-reactive protein (CRP) and α1-acid glycoprotein (AAG) levels were serially measured in the plasma of five dogs experimentally inoculated with *E. canis* and 10 sham-inoculated or noninoculated control dogs. The CRP concentration was measured by a canine-specific capture enzyme-linked immunosorbent assay, and the AAG concentration was measured by a canine-specific radial immunodiffusion method. In all *E. canis*-inoculated dogs, a 3.3- to 6.5-fold increase in the plasma CRP concentration and a 1.9- to 8.6-fold increase in the plasma AAG concentration over the preinoculation level occurred at days 4 to 6 postexposure. Despite the persistence of *E. canis* and high antibody titers, both CRP and AAG concentrations gradually declined to preexposure levels by day 34 postexposure. *E. canis*-infected dogs had mild transient clinical signs which resolved without treatment by day 14 postexposure. The CRP and AAG concentrations in control inoculated or noninoculated dogs remained within the normal range throughout the experimental period. Of 12 dogs naturally infected with *E. canis*, 75% had greater than 50 μg of CRP per ml and 83% had greater than 500 μg of AAG per ml. All of these 12 dogs had chronic and severe clinical signs of canine ehrlichiosis. Thus, elevations in the levels of acute-phase proteins occur in both acute and chronic canine ehrlichiosis. Determination of CRP and AAG concentrations may help in assessing the severity of inflammatory damage in dogs with *E. canis* infections.

Ehrlichial organisms belonging to the family *Rickettsiaceae* are obligate intracellular bacteria of either monocytes and macrophages or granulocytes of various species of animals including humans (21). *Ehrlichia canis* parasitizes monocytes and macrophages, causing canine ehrlichiosis or canine tropical pancytopenia. Canine ehrlichiosis consists of acute, subclinical, and chronic phases (25). At 1 to 3 weeks postexposure, mild clinical signs may appear. The acute phase of the disease consists of transient fever, serous nasal and ocular discharges, anorexia, depression, and weight loss (10). Most dogs recover from the acute phase of the disease without treatment; however, they may remain infected. Hematologic findings include transient thrombocytopenia and nonregenerative anemia. After several months to years of subclinical infection, dogs may develop the severe pancytopenic stage of chronic infection, which has a poor prognosis, despite therapy. Death is usually caused by complications of secondary infection or hemorrhage (9, 25).

C-reactive protein (CRP) and α1-acid glycoprotein (AAG) are acute-phase proteins which are synthesized in the liver following tissue damage caused by infection, inflammation, or trauma. In human medicine, quantitative CRP and AAG measurements have become increasingly important as tests for the rapid diagnosis of serious infectious diseases, such as sepsis, meningitis, and pneumonia (15, 17). Quantitative CRP and AAG measurements have also been used to detect inflammatory conditions which otherwise might not be recognized and to monitor the progress of response to antibacterial treatment. CRP and AAG levels fall rapidly following effective therapy (24). It is unknown whether acute-phase proteins are produced during infection with rickettsia or other intracellular bacteria.

In the study described here, five healthy dogs were inoculated with *E. canis*, and clinical signs, antibody titers to *E. canis*, and variations in plasma CRP and AAG concentrations were monitored by using canine-specific assays. The sera from 12 naturally infected dogs were also evaluated for their CRP and AAG concentrations. The results indicate that *E. canis* infection causes an elevation in CRP and AAG levels.

**MATERIALS AND METHODS**

Preparation of *E. canis* and AS145 strain-infected dog macrophages. *E. canis* Oklahoma and AS145 were cultured in a dog macrophage cell line (DH82) in minimum essential medium (MEM) containing 10% fetal bovine serum and 2 mM l-glutamine in 5% CO2-air as described previously (22). Strain AS145 is an ehrlichial strain originally isolated from the spleen of a wild mouse in Japan (11).

