FULL PAPER *Internal Medicine*

Evaluation of Serum Amyloid A and Surfactant Protein D in Sera for Identification of the Clinical Condition of Horses with Bacterial Pneumonia

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(Received 22 February 2007/Accepted 23 April 2007)

ABSTRACT. In the present study, the concentrations of serum amyloid A and surfactant protein D in sera were measured to evaluate them for identification of the clinical condition of horses with bacterial pneumonia. The study utilized 185 clinically healthy control thoroughbreds and 9 thoroughbreds for experimental infectious study with *S. zooepidemicus*. Blood samples were collected from the 185 healthy control thoroughbreds. The 9 thoroughbreds were experimentally infected *S. zooepidemicus* using an endoscopic injection to a lung lobe and were then observed of clinical conditions. Blood samples were collected before inoculation and on the 1–15th, 22nd, and 29th days after inoculation (follow-up group). The levels of SAA and SP-D in the healthy control thoroughbreds were very low. In the follow-up group, the levels of SAA and SP-D changed in parallel with the horses' clinical condition. The pyrexia observed after bacterial inoculation faded by the 11th day, and the changes in SAA and SP-D occurred simultaneous to disappearance of the clinical signs. Measurement of SAA and SP-D proved useful for monitoring the clinical condition of the horses with bacterial pneumonia. Changes in the SP-D value were preceded by changes in the SAA value. Since the changes in SP-D occurred approximately simultaneous to the changes in the horses' clinical signs, we believe that they reflect the condition of the alveolar membranes. We conclude that measurement of SAA and SP-D in sera is useful for identification of the clinical condition of horses with bacterial pneumonia.

KEY WORDS: bacterial pneumonia, clinical condition, equine, serum amyloid A, surfactant protein D.

J. Vet. Med. Sci. 69(8): 827–830, 2007

Training is delayed when a racehorse suffers from pneumonia. Pneumonia is a clinically important and occasionally fatal disease. Currently, identification of the clinical condition of a racehorse with pneumonia is usually obtained based on clinical signs, white blood cell (WBC) count in peripheral blood, and the plasma fibrinogen (Fbg) concentration [2, 7–9, 18]. Although clinical signs are a very important index, they can be influenced by clinical experience and lack of objectivity. WBC counts in peripheral blood, which are believed to reflect the state of inflammation, are frequently used in diagnosis of infectious diseases [14]. Moreover, a previous report stated that the changes in WBC are more significant than those in Fbg in the case of infection [2]. However, depending on the clinical conditions of the infectious disease, there are many cases in which peripheral WBC counts decrease despite the presence of a suspected severe infection [7]. There is a comparatively long time lag between the degree of inflammation and change in Fbg concentration. It has been pointed out that the changes in Fbg concentration are not necessarily in agreement with the actual progress of a disease [2, 7, 8].

In recent years, serum amyloid A (SAA) is increasingly being employed as an inflammation marker in human medical treatment because it closely reflects changes in clinical condition [10, 11]. It has been reported that SAA is superior to other acute phase proteins as an inflammation marker because SAA is the most sensitive acute phase protein in the horse compared with other acute phase proteins [7, 8, 12–14]. SAA is produced by the liver in response to increased cytokine production [10, 11]. Although its usefulness has also been reported in equine medicine, up to now there has been no detailed analysis of its relevance to the clinical condition of bacterial pneumonia.

On the other hand, surfactant protein levels in sera are used clinically as alveolar damage markers [4–6]. Surfactant protein (SP), which is initially only present in the alveoli, is thought to leak out into the bloodstream if the alveolar basal membrane is damaged [1, 5, 6]. SP-D in the bloodstream is known to more dynamically reflect alveolar basal membrane damage than SP-A in sera [5, 6]. Based on these things, measurement of the SP-D concentration in sera has been considered necessary for understanding pulmonary condition in the presence of bacterial pneumonia.

In this study, the SAA measurement system currently used in human medical treatment was applied to the horse after first setting an equine SAA standard. We compared measurement of SAA with Fbg to determine whether SAA is useful as an inflammation marker when horse contract bacterial pneumonia. Moreover, its relevance to clinical condition after the horses contracted bacterial pneumonia was examined simultaneously by observing the changes in the bloodstream levels of SP-D.

