

SERUM C-REACTIVE PROTEIN (CRP) MEASUREMENT IN DOGS WITH ALTERED HEMATOLOGICAL PARAMETERS

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ABSTRACT

The natural immune response to infectious agents is partially mediated by a group of proteins named acute phase proteins; one of the major and most important proteins from the acute phase is the C-Reactive Protein (CRP). The aim of the present study was to investigate the correlation between alterations on white blood cell counts (WBC) and the presence of circulating CRP. Blood samples from 70 dogs were submitted to hematological examination and detection of CRP; in addition, blood samples from 12 dogs experimentally inoculated with an inactivated sample of *Micrococcus luteus* (ATCC 7468) were also analyzed. According to the hematological parameters and CRP values, the samples were classified in 5 different groups: group A) dogs without CRP and normal WBC; group B) dogs with elevated levels of CRP

but normal WBC; group C) dogs with elevated CRP values and altered WBC (neutrophilia and leukocytosis); group D) dogs with both CRP and increased neutrophils values; and group E) dogs with elevated CRP values but with leukopenia. Dogs experimentally inoculated with *M. luteus* had a significant increase in CRP values between 24 and 48 h p.i., which declined up to the 10th day when the experiment was completed. In these dogs, a discrete neutrophilia and leukocytosis was observed coincidentally with the CRP elevation. Thus, it can be suggested that the detection of CRP in the blood of dogs should be used as an indication of an infection or inflammation and, because of the low amount of blood needed, low cost and easiness to perform the test, monitoring CRP levels might be useful to evaluate the recovery from an infection disease.

KEYWORDS: acute phase proteins; blood cell count; diagnosis; inflammation.

DETERMINAÇÃO DOS NÍVEIS SÉRICOS DE PROTEÍNA C-REATIVA (CRP) EM CÃES COM ALTERAÇÕES DOS PARÂMETROS HEMATOLÓGICOS

RESUMO

O sistema imune natural responde ao processo infeccioso ou inflamatório produzindo uma série de proteínas inflamatórias, com atividade inespecífica, denominadas de proteínas de fase aguda, entre as quais se destaca a Proteína C-Reativa (CRP). O presente estudo teve como objetivo investigar a correlação entre as alterações dos parâmetros hematológicos em cães e a presença de CRP na circulação sanguínea. Amostras de sangue de 70 cães atendidos em um Hospital Veterinário Universitário foram submetidas ao hemograma e à quantificação da CRP; em um segundo momento, amostras de sangue de cães

inoculados experimentalmente com uma suspensão inativada de *Micrococcus luteus* (ATCC 7468) foram submetidas à mesma avaliação. De acordo com os parâmetros hematológicos e a presença ou não de CRP, pode-se classificar os animais em cinco grupos distintos: grupo A) cães negativos para a CRP e hemograma normal; grupo B) cães positivos para a CRP e hemograma normal; grupo C) cães positivos para a presença de CRP e hemograma alterado (neutrofilia e leucocitose); grupo D) cães positivos para a CRP, com neutrofilia; grupo E) cães positivos para a CRP e leucopenia. Cães inoculados

experimentalmente com *M. luteus* apresentaram um aumento significativo na concentração de CRP, entre 24 e 48 horas após inoculação, com um decréscimo nas concentrações gradativamente até o 10º dia. Nesses animais, observou-se uma discreta neutrofilia e leucocitose coincidindo com o pico nos valores da CRP. Baseado nos resultados acima, concluiu-se que a

determinação da presença da CRP em cães pode ser utilizada como uma importante ferramenta no diagnóstico veterinário. Haja vista a rapidez com que o teste é executado, o baixo custo e a necessidade de pequena quantidade de amostras, a utilização da CRP pode auxiliar o monitoramento da evolução de um processo infeccioso/inflamatório.

PALAVRAS-CHAVE: diagnóstico; inflamação; hemograma; proteínas de fase aguda.