Analysis of dogs experimentally infected with *E. canis*. A total of 15 1- to 2-year-old German Shepherd-mixed breed dogs were purchased from Biomedical Associates, Inc., Friedensburg, Pa. Dogs were either colony bred or originally acquired at a pound by Biomedical Associates after a veterinarian’s health certification and rabies vaccination. These dogs had been quarantined for more than 1 year in the Biomedical Associates facility. During the quarantine period, each dog was vaccinated (according to the manufacturer’s instructions) against distemper, hepatitis, parvovirus infection, and leptospirosis and several dewormings were done according to the manufacturer’s instructions. Dogs weighed 40 to 70 lb (18 to 32 kg) at the time of initiation of the study. At 15 days before and day 0 of the experiment, all dogs were seronegative for *E. canis*, as determined by the indirect fluorescent-antibody (IFA) test (22). Each of five dogs was intravenously inoculated with
10⁷ *E. canis*-infected DH82 cells suspended in 5 ml of MEM. Heparinized blood (20 ml) was collected from the jugular vein every 2 days during the first 2 weeks postexposure and weekly thereafter to monitor specific antibody titers, acute-phase protein levels in plasma, and the presence of *E. canis* in the blood. Blood specimens were also collected weekly from three dogs which were kept indoors during the experimental period (noninoculated), four control dogs which were inoculated with 5 ml of MEM (sham inoculated), and three dogs which had been inoculated with 10⁷ strain AS145-infected DH82 cells (ehrlichia-infected DH82 cell control).

**Analysis of dogs naturally infected with *E. canis***. Serum samples from 12 dogs that were naturally infected with *E. canis* and that were seropositive (≥1:20 by the IFA test (22)) were evaluated for their CRP and AAG concentrations. Serum specimens from these dogs were submitted to our laboratory for *E. canis* serology testing by the IFA test during the years 1991 to 1993. Only dogs for which additional etiologies were ruled out by laboratory testing were selected.

**IFA test and culture of *E. canis* and AS145 strain**. Antibody titration of serum and plasma specimens from dogs exposed to *E. canis* and AS145 was performed by the IFA test by using acetone-fixed *E. canis* or AS145 cultured in DH82 cells, respectively, as the antigen (11, 22). The presence of erlichial organisms in the blood was determined by overlaying mononuclear cell fractions obtained by Histopaque 1077 (Sigma, St. Louis, Mo) on DH82 cell monolayers and culturing them for up to 2 months as described previously (22).

**CRP determination**. The CRP concentrations in the plasma of dogs were quantified by capture enzyme-linked immunosorbent assay (ELISA) by using purified canine CRP, rabbit anti-dog CRP immunoglobulin G (IgG), and peroxidase-conjugated rabbit anti-dog CRP IgG prepared as described previously (29). Canine CRP protein was isolated by ion-exchange chromatography by using DEAE-Sephaloc and DEAE-Sephadex A-50 from acute-phase serum obtained from beagle dogs 24 h after surgical stimulation (29). After removing the IgG from the crude CRP fraction by protein A-Sepharose CL4B affinity chromatography, the flowthrough fraction was separated by agar gel electrophoresis, and the CRP at the cathodic side (1.0 cm from the sample well) was collected. The purified canine CRP did not react with the anti-dog normal whole serum in immunoelectrophoresis but formed a single distinct precipitin line with anti-canine CRP serum (29). Rabbit anti-dog CRP serum was prepared by subcutaneous injection of two rabbits with purified canine CRP mixed in an equal volume of complete Freund’s adjuvant (Difco Laboratories, Detroit, Mich.). The antisera was absorbed by the normal canine serum globulin fraction by affinity chromatography and was used as antisera specific to CRP. An immunosorbent column (2 by 15 cm) of CNBr-activated Sepharose 4B coupled to the globulin fraction of normal canine serum was used for absorption of the anti-dog CRP serum (29). The rabbit anti-dog CRP IgG antibody labeled with horseradish peroxidase (Sigma type VI; R230; Sigma) was prepared by the method of Nakane and Kawai (18). Ninety-six-well ELISA plates were coated with the rabbit anti-dog CRP IgG diluted 1:600 with 0.05 M bicarbonate buffer (pH 9.6) at 100 μl per well. After blocking with 1% bovine serum albumin in bicarbonate buffer (pH 9.6) and rinsing, 100 μl (per well) of serially diluted dog serum with a known CRP concentration (444 μg of CRP per ml) and the test sera were added. The plates were incubated at 37°C for 2 h and washed three times with phosphate-buffered saline (PBS; pH 7.2) containing 0.1% Tween 20. The horseradish peroxidase-conjugated anti-dog CRP IgG diluted 1:600 in PBS was added at 100 μg per well, and the plates were incubated at 37°C for 2 h. As a substrate of horseradish peroxidase, 2,2'-azino-di(3-ethyl-benzothiazoline sulfonic acid-6) (Sigma) dissolved at a concentration of 500 μg/ml in 0.05 M citrate buffer (pH 4.2) containing 0.03% hydrogen peroxide was added. After incubation for 10 min at room temperature, the A₄₉₄ was measured. The results were compared with a standard curve obtained with serially diluted specimens from dogs with known CRP concentrations.