MATERIALS AND METHODS

Horses: This study examined 185 clinically healthy racehorses (104 males, 81 females; mean ± SD, 2.5 ± 0.8 years old) trained with a sufficient load for racing to determine

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physically normal values. Nine thoroughbreds (9 females; 1.0 ± 0.0 years old) were used in an experimental infection study employing *Streptococcus equi* subsp. *zooepidemicus* (*S. zooepidemicus*). The Animal Use and Care Committee and Animal Welfare and Ethics Committee of the Japan Racing Association's Equine Research Institute approved the protocols for this study.

Blood sampling for the healthy horses: Blood samples were collected from the jugular veins of the racehorses while in a resting state more than two hours after completion of training using blood collection tubes containing sodium citrate buffer (VT-050DK, Terumo, Tokyo, Japan), blood collection tubes containing EDTA (VP-C050, Terumo) or plain blood collection tubes (VP-P100K, Terumo; healthy group).

Experimental infection study: Nine thoroughbreds were experimentally infected with 1×10^9 CFU of an overnight culture of *S. zooepidemicus* by endoscopic injection to a lung lobe [19]. The 9 thoroughbreds were observed for clinical signs, and blood samples were collected before inoculation and on the 1–15th, 22nd, and 29th day after inoculation (follow-up group). The follow-up group was administered an antimicrobial agent (Cephalothin sodium, Coaxin, Tobishi, Tokyo, Japan) from 24 hr after the *S. zooepidemicus* inoculation until their clinical signs disappeared. Blood was sampled using the same methods as that for the healthy group. The follow-up group was examined twice daily for clinical signs (decrease in activity or appetite, rhinorrhea, cough, enlargement of lymph nodes, abnormal chest sounds) and rectal temperature.

Measurement of rectal temperature, WBC counts, Fbg concentrations, SAA concentrations, and SP-D concentrations in peripheral blood: Rectal temperature was measured using a mercury thermometer. The WBC counts of the blood collected into blood collection tubes containing EDTA were measured using an automatic blood-cell counter (K-4500, Sysmex, Kobe, Hyogo, Japan).

The plasma was separated from samples collected into blood collection tubes containing sodium citrate buffer by centrifugation (2,000 × g, 10 min, 4°C). The Fbg concentration in the obtained plasma was measured by the salting-out method (Iatroset Fbg, Mitsubishi Kagaku Iatron, Tokyo, Japan) using human fibrinogen as the standard.

Sera were separated from the samples collected into plain blood collection tubes by centrifugation (2,000 × g, 10 min, 4°C). The SAA concentration in the sera thus obtained was measured by the latex agglutination method using equine standard sera with SAA concentrations ranging from 0.8 to 400.0 μg/m*l* that was produced by the methods described in previous reports as the standard [12, 15, 16]. The assay was performed on an automated analyzer (Hitachi 7020, Hitachi, Tokyo, Japan) using polyclonal rabbit and monoclonal murine antibodies covalently bound to polystyrene latex particles (LZ test "Eiken" SAA, Eiken Chemical, Tokyo, Japan). The coefficients of variation of this assay at 35.5 μg/m*l* (n=20) and 257.3 μg/m*l* (n=20) were 0.9 and 0.8%, respectively. The assay detection limit was ≥0.1 μg/m*l*. The concentrations of SP-D in sera were measured using a two-site sandwich ELISA method as described previously [3]. The dilution rate of the sera was 20 times as suggested by the results of preliminary experiments (data not shown). The coefficients of variation of this assay at 5.0 *n*g/m*l* (n=20) and 31.0 *n*g/m*l* (n=20) were 3.3 and 1.9%, respectively. The assay detection limit was ≥0.4 *n*g/m*l*.

Statistical analyses: Data are expressed as the mean ± SD. The data were analyzed using one-way ANOVA for repeated measures followed by a Dunnet test to compare the post-value with the pre-value. Values of $p<0.05$ were considered significant.

