

Recent advances and future prospects for the use of acute phase proteins as markers of disease in animals

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SUMMARY

Acute phase proteins such as haptoglobin, serum amyloid A and C-reactive protein are plasma proteins which increase in concentration following infection, inflammation or trauma. Investigations over the last decade have shown that the quantification of their concentration in plasma or serum can provide valuable diagnostic information in the detection, prognosis and monitoring of disease. Species differences in the acute phase protein response profile mean that each species should be examined individually and that immunoassays for the proteins should be carefully validated before use. Acute phase protein analysis is becoming a common procedure in clinical and experimental investigations of infectious disease in farm and companion animals. Assessment of the concentration of major and moderate acute phase proteins provides a means to estimate the combined effect of the pro-inflammatory cytokine stimulation of systemic functions. In the future, measurement of these proteins could have further applications in the identification of diseased animals at slaughter and for monitoring the presence of sub-clinical disease leading to poor growth rates on farms. These applications will be facilitated by current developments in diagnostic biotechnology. Future prospects for investigation of the acute phase proteins in companion animals include assessment of the effects of the acute phase response on the pharmacokinetics of veterinary drugs and on association between sub-clinical levels of the proteins and chronic diseases.

KEY-WORDS : acute phase proteins - haptoglobin - serum amyloid A - immunoassay - infection - meat inspection - cattle - pigs - dogs - cat.

RÉSUMÉ

Progrès récents et perspectives d'utilisation des protéines de la réaction inflammatoire aiguë comme marqueurs des affections chez les animaux. Par P.D. ECKERSALL.

Les protéines de la réaction inflammatoire aiguë, comme l'haptoglobine, la protéine amyloïde A et la protéine C réactive, sont des protéines plasmatiques dont la concentration augmente après une infection, une inflammation ou un traumatisme. Les recherches des dix dernières années ont montré que la mesure de leur concentration plasmatique pouvait fournir une information utile pour la détection, le pronostic et la surveillance de certaines affections. Les différences interspécifiques des profils de protéines de la phase aiguë impliquent que chaque espèce doit être considérée séparément et que les tests immunologiques pour les protéines doivent être validés soigneusement avant utilisation. L'analyse des protéines de la phase aiguë devient une procédure usuelle en clinique et en recherche dans les maladies des animaux de compagnie et de ferme. L'évaluation des protéines de la réaction inflammatoire aiguë permet d'estimer l'effet combiné de stimulation des fonctions de l'organisme par les cytokines pro-inflammatoires. Dans le futur, la mesure de ces protéines pourrait avoir des applications dans l'identification des animaux malades à l'abattoir, dans la surveillance de la présence de maladies subcliniques entraînant des retards de croissance. Ces applications seront facilitées par les développements des biotechnologies diagnostiques. Des développements futurs de la recherche sur les protéines de la réaction inflammatoire aiguë chez les animaux de compagnie portent sur l'évaluation des effets de la réponse à la phase aiguë sur la pharmacocinétique des médicaments vétérinaires et sur l'association entre des variations sub-cliniques des concentrations de ces protéines et des affections chroniques.

MOTS-CLÉS : protéines de la réaction inflammatoire aiguë - haptoglobine - protéine amyloïde A - dosage immunologique - infection - inspection des viandes - bovins - porc - chien - chat.

Introduction

Animals undergoing external or internal challenge to their state of health mount a vigorous response including activation of both the innate and acquired immune systems. The

acquired immune system leads eventually to the development of specific cellular and humoral immune responses but at the time of the initial challenge the survival of the host depends on the ability of the innate responses to combat the causes of disease. Among the systems evolved to contribute

to this non-specific innate immune response are the varied reactions of the acute phase response which encompass a wide range of pathophysiological responses such as pyrexia, leukocytosis, hormone alterations, serum trace element and muscle protein depletion [48, 94] combining to minimise tissue damage while enhancing the repair process. These systemic responses to disease are accompanied by an increase in the circulating concentrations of a number of plasma proteins which are known collectively as the acute phase proteins (APP) [4, 63, 21]. The circulating concentration of these proteins is related to the severity of the underlying condition and thus quantification of their concentration provides a ready means of evaluating the presence and extent of the disease processes causing the response. In human medicine and increasingly in veterinary medicine, determination of the plasma concentration of these proteins gives valuable clinical information on infection and inflammatory lesions [87, 55, 29, 30].