**AAG determination**. The plasma AAG concentrations in dogs were determined by the single radial immunodiffusion method of Mancini et al. (16), as directed by the manufacturer of the kit (Saikin Kagaku Institute Co., Ltd., Sendai, Japan). Serum samples (5 μl) were applied in each well in the agarose gel containing anti-canine AAG rabbit serum. After 24 h of incubation at room temperature (23 to 27°C) in a humid chamber, the diameter of the precipitin ring was measured to an accuracy of 0.1 mm. The results were compared with those obtained from a reference curve created in each assay with standard solutions of 2,000 and 500 μg of canine AAG per ml according to the manufacturer’s instructions. Serum samples which contained greater than 2,000 μg/ml were diluted two- to fivefold with PBS and were reassayed.

### RESULTS

There were no significant increases in the CRP or AAG concentrations in the three nontreated dogs, four sham-inoculated dogs, and three dogs inoculated with strain AS145-infected DH82 cells in comparison with those in healthy dogs (Table 1). Strain AS145 is ultrastructurally and antigenically most closely related to *E. canis*, as determined by both the IFA test and Western immunoblot analysis (11). Strain AS145 did not establish infection in our experimentally infected dogs, as determined by weekly cultivation of the peripheral blood mononuclear cell fraction of the dogs and the IFA test results obtained with the sera. Strain AS145 was not resolated from the mononuclear cell fraction of any of the three dogs through-

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>CRP (μg/ml)</th>
<th>AAG (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noninoculated</td>
<td>3 ± 1</td>
<td>393 ± 150</td>
</tr>
<tr>
<td>Day 0</td>
<td>2 ± 1</td>
<td>393 ± 150</td>
</tr>
<tr>
<td>Day 14</td>
<td>0 ± 0</td>
<td>290 ± 185</td>
</tr>
<tr>
<td>Day 20</td>
<td>0 ± 0</td>
<td>216 ± 118</td>
</tr>
<tr>
<td>Day 28</td>
<td>0 ± 0</td>
<td>180 ± 102</td>
</tr>
<tr>
<td>Day 31</td>
<td>0 ± 0</td>
<td>140 ± 64</td>
</tr>
</tbody>
</table>

* DH82 cells inoculated with strain AS145 (n = 3)

### TABLE 1. CRP and AAG concentrations in sera of control dogs

- **Conc (μg/ml)**
- **Conc (μg/ml)**

- **Noninoculated (n = 3)**
  - Day 0: 3 ± 1
  - Day 14: 2 ± 0
  - Day 28: 6 ± 6
  - Day 42: 18 ± 20

- **Sham inoculated (n = 4)**
  - Day 0: 11 ± 8
  - Day 13: 8 ± 4
  - Day 31: 6 ± 6

- **DH82 cells inoculated with strain AS145 (n = 3)**
  - Day 0: 3 ± 1
  - Day 6: 2 ± 1
  - Day 13: 0 ± 0
  - Day 20: 0 ± 0
  - Day 28: 0 ± 0
  - Day 34: 0 ± 0

*CRP concentrations in healthy dogs are reported to be 8.4 ± 4.9 μg/ml (26) and is particularly high in dogs with various infectious diseases (23) and following surgery (5).