RESULTS

Healthy group: The SAA concentration of the healthy group was 0.1 ± 0.1 μg/m*l*. The SAA concentration of 76 (41.1%) of the 185 samples was 0 μg/m*l*; the highest samples only reached 1.0 μg/m*l*. The Fbg concentration of the healthy group was 246.0 ± 33.1 mg/d*l* with a range of 200–300 mg/d*l*. The SP-D concentration of the healthy group was 0.2 ± 0.3 *n*g/m*l* with a range of 0–1.0 *n*g/m*l* .

Experimental infection study: Aggravation of clinical signs, including high rectal temperature, was observed after bacterial inoculation of the follow-up group on the 1st day. There was a significant increase in Fbg, SAA, and SP-D at the time of bacterial inoculation and 24 hr later in the follow-up group (Fig. 1). The peak values of each index were obtained on the following days after bacterial inoculation: days 4–5 for Fbg, day 3 for SAA, and day 2 for SP-D. The days of recovery after bacterial inoculation for these indexes were as follows: day 22 for Fbg, day 15 for SAA, and day 13 for SP-D. The pyrexia observed after bacterial inoculation returned to normal on the 11th day after bacterial inoculation, and the clinical signs also disappeared at this time (Fig. 1). Treatment with antimicrobial agents was halted based on a clinical assessment 12 days after bacterial inoculation.

DISCUSSION

SAA is an acute-phase reactivity protein produced in the liver in response to detection of cytokines resulting from an inflammatory outbreak. The following knowledge has been obtained concerning SAA from study of humans and other animals: 1) the normal value is very low; 2) changes in clinical condition elicit a rapid response; and 3) the rate of synthesis is the only factor affecting blood concentration [7, 8, 10–16]. In the present study, the SAA concentration was determined using a latex agglomeration immunoassay in an automated analyzer and employing equine standard sera with SAA concentrations ranging from 0.8 to 400.0 μg/m*l* as the standard [12, 15, 16]. Most SAA in serum is associated with high-density lipoprotein particles. Purified SAA is difficult to solubilize and is unstable in physiologic solution, possibly because of its hydrophobic characteristics [15]. Therefore, a pure unlyophilized SAA-rich fraction was used in the present study that was easily diluted with

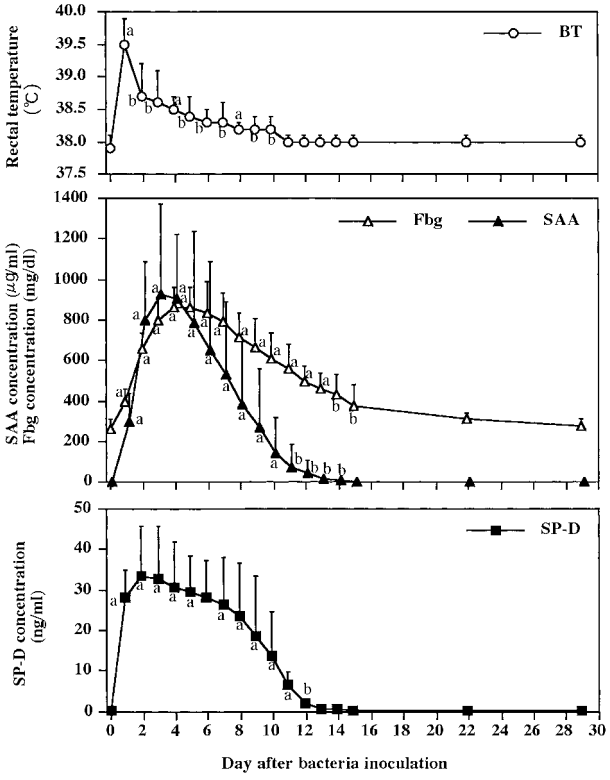

Fig. 1. Changes in rectal temperature and SAA, Fbg, and SP-D concentrations in the follow-up group after bacterial inoculation. The Fbg, SAA, and SP-D values of the follow-up group increased significantly 24 hr after bacterial inoculation. The peak values for each index were reached on the following days after bacterial inoculation: days 4–5 for Fbg, day 3 for SAA, and day 2 for SP-D. The day of recovery after bacterial inoculation was day 22 for Fbg, day 15 for SAA, and day 13 for SP-D. The pyrexia observed after bacterial inoculation returned to normal on the 11th day after bacterial inoculation, and the clinical signs also disappeared at this time. Treatment with antimicrobial agents was halted based on a clinical assessment 12 days after bacterial inoculation. Data are expressed as the mean ± SD. [a] Significantly different ($p<0.01$) from before bacteria inoculation (0 day). [b] Significantly different ($p<0.05$) from before bacteria inoculation (0 day).