The last decade has seen growing interest in monitoring the acute phase response in animals for clinical and experimental purposes. This has resulted in many recent advances in our knowledge of this important host response and has also opened up many intriguing questions where research is needed to provide answers which could lead to diagnostic procedures of great potential benefit to veterinary medicine and animal production.

Stimulation of the acute phase protein response

The acute phase response is stimulated by the release of cytokines such as interleukin-1 (IL-1), interleukin-6 (IL-6) and tumour necrosis factor α (TNF α) from macrophages and monocytes at the site of inflammatory lesions or infection [8, 19, 33]. Initial release of these pro-inflammatory cytokines is augmented by their paracrine actions which cause further stimulation of cytokine release and eventually results in a systemic release of cytokine. Increases in the circulation of the cytokines then stimulates the hepatic acute phase response [63].

The mechanism for stimulation of the hepatic production of the acute phase protein by pro-inflammatory cytokines has been extensively studied and reported elsewhere [33]. Induction of the acute phase proteins by IL-6, following binding to the IL-6 receptor, is via the phosphorylation of the transcription factor, NF-IL6 which is then translocated to the nucleus where it mediates the transcription of acute phase genes [47]. IL1 and TNF α , after linking to their respective receptors cause phosphorylation and degradation of I κ B, the inhibitor of transcription factor NF κ B leading to release of NF κ B and subsequent activation of acute phase genes in the nucleus [47].

Acute phase proteins

The first acute phase protein to be recognised, over fifty years ago [89], was C-reactive protein (CRP), which in man has become the most important analyte providing diagnostic

information on the presence of inflammatory lesions, on the prognosis of the condition and on the response to treatment [70, 27]. CRP in man is a major APP, with its concentration increasing over 200 times from a low, virtually negligible, normal level. Other recognised acute phase proteins are serum amyloid A (SAA), haptoglobin (Hp), α_1 -acid glycoprotein (AGP), fibrinogen (Fb) α_1 -anti protease (AP) and ceruloplasmin (Cp) [87]. Of these, SAA is a major acute phase protein, while Hp, AGP, Fb and AP are moderate acute phase proteins as their serum concentration increases only 2-3 times during the response.

In animals, investigations over the last decade have confirmed that measurement of these proteins in serum or plasma can identify the presence of infection or inflammatory lesions. However, there are substantial differences between species in the relative changes in acute phase protein production following stimulation. Thus while CRP is a major APP in man, dog and pig, in ruminants its serum concentration is hardly altered by the presence of infection or inflammation. In contrast, Hp is a major APP in ruminants in which species it has a negligible circulating level in normal animals, but increases over 100 fold on stimulation. In contrast in dogs, Hp is a constitutive serum protein and moderate APP [15, 16, 23].

While most studies in animals have focused on the pathophysiology of the *in vivo* response, a number of investigations of the biochemistry of the acute phase proteins in animals has received attention. It is known that SAA associates with lipoprotein in the circulation but recently two low molecular weight proteins related to bovine SAA have also been identified in association with lipoprotein during an acute phase response [96] in cattle. The same group has also detected the presence of Hp in the high density lipoprotein fraction suggesting a role in lipid metabolism or possibly related to its antioxidant activity [53]. The glycosylation pattern of Hp has been shown to vary with disease in a study which examined the microheterogeneity of this acute phase protein by isoelectric focusing and demonstrated an increase in the fucose content of the Hp during disease particularly associated with anaemia [2].

Advances in the analysis of acute phase protein

Although a number of research groups around the world have established ELISA assays for bovine Hp [28, 100, 80, 62, 66], the greater convenience of methods based on the binding of Hp to haemoglobin (Hb) and the potential for the development of automation, for instance with a robotic system to semi-automate the assay [84], has led to Hp assays becoming a routine biochemical test in a number of veterinary diagnostic laboratories. Further advance came from investigation of the biochemical interactions involved in the Hp-Hb binding assay suggesting that serum albumin can cause false positive results at low Hp levels. A novel reagent cocktail was devised to eliminate this interference and allow the development of a more robust automated biochemical assay system [24], which is the basis for the first commercial

biochemical assay kit for serum Hp¹. Indeed the commercial availability² of assays for animal acute phase proteins, such as SAA, AGP and CRP as well as Hp, is likely to have a major role in further expanding the use of APP quantification in experimental investigation and diagnosis. A novel method for estimation of serum Hp by the use of capillary zone electrophoresis [73] has also been described.