* AAG concentrations in the sera of healthy dogs are reported to be 374 ± 37 μg/ml and 1,500 ± 266 μg/ml in dogs with inflammatory diseases (1).
out the experiment. The IFA test titers were negative throughout the 5 weeks of the experiment except at 3 weeks postinfection, when the homologous titer against strain AS145 was 1:20. Rectal temperature, appetite, attitude, body weight, blood leukocyte count, and thrombocyte count were normal in dogs inoculated with strain AS145 throughout the study. Thus, strain AS145 was used as a control for the infection of dogs with infected DH82 cells.

In the dogs experimentally infected with *E. canis*, plasma CRP levels were within the reported control range in all five dogs a few minutes prior to inoculation (day 0) and rose gradually to a peak between days 4 and 6 postinoculation (Fig. 1). The maximum plasma CRP concentrations were 3.3- to 6.5-fold greater than those in the plasma of preexposure controls. Similar results were found for plasma AAG concentrations. Peak plasma AAG concentrations were also attained between 4 and 6 days postinoculation. The maximum plasma AAG levels were 1.9- to 8.6-fold greater than the levels in the plasma of preinoculation controls for each dog (Fig. 2). Of the five infected dogs, dog 320 showed a relatively high AAG concentration (greater than 600 µg/ml) throughout the experimental period and responded to *E. canis* exposure with a higher peak AAG concentration than the other dogs in the study (Fig. 2). Both plasma CRP and AAG levels declined to preinfection levels by day 34.

Overall, clinical signs in the five infected dogs were quite mild and transient. A temperature of greater than 103.4°F (39.7°C) lasting 1 to 2 days was recorded in all animals between days 3 and 9 postinfection. A moderate reduction in platelet counts was observed in all five dogs starting on day 8 and continuing to day 13 postinfection (data not shown). Dog 303 had the most severe thrombocytopenia (19 × 10³ platelets per µl) at day 13 postinfection. All five dogs lost 5 to 10 lb (2.3 to 4.5 kg) of body weight during the 2-month period. *E. canis* was reisolated from the peripheral blood mononuclear cell fractions of all five dogs every week throughout the 2-month postinfection period.

All *E. canis*-infected dogs seroconverted at days 2 to 4 postexposure, and antibody titers reached a plateau at day 10 postexposure and remained high in all five dogs (Fig. 3). Of 12 serum specimens from naturally infected dogs, 10 (83%) had an AAG level of greater than 500 µg/ml and 9 (75%) had a CRP level of greater than 50 µg/ml (Table 2). All of these dogs had severe clinical signs; therefore, they were under a veterinarian’s care. All serum specimens were collected after several weeks to months of illness at stages beyond the acute stage of illness. The highest AAG levels (>6,000 µg/ml) were seen in dogs with moderate antibody titers (1:2,560 and 1:1,280) rather than in dogs with the highest antibody titer (1:10,240).

**FIG. 1.** Changes in plasma CRP concentration in dogs following inoculation with *E. canis*. Dashed line, mean CRP concentration in the serum of healthy dogs (28). The five bars represent results for dogs 011, 340, 307, 303, and 320, from left to right, respectively.

**DISCUSSION**

Results of the present study demonstrated that the levels of two acute-phase proteins, CRP and AAG, in plasma were increased in dogs infected with *E. canis* at the acute stage of infection. CRP levels were previously reported (8) to be increased in control dogs which were sham injected with sterile saline or even those which had been given only a hypodermic needle puncture in one study with human CRP assay reagents. With canine-specific reagents, however, the plasma of our control dogs, which were inoculated with DH82 cells infected with strain AS145 (mouse isolate) (11), MEM, or noninoculated, did not show significant increases in CRP or AAG levels. Thus, it is unlikely that the increases in CRP and AAG levels...
FIG. 2. Changes in plasma AAG concentration in dogs following inoculation with *E. canis*. Dashed line, mean AAG concentration in the serum of healthy dogs (1). The five bars represent results for dogs 011, 340, 307, 303, and 320, from left to right, respectively.

observed in *E. canis*-infected dogs were due to inoculation of DH82 cells or MEM or to blood collection procedures.