physiological saline. This method was simple and easy, and it produced results in only 10 min. Furthermore, this method was a prospective assay because only a serum sample was used; however, the plasma levels were also measurable (data not shown).

The measurement of SP-D in serum was applied to assay of bronchoalveolar lavage fluid using a test that we developed [3]. In this assay, the highest precision was obtained at a sample dilution rate of 1:20 (data not shown). Normally, SP-D is only present in alveoli [17]. However, when the permeability of the alveoli increases as a result of damage, it appears to leak out into the bloodstream [5, 6].

The SAA value of the healthy group was 0.1 ± 0.1 μg/m*l*, and since the values of all the samples fell within 0–1.0 μg/m*l*, we selected 0–1.0 μg/m*l* as the physiological reference value in this method. Although the SAA value was clearly low even when compared with the results of previous reports [7, 12, 15], we believed that this was due to the fact that the racehorses were young thoroughbreds that were healthy, the measuring methods were different, the sample used as the healthy group was favorable clinically, and the groups of horses used contained comparatively little variation in the previous reports. The same trend was observed for Fbg [18], i.e., the physiologic reference value was lower than in previous reports [2, 7, 8].

The SP-D value of the healthy group was 0.2 ± 0.3 *n*g/m*l*, and since the values of all the samples fell within 0–1.0 *n*g/m*l*, we selected 0–1.0 *n*g/m*l* as the physiological reference value for this method. Although this value is lower than the human physiological reference value [5], we concluded this to be due to a difference in species or the measurement system.

The SAA and Fbg levels peaked and then fell again after *S. zooepidemicus* inoculation in the follow-up group; however, the rate and amplitude of increase 24 hr after inoculation was conspicuously higher for SAA than Fbg. The SAA value also reached the peak value earlier than the Fbg value. It subsequently decreased gradually and in most cases returned to a normal value with disappearance of the clinical signs. This result indicates that SAA reacts more rapidly than Fbg to changes in clinical signs in horses. Hülten *et al.* reported in a study of experimentally induced arthritis that the SAA value rose earlier than the Fbg value and that it reached a peak at 48 hr compared with 72 hr, respectively [8]. These results suggest that measurement of SAA is useful as a method of anticipating the clinical condition of a horse with bacterial pneumonia.

Changes in the SP-D value also preceded changes in the SAA value. It appears that SP-D is detected in the bloodstream due to leakage from alveoli [5], while SAA is produced in the liver in response to increased levels of cytokines [10, 11]. Honda *et al.* reported that the SP-D concentration in sera increases if the alveolar membrane is damaged [5, 6]. Fujita *et al.* reported that SP-D in serum can serve as a biomarker of lung inflammation in both acute and chronic injury in mice [1]. In other words, it is thought that the SP-D concentration in sera reflects the condition of the alveolar membrane. In this study, the changes of SP-D in sera may reflect the condition of the alveolar membranes because the changes in SP-D were consistent with the changes in clinical signs.

Measurement of SAA and SP-D proved useful for monitoring the clinical conditions of horses with bacterial pneumonia. Changes in the SP-D value were preceded by changes in the SAA value. Since the changes in SP-D were in accordance with the changes in the horses' clinical condition, we believe SP-D reflects the condition of the alveolar membranes. We conclude that measurement of SAA and SP-D in sera is useful for identification of the clinical condition of horses with bacterial pneumonia.

ACKNOWLEDGEMENTS. The authors thank Dr. Fujinaga and Dr. Okumura (Laboratory of Veterinary Surgery, Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan) for kindly providing purified equine SAA and polyclonal antibody for equine SAA.

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