Immunoassays for the estimation of Hp in pigs have been described using a nephelometric approach [57, 58] while the similar technique of turbidimetry has been used to measure Hp in equine and canine serum [92]. However these assays depend on the cross reactivity of antiserum to human Hp with the analogous protein in animal serum and must to be properly validated before use.

Serum amyloid A is a major APP in most species where it has been monitored, but the use of assays for SAA has not been as widespread as those for Hp, probably due to the difficulty of measuring the former. However, an ELISA for equine SAA using chemiluminescent substrate has recently been established [43], while a monoclonal antiserum against human SAA [61] has been effectively used in a sandwich ELISA to monitor canine SAA [101].

Advances in farm animal acute phase proteins

Much of the recent attention on Hp assessment in ruminants has resulted not only from the methodological advances described above but also because it has been recognised as a valuable marker of disease in these species. Hp has the characteristics of a major APP in ruminants where, as an indicator of inflammation, its assay provides valuable additional information to more traditional haematological investigations [81, 14]. Application of Hp analysis in disease investigation in cattle has shown that it has a major diagnostic contribution to make in investigations of pasteurellosis and pneumonia [17, 41, 12, 28, 95], mastitis [35, 78], foot and mouth disease virus [39], fatty liver syndrome [66], surgically treated abdominal disorders [37] and in ewes with dystocia [3]. Haptoglobin has even been identified in bronchoalveolar lavage fluid from calves with experimental pasteurellosis, although the conclusion was made that the Hp in the lavage fluid resulted from leakage from the circulation across the blood-lung barrier [52].

Studies using the APP as to indicate the health status of cattle have largely focused on determination of the serum concentration of Hp due to the wider availability of the assays for this protein. Thus Hp was raised in cattle at slaughter which had been classified as casualty animals needing close examination [77] while Hp and AGP were raised in emergency slaughtered cattle [36] indicating that examination of the APP could aid in improving food safety.

A current research issue on the use of acute phase protein in farm animal medicine is whether the diagnostic value would be increased by monitoring a number of acute phase proteins. In addition to Hp, SAA has been suggested as being a valuable acute phase protein for assessment in bovine medicine [1, 40, 41]. Likewise AGP has been identified as an APP in cattle [86, 45, 65]. Recently Hp, SAA and AGP were estimated in plasma from 81 cattle in which a detailed *post-mortem* confirmed the presence of inflammation and the APP concentrations were compared between the cases classified as having acute or chronic inflammatory lesions [42]. In the 81 animals with inflammation, it was apparent that SAA and Hp were raised in acute rather than chronic cases while in the latter group AGP was more likely to be elevated.

As described in a presentation to the National Academy of Sciences (USA), the assessment of APP is a most valuable analyte to monitor in studies of animal models of infection or inflammation³ when the APP can give quantitative data on the total response to stimulation by the pro-inflammatory cytokine pathways [48]. The use of Hp is becoming established in a wide variety of such studies where information on the acute phase response adds substantial value to the interpretation of the experimental outcome. Hp assessment identified the effectiveness of antibiotic treatment in feedlot cattle [95] and in cattle suffering from toxic puerperal metritis [82, 83]. Monitoring the concentration of serum Hp has also been used to assess the host response to experimentally induced mastitis [38], the response to a prolonged low dose of lipopolysaccharide [93] and in an antigen induced model of arthritis in sheep [34]. Interaction between the acute phase response and reproductive biology has been highlighted with identification of a classical SAA response during the expulsive stage of parturition although not during luteolysis [54]. Hp was also raised in ewes *post-partum* with the presence of interuterine bacterial infection causing even higher Hp levels [75] while assessment of serum Hp concentration allowed quantitative measurement of an experimental inflammatory event during pregnancy in ewes in a study designed to assess the effect of inflammation on plasma follistatin concentrations [74].

Analysis of acute phase proteins in pig production and medicine has become an area of increasing interest in relation to assessment of levels of immunological stress related to sub-clinical infection and thus to herd health status and as a potential marker for disease at slaughter. Sub-clinical infections increase the levels of the pro-inflammatory cytokines [48] but as these are difficult to measure and are in the circulation for only a short space of time it is more appropriate to monitor the APP as biomarkers for such immunological stress. Indeed it has been shown that measuring the serum Hp concentration identifies the presence of sub-clinical infection which leads to a reduced growth rate [26] and there are indications that the mean of herd Hp levels can be related to the state of hygiene of the production facilities [57, 59].