INTRODUÇÃO

The acute phase response is a complex and nonspecific response of the organism, which, generally, develops and ends shortly after any tissue injury (MURATA et al. 2004). The origin of this mechanism can be attributed to an immune, infectious, neoplastic or traumatic response (MURATA et al., 2004; PEPYS & HIRSCHFIEL, 2003; CERÓN et al., 2005). The acute phase response is considered part of the natural immune response of the body's defense, responsible for the survival of the host during the critical and early phase, due to exposure to different microorganisms (MURATA et al., 2004; PETERSEN et al., 2004; CERÓN et al., 2005).

Systemic changes of the acute phase, during the inflammatory process, include fever, increase in the number of circulating leukocytes, changes in blood cortisol levels and variations in plasma concentrations of a group of proteins known as acute phase proteins (MARTÍNEZ-SUBIELA et al., 2001; CECILIANI et al., 2002). The acute phase proteins (APP) are classified according to their positive or negative regulatory character when the concentrations detected in the bloodstream of the body are considered (MURATA et al., 2004). Albumin and transferrin are in the group of proteins with negative regulatory character. The positive character APPs are classified as glycoproteins, synthesized mainly by hepatocytes in the liver under the action of proinflammatory cytokines. Hepatoglobine, serum amyloid A, ceruloplasmin, alpha-1-acid glycoprotein, fibrinogen and C-reactive protein belong to this group (MURATA et al., 2004; EKERSALL et al., 2011). Among these proteins, C-Reactive protein (CRP) stands out. It is synthesized in the liver under the influence of cytokines, such as tumor necrosis factor (TNF), interferon- γ (IFN- γ) and interleukin 1 (IL-1) and 6 (IL-6), which are produced by a specific group of cells, mainly by macrophages in response to external stimuli (CERÓN et al. 2005). CRP belongs to the pentraxin family and is a strong

indicator of inflammation and/or infection in veterinary medicine (RIKIHISA et al., 1994; YAMAMOTO et al., 1994; HAYASHI et al., 2001; SHIMADA et al., 2002; EKERSALL & BELL, 2010). The CRP has an important role in the interaction between the innate and specific immune response, acting in opsonization, interaction with specific receptors in phagocytosis, activation of the classical complement pathway, cytokine synthesis, and, finally, acting in the regulation of the host immune response (DU CLOS & MOLD, 2001). On the other hand, cytokines produced during an inflammatory/infectious process provide different stimuli to bone marrow, changing the hematological parameters which are key to evaluate the presence or absence of infections in the veterinary clinic, especially in small animals (JAIN et al., 2011).

In veterinary diagnostics, the complete blood count invariably requires samples collected properly and exhibiting excellent quality standards, avoiding thus the generation of inconsistent and questionable results. This process requires significant and frequent amounts of blood (1 to 3 mL) and the total absence of fibrin, which is not always possible or feasible, especially in case of small breed and/or aggressive animals. In such cases, the use of other fast, practical and low-cost biomarkers of infectious and/or inflammatory process plays a key role in both the development of diagnosis, prognosis and monitoring of antimicrobial therapy in dogs (EKERSALL & BELL, 2010). Currently, the literature about the interaction between changes in hematological parameters and the presence of APP, mainly C-Reactive Protein, is scarce and/or inconsistent (BURTON et al., 1994).

In Brazil, there are no available kits specific for the canine species that could be used in C-Reactive Protein detection and considering the difficulties of importing such material, either by customs restrictions or the high cost, the use of this tool becomes impractical, especially in

veterinary clinics. In the last decade, the international scientific community has demonstrated the possibility of using heterologous reagents, particularly those used for humans, for the detection of CRP in dogs. In these studies, C-Reactive Protein is measured by automated techniques such as the immunoturbidimetry, widely used in humans, and more recently by immune-enzymatic assays and highly sensitive CRP kits (KJELGAARD-HANSEN et al., 2003; FRANSSON et al., 2007; MUNHOZ et al., 2009; KLENNER et al., 2010; PAIN et al., 2013). As the vast majority of veterinary hospitals and especially the Brazilian veterinary clinics lack the structure and the equipment required to carry out these techniques, the availability of a fast and low cost technique could help considerably both the diagnosis of infections and the prognosis and monitoring of treatment of dogs.