Only a few kinetic studies have been done on the CRP response in dogs (6, 7, 27). Maximum plasma CRP protein levels were lower and the kinetics of the CRP response were slower in dogs infected with *E. canis* than in dogs infected with *Bordetella bronchiseptica* or in dogs that had undergone surgery. In the last two cases, the peak CRP responses were

FIG. 3. Changes in antibody titer to *E. canis* in dogs following inoculation with *E. canis* determined by the IFA test. The five bars represent results for dogs 011, 340, 307, 303, and 320, from left to right, respectively.
TABLE 2. CRP and AAG concentrations in sera of dogs seropositive for *E. canis*

<table>
<thead>
<tr>
<th>Dog no.</th>
<th>Anti-<em>E. canis</em> titer</th>
<th>CRP (µg/ml)</th>
<th>AAG (µg/ml)</th>
<th>Clinical signs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>160</td>
<td>0</td>
<td>200</td>
<td>Anorexia, central nervous system deficit</td>
</tr>
<tr>
<td>2</td>
<td>160</td>
<td>82</td>
<td>2,000</td>
<td>Fever, anorexia, icterus, weight loss</td>
</tr>
<tr>
<td>3</td>
<td>320</td>
<td>53</td>
<td>660</td>
<td>Glomerulonephritis, pulmonary nodule</td>
</tr>
<tr>
<td>4</td>
<td>320</td>
<td>57</td>
<td>3,000</td>
<td>Seizure, lethargy, and polyarthritis</td>
</tr>
<tr>
<td>5</td>
<td>640</td>
<td>69</td>
<td>1,480</td>
<td>Anemia with autotoliginin, ascites, and splenomegaly</td>
</tr>
<tr>
<td>6</td>
<td>1,280</td>
<td>50</td>
<td>6,000</td>
<td>Intraocular hemorrhage, thrombocytopenia</td>
</tr>
<tr>
<td>7</td>
<td>2,560</td>
<td>0</td>
<td>7,800</td>
<td>Anorexia, lethargy, diarrhea</td>
</tr>
<tr>
<td>8</td>
<td>10,240</td>
<td>86</td>
<td>1,720</td>
<td>Splenomegaly, epistaxis, polydypsis</td>
</tr>
<tr>
<td>9</td>
<td>10,240</td>
<td>66</td>
<td>1,480</td>
<td>Fever, anorexia, weight loss, neutropenia, anemia, thrombocytopenia</td>
</tr>
<tr>
<td>10</td>
<td>10,240</td>
<td>16</td>
<td>340</td>
<td>Inginal lymphadenopathy, diffuse muscle atrophy, anemia, diarrhea, anorexia, weight loss, hypergamma-globulinemia, leukopenia, thrombocytopenia</td>
</tr>
<tr>
<td>11</td>
<td>10,240</td>
<td>56</td>
<td>1,240</td>
<td>Thrombocytopenia, nonregenerative anemia</td>
</tr>
<tr>
<td>12</td>
<td>10,240</td>
<td>58</td>
<td>840</td>
<td>Anorexia, diarrhea, enlarged liver, spleen, lymph nodes, anemia</td>
</tr>
</tbody>
</table>

observed within 1 day postinfection or treatment and the maximum plasma CRP concentrations reached 100 µg/ml (6) or 200 to 300 µg/ml (27). In contrast, in dogs infected with a facultative intracellular parasite, *Trypanosoma brucei*, a CRP response of approximately 200 µg/ml began at day 10 postinfection (19). The relatively mild acute clinical signs concomitant with the slow growth rate of *E. canis*, the intracellular location of the microorganism, and the resultant slow and mild tissue damage may explain this difference in CRP responses. None of the naturally or experimentally infected dogs had more than 100 µg of CRP per ml of serum. Thus, diagnostically, extremely high plasma CRP levels (>100 µg/ml) may suggest an additional source of inflammation or infection.