1. Tridelta Development Ltd, Greystones, Co Wicklow, Ireland.

2. As above and Saikin Kagaku Institute, Sendai, Japan ; Cardiotech Services, Louisville, USA.

3. Olfert, E.D. and Godson, D., Endpoints in infectious disease animal models, <http://altweb.jhsph.edu/science/meetings/pain/olfert.htm> 1998.

Recently, APP were measured in porcine serum following the stimulation of the acute phase response with turpentine injection [56, 23]. Porcine Hp, and CRP were identified as major acute phase proteins with significantly raised concentration after 24 hours, a peak by 48 hours and a return to normal levels within 6 days. In addition a novel porcine APP, called Major Acute Phase protein (MAP) which had a significant and rapid increase on stimulation, was identified in pigs [56]. AGP was also reported as an acute phase protein in pigs, with infectious diseases such as pneumonia or meningitis [46].

As is the case with investigation of disease in ruminants, the acute phase response is being increasingly used as means of quantifying the response of pigs to experimental infection. The Hp response in particular is providing a valuable biomarker and end-point for investigation of infection with *Actinobacillus pleuropneumoniae* [31], *Bordetella bronchiseptica* and *Pastuerella multocida* type D [91]. In a more detailed study of *A. pleuropneumoniae* infection, the circulating concentrations of Hp and MAP had the greatest increases with serum concentrations capable of 40 and 12 fold increases respectively over the pre-challenge concentrations [32]. Similarly Hp concentration was raised in pigs experimentally infected with *Toxoplasma gondii* [50]. However, although Hp was raised in response to *Mycoplasma hyorhinis*, there was no difference in the response between lines of pigs known to have high or low immune responses to infection [60].

Advances in companion animal acute phase proteins

In experimental study and diagnosis of canine disease, CRP has the characteristic response of a major APP and has received most attention. Using an immunoturbidimetric assay for CRP [22], a study of 67 cases involving inflammatory reactions showed a significant, but weak, correlation between CRP concentration and band neutrophil count [9]. The range of the CRP response was greater than the response in band neutrophils so that CRP assay could be more indicative of the extent of the inflammatory lesion. Using an ELISA, serum CRP was found to be raised in various disorders being especially high in Leptospirosis, bacterial and haemorrhagic enteritis, parvovirus infection and in the presence of tumours, while surgery also led to major increases [99]. The ELISA was also used to quantify the physiological range of CRP [69] revealing that there is no circadian rhythm in this analyte but that individual differences do occur in normal CRP concentrations. Serum CRP was found to rise up to 95 fold on experimental infection of dogs with *Bordetella bronchiseptica* [98]. An alternative assay system, the capillary reverse passive latex agglutination test has been introduced with the potential for use as a rapid diagnostic aid to quantification of the acute phase response in dogs [85].

The alternative major canine APP is SAA and antibody against this protein has been produced, so that it is conceivable that immunoassays for canine SAA will prove to be useful method of choice for monitoring the response in dogs

[97]. Indeed, monitoring SAA has been valuable as a biomarker of infection during assessment of the response to experimental infection with canine parvovirus during studies of the efficacy of vaccines to this disease [101].

Haptoglobin [15] and AGP [67] have been recognised among the moderate APP in dogs. Assays for the latter were shown to be able to differentiate between healthy animals and those with clinical or sub-clinical disease in a screen of animals for experimental use [67]. While both AGP and CRP were raised in dogs with *Ehrlichia canis* [76]. For haptoglobin an immunotubidimetric assay based on antiserum to human Hp was validated and shown to be useful in diagnosis of canine inflammation [92]. In contrast, electrophoretic separation and quantification of APP revealed that Hp and α -1 antitrypsin were low in dogs with terminal liver cirrhosis whereas normal or elevated levels indicated a more favorable prognosis [79]. In dogs with tumours a correlation has been found between increased AGP concentrations and total sialic acid content, which is known to be raised in these animals [88].