The objective of this study was to evaluate the presence and kinetics of CRP activity by latex agglutination technique and its possible association with hematological disorders in canines, in order to spread the idea of its use as an adjunctive method for the determination of inflammatory process and action the monitoring or therapies.

MATERIAL AND METHODS

The study was divided into two stages. The first stage was conducted to test the hypothesis of association between the two techniques. We used 70 blood samples obtained from dogs, patients of the Veterinary Hospital of the University of Passo Fundo, UPF-RS. We sent the samples to the laboratory of Clinical Pathology for complete blood count and serum biochemistry carry-out; subsequently we used the samples for detection and quantification of CRP. We compared the results of the hematological tests, from the complete blood count, for CRP presence and quantity.

In the second stage, twelve mongrel (male and female) dogs of the Central Laboratory of the Institution were used. The animals were previously tested, and presented complete blood count with no alteration and negative result for CRP, they were, then, inoculated intramuscularly with a bacterial antigen (*Micrococcus luteus* ATCC 7468, 1 mL, optical density of 0.5/OD_{492nm}), heat-inactivated at 60 °C for 30 min in order to induce an inflammatory response. We collected blood samples at intervals of two to three days for

determination of hematological and C-Reactive Protein parameters for a period of 10 days.

This study was conducted in accordance with the standards of COBEA and with the Ethics Committee in Research – CEP at the time of the study.

We collected the samples in tubes with and without EDTA for complete blood count and CRP, respectively. We performed the complete blood count using an automated method and we obtained differential leukocyte count using blood smears stained by the rapid panoptic (WEISS & TVEDTEN, 2004), followed by differential counting by optical microscopy (1000X), using reference values previously cited in the literature (MEDÁILLE et al., 2006; BECKER et al., 2008).

We assessed CRP presence and quantification using a latex agglutination kit, used in humans, according to the manufacturer's recommendations (In Vitro Diagnóstica Corporation): serum samples (25µL) were mixed with an equal volume of the reagent on a glass plate. The presence of a distinct agglutination was indicative of the presence of CRP at a minimal concentration of 6 µg/mL (lower sensitivity limit for human serum); positive and negative controls were used during the tests. Subsequently, the positive samples were serially diluted (factor two) in saline solution (0.9%; dilutions from 1:2 to 1:256, equivalent to serum concentrations of 12 to 1536 µg/mL, respectively) to determine the amount of CRP in serum samples.

To test the hypothesis of correlation between the two tests, data from CRP and blood counts were tabulated and submitted to statistical analysis by the Pearson's equation for determining the correlation coefficient followed by the degree of agreement between tests by coefficient Kappa.

RESULTS AND DISCUSSION

The results obtained in the first stage of the study revealed an association between changes in hematological parameters and the presence or absence of CRP, and the animals were divided into five groups: group A) dogs negative for CRP and normal complete blood count; group B) dogs positive for CRP and normal complete blood count; group C) dogs positive for CRP and altered complete blood count (neutrophilia and leukocytosis); group D) dogs positive for CRP, with neutrophilia; group E) dogs positive for CRP with leukopenia (Table 1).

In the second stage of the experiment, we observed that dogs inoculated with *M. luteus* presented a peak in the CRP levels between 24-48 hours post-induction, values that decreased rapidly within days of inoculation (Figure 1). It is noteworthy that the peak of CRP coincided with a

slight leukocytosis followed by neutrophilia (Figure 2). However, it was observed that the serum levels of CRP increase or decrease faster and more intensely than the discrete changes in the blood.