The time course and increased concentration of AAG in dogs with experimental *E. canis* infection are similar to those reported by others in various inflammatory diseases of dogs and humans (6, 13). In contrast to the CRP concentration, extremely high AAG levels (>3,000 µg/ml) were observed in some of the naturally infected dogs, suggesting that the regulatory mechanisms of CRP and AAG production and clearance are independent. The naturally infected dogs examined in the present study were representative of dogs that veterinarians actually encounter in Ohio. In general, the naturally infected dogs had more severe clinical signs for longer periods of time than those in the experimentally infected dogs used in the study. This may account for the higher AAG levels that we observed in naturally infected dogs. This suggests that when dogs recover from the acute stages of infection, acute-phase proteins may drop to the control values. However, they may rise again to higher levels when some of the dogs develop the chronic stages of canine ehrlichiosis. The reason for the constantly high basal plasma AAG concentration but not plasma CRP concentration in dog 320 is unknown. Although this is still within the variation seen in “normal” dogs (1), she might have had an unrecognized subclinical disease.

In dogs with *Tyrpanosoma brucei* infections, the serum CRP concentration is sustained at a high level as long as the parasite persists in the dog (19). Only after successful elimination of the parasite by chemotherapy do the CRP levels decline. However, in dogs with *E. canis* infections, despite the persistence of *E. canis* in the blood throughout the experimental period, both CRP and AAG concentrations declined and clinical signs resolved without treatment, suggesting self-healing of the initial damage caused by the *E. canis* infection or the induction of a compensatory mechanism. Thus, CRP or AAG cannot be used as an indicator of the elimination of *E. canis* by antibiotic therapy.

Kupffer cell hypertrophy and hyperplasia in the liver and increased activity of liver-specific enzymes such as alanine transaminase and alkaline phosphatase have been reported in dogs with acute-phase, experimentally induced ehrlichiosis (8, 20, 26). High levels of activity of liver enzymes were detected in the sera of approximately one-third of naturally infected dogs with mild clinical signs of disease (12, 25). These increased enzyme concentrations in dogs with *E. canis* infection may correlate with increased levels of CRP or AAG synthesis in the liver, thus causing high plasma CRP or AAG levels in infected dogs. Capsi et al. (3) showed a correlation between serum alkaline phosphatase and CRP levels. Other liver enzymes, such as aspartate aminotransferase, however, are not elevated in dogs with high plasma CRP levels (3).

Whether these elevated levels of acute-phase proteins in plasma influence the infectivities of blood monocytes with *E. canis* is unknown. CRP was reported to cause the activation of mouse macrophages for tumor cell killing (30). Thus, increased plasma CRP concentrations in the acute stages of *E. canis* infection may help to kill *E. canis* in the macrophages of infected dogs, controlling the infection at a subclinical level. AAG at 0.5 and 2 mg/ml suppresses mouse (2) and human (4) lymphocyte proliferation, respectively. At a concentration of 0.3 mg/ml, AAG prevents human polymorphonuclear leukocyte activation (14). Thus, high plasma AAG levels in dogs with *E. canis* infection may help *E. canis* to survive in the dog by inducing nonspecific immunosuppression. The immunosuppression caused by the high plasma AAG levels may be the reason why naturally infected dogs with extremely high plasma AAG levels (>3,000 µg/ml) did not have high IFA test titers.

On the basis of our results, in addition to various bacterial, viral, and parasitic infections, *E. canis* infection must be suspected if plasma CRP and AAG levels are high. Both CRP and AAG assays each require only a small (5 µl) sample of serum, and results can be obtained very quickly (within 1 day). Assay of CRP and AAG levels may help in assessing the severity of inflammatory damages in *E. canis*-seropositive dogs, and veterinarians might use this information to decide whether to use anti-inflammatory therapy (25) in addition to antibiotic therapy.

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