It is possible that the application of APP measurement in small animal medicine will be of value in differential diagnosis where the relative size of inflammatory reaction is of importance. While the use of APP analysis in feline medicine has not been the subject of as much study as in the dog, there is evidence from this species that APP investigation can provide such valuable diagnostic information. The quantification of feline AGP in the diagnosis of feline infectious peritonitis [20] has allowed an improvement in the clinical specificity and sensitivity of the diagnosis of this disease. As the prognosis of cats with this infection is very poor it is important to differentiate it from conditions with similar clinical signs, such as myopathies. The determination of the level of AGP in serum or peritoneal exudate assisted in identification of the inflammatory nature of the peritoneal fluid accumulation and in this role was more effective than assessment of Hp concentration. Feline SAA has been sequenced and expressed allowing the development of specific antibodies [68] and comparison to other APP in diagnosis of disease in cats. SAA was found to be the earliest of the APP to respond to stimulation in this species with Hp and AGP rising after a short delay, but the concentration of CRP was not significantly altered during the response [51].

Future research in animal acute phase proteins

Research into the pathophysiology of infection and inflammation in animals, as revealed by assessment of the serum APP concentrations, has come a long way since interest first developed in this fascinating aspect of the host non-specific defense mechanism. However, there are many more avenues of enquiry which can be pursued to deepen our knowledge of the acute phase response and also to develop novel applications for the APP as biomarkers for the presence of disease in animals.

In clinical medicine there has been renewed interest in the use of monitoring low level changes in APP following the

discovery that small increases in CRP concentration, previously considered to be irrelevant, were associated with predisposition to coronary heart disease [27]. While this condition has less importance in veterinary medicine, the subsequent hypothesis that diabetes mellitus could be related to the chronic stimulation of the innate immune system with associated increases in APP [72] has implications for the diagnosis and treatment of diabetes mellitus in dogs and is worthy of further investigation. Pharmacokinetic studies in man have shown that altered plasma concentrations of proteins, which have a high affinity for drugs (eg albumin and AGP), during an acute phase response can cause significant change to drug disposition. Initial investigations in dogs have revealed that there is a 99 % fall in the availability of unbound quinidine in serum with raised AGP concentrations [44] and further investigation should be undertaken to examine the effects of the acute phase response on the efficacy of veterinary drugs in the different species.

In relation to production of farm animals, as described above, there is evidence that the use of assays for Hp at slaughter could assist meat inspectors identify animals with current infection and hidden pathological lesions, thus improve the safety of food for public health [77]. This may be of particular benefit to inspection of poultry where the volume of carcasses to be inspected is greater. Initial studies have established that the acute phase response does occur in poultry, much as in other species and that SAA is likely to be one of the more useful APP [10, 11]. Current research on the application of APP analysis at slaughter as a means of improving food safety is establishing the diagnostic value of this procedure. Implementation of these assays as a practical step in the food chain will however depend on their feasibility under routine inspection conditions. Fortunately diagnostic biotechnology has been making rapid advances over the last few years and developments in nanotechnology by miniaturizing assay procedures to ultra low volume [6, 7] and in the use of biosensor technology [49] are likely to provide the necessary techniques. Indeed novel developments in the use of biosensors have been described for application to testing at slaughter for the detection of drug residues [5, 25] and salmonella infection [18]. Similarly, the potential of biosensors allied to robotic sampling is being assessed for the development of automated milk sampling and monitoring systems incorporating progesterone assays [71, 13] and ketone determination [64]. Incorporation of immunoassays for the acute phase proteins into such systems would allow the APP assays to become primary markers of disease of great value.

Thus, applications for the use of APP analyses are not restricted to their use in diagnosis, prognosis and monitoring of treatment during infection and inflammation. A further likely use of the APP as markers for clinical and sub-clinical disease in production animals is that their assay will have a role to play in monitoring health for optimal growth. Public pressure to reduce the use of antibiotics as growth promoters will necessitate systems to identify individuals or herds with sub-clinical infection and APP quantification should have an important role to play in maintaining health of farm animals. In this respect, it is likely that more emphasis will be placed on small changes in the serum APP concentrations and use of

an acute phase index, incorporating positive and negative APP could be the means to identify small but significant changes from health to disease [90].

However, with all the exciting applications for quantitative APP assay it is essential that methods established in laboratories around the world produce comparable results and this will only be possible if all assays are calibrated against a common standard. Fortunately this process is underway and within a few years suitable reference preparations will be available for calibration of APP assays in cattle and pigs. The harmonization of assay calibration between laboratories will be a major practical advance for the future prospects in the application of APP assays to animal health and welfare.

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