Table 1. Relationship between the parameters of complete blood count and serum C-Reactive Protein (CRP, µg/mL) in (male and female) dogs attended at the Veterinary Hospital, classified into five distinct groups, according to the results of the complete blood count and the CRP concentration

Group	n	C-Reactive Protein (µg/mL)	Neutrophis Reference interval* (3000 – 11500)	Total leukocytes Reference interval (6000 -17000)
A	11	Negative**	3786 – 11130	6650 – 14752
B	28	6.0 – 768.0	3168 – 10959	6060 – 16860
C	22	12.0 - 1536.0	12372 – 50406	17280 – 69773
D	6	12.0 - 384.0	11860 – 14201	13500 – 16310
E	3	6.0 – 768.0	878 – 4283	4360 – 5160

^Anegative for CRP and normal complete blood count, ^Bpositive for CRP and normal normal complete blood count, ^Cpositive for CRP and altered complete blood count (neutrophilia and leukocytosis), ^Dpositive for CRP and neutrophilia, ^Epositive for CRP and leukopenia; ⁿ number of samples. * No./µL - WEISS & TVEDTEN (2004); ** (CRP <6µg/mL).

The temporal response of CRP to inoculation of *M. luteus* was similar to that previously observed for infection by *Bordetella bronchiseptica* (YAMAMOTO et al. 1994) or by intramuscular casein administration (PARRA et al. 2005), but it was much faster than that observed when there was experimental infection by *Ehrlichia canis* (RIKIHISA et al., 1994; SHIMADA et al., 2002) or *Trypanosoma brucei* (NDUNG'G et al. 1991). This time difference is primarily due to the nature of the inoculated antigen; both the inactivated inoculum of *M. luteus* or the intramuscular inoculation of casein (PARRA et al., 2005), and the active infection by *B. bronchiseptica* (YAMAMOTO et al. 1994) remain outside the cells (exogenous antigen) and are

immediately recognized by macrophages, generating the necessary stimulus for the secretion of CRP. On the other hand, the challenge by *E. canis* and *T. brucei* slowly replicate within phagocytic cells, particularly monocytes and macrophages, and the stimulus to the production of pro-inflammatory cytokines, which induce CRP, occurs only after the release of a large quantity of microorganisms in the bloodstream. However, the formation of the cell wall of the microorganisms presents peculiar characteristics, with greater or smaller presence or with absence of lipopolysaccharide and therefore resulting in different forms of stimulation of macrophages and thus of the cytokine responsible for mediating CRP production.

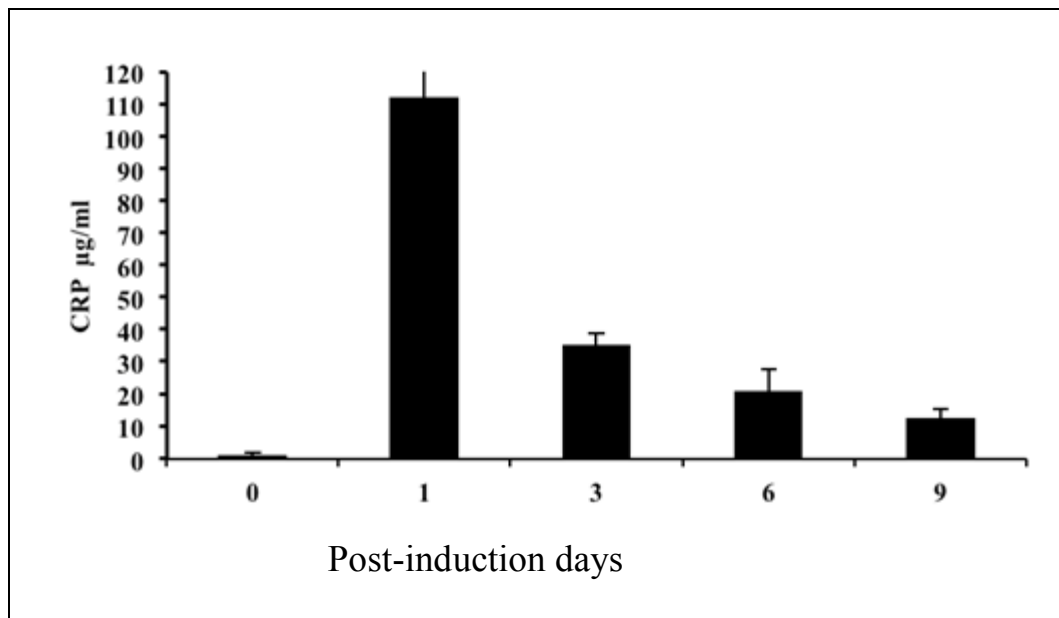


Figure 1. Temporal response of C-Reactive Protein (CRP) to antigenic stimulation. Blood samples were previously collected for determination of serum CRP (day 0). After intramuscular inoculation with a suspension of inactivated *Micrococcus luteus* (ATCC 7468), samples were collected on alternate days for CRP determination.

Due to the different methods used for determination of C-Reactive Protein, it is not possible to establish a true relationship between the amount ($\mu\text{g/ml}$) of CRP detected in different studies, mainly due to the fact that the method used in this work (agglutination latex) presents lower sensitivity than the immunoenzymatic assay (ELISA) and the immunoturbidimetric assay, often used in other studies (NDUNG'G et al., 1991; RIKIHISA et al., 1994; SHIMADA et al., 2002; PARRA et al., 2005). However, on reports on infectious bacterial antigen (*B. bronchiseptica*), the average levels detected ($498 \pm 132 \mu\text{g/ml}$) were slightly higher, while infection by *E. canis* (peak $40 \mu\text{g/ml}$) and inoculation of casein (average of $44.89 \mu\text{g/ml}$) induced lower serum CRP levels than the peak detected in this sample ($192 \mu\text{g/ml}$). Furthermore, it should be considered that, in the present study, we used inactivated inoculum compared to the infectious inoculum of *B. bronchiseptica* (YAMAMOTO et al. 1994), *E. canis* (RIKIHISA et al. 1994; SHIMADA et al. 2002) and *T. brucei* (NDUNG'G et al. 1991).

When comparing serum CRP in dogs of groups C and D, we verified that an active infectious process induced serum CRP levels similar to

experimental infection with *B. bronchiseptica* (YAMAMOTO et al. 1994) regardless of the sensitivity of the assay used. Moreover, dogs with diverse clinical pathological cases (Leishmaniasis, pyometra, sepsis, mammary tumor, among others) had serum CRP ranging from 29.98 to $1536 \mu\text{g/mL}$; on the other hand, healthy dogs had higher serum CRP levels, between 0.0 and 3.47 g/mL (PARRA et al. 2005; BASSO et al. 2007). These levels could not be detected in this experiment due to the sensitivity of the assay we used. Nevertheless, we demonstrated a significant positive correlation between the CRP detection by both methods (latex agglutination and ELISA), as described by VEIGA et al. (2009).

In this study, we found a strong positive correlation between the results of the CRP and the complete blood count (leukocytosis and neutrophilia) ($p = 0.77$ and $p = 0.93$, respectively). When we tested the values of agreement between both tests we found a Kappa value of 0.53 , which is considered moderate. Thus, the data of this study allow us to conclude that the latex agglutination method could be an alternative in the monitoring of the infectious process and guidance of therapeutic procedures.

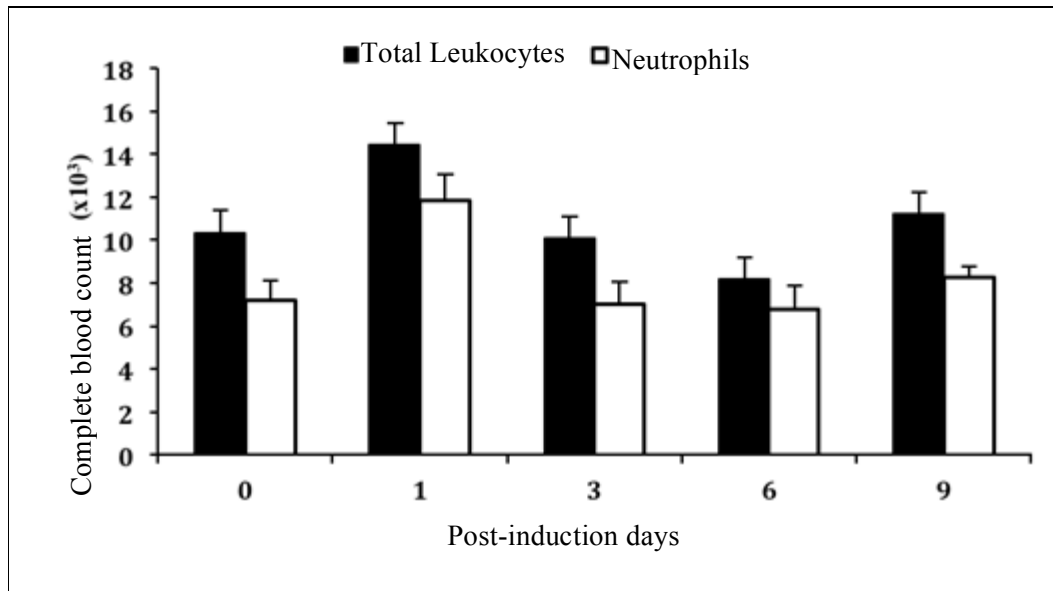


Figure 2. Variations of total leukocytes count and neutrophils ($1 \times 10^3/\mu\text{L}$) in dogs inoculated with a suspension of inactivated *Micrococcus luteus* (ATCC 7468). Blood samples were collected for analysis of hematological parameters prior to inoculation (day 0) and after inoculation.

The fast variation (increase and decrease) in CRP levels detected in dogs experimentally inoculated with *M. luteus* is due to the fact that the CRP is induced immediately and only in response to the pro-inflammatory cytokines, TNF, IL-1 and IL-6, which are produced in large amounts by macrophages in response to antigens (JAIN et al., 2011). When cytokines are stimulated, these levels can increase by 100 to 1000 times the baseline. On the other hand, the C-Reactive Protein has short half-life in the bloodstream, and their production is immediately interrupted when the antigenic stimulus ceases – removal of the infectious focus (EKERSALL & BELL, 2010). In contrast, bone marrow response to cytokines is slower and its effects extend for a longer period of time due to the relatively long half-life of leukocytes, which is translated by leukocytosis and neutrophilia (WEISS & TVEDTEN, 2004), usually observed in dogs with an infectious process in the resolution phase. Indeed, observing the results of CRP determination and complete blood count of the dogs in this experiment, we verified that the dogs of group B (CRP positive / normal complete blood count) showed the beginning of an infection or a discrete infectious process as that observed by inoculation of *M. luteus*, but not yet detected by the complete blood count. The dogs of groups C and D (altered CRP and leukocytosis and or neutrophilia) were visibly in an infection or inflammatory process either in progress or resolution phase, respectively. Finally, the data of group E suggested a possible early viral infection, indicated

in clinical diagnosis (personal communication – data not shown).

The intense response detected for CRP, combined with leukopenia, has recently been demonstrated in experimental studies with canine parvovirus, where the CRP presents itself as a strong and excellent biomarker indicative of infection prognosis (GODDARD et al., 2008; KOKATURK et al., 2010). In fact, these interpretations are consistent with the clinical case of each animal, according to data obtained from the records of the hematological tests.

CONCLUSION

The CRP detection in the serum of dogs is a clear indication of the presence of an infection or inflammation in the animal. CRP kinetics consists of an intense, fast and short response. Depending on the stage of the infection or inflammation, the peak in CRP levels in the blood can be observed along with hematological disorders (leukocytosis, neutrophilia, and in cases of viral infections, leukopenia). CRP determination does not exempt from complete blood count; however, the evaluation of the presence or absence of CRP can be an important tool in monitoring the progress of an infection or inflammation, aiding in predicting mortality. The latex agglutination test can be performed quickly with a significantly small amount of sample, and consequently it can be determined with greater

frequency. The technique is easy to perform, does not require sophisticated equipment, absent in veterinary clinics, and, mainly, has significantly reduced cost - around one Brazilian Real, compared to the other tests routinely used in laboratories of Veterinary Clinical Pathology.

SOURCES OF ACQUISITION

^a In vitro Diagnóstica Corporation, Itabira, MG

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