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The protozoan parasite, *Theileria annulata*, induces a distinct acute phase protein response in cattle that is associated with pathology

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Abstract

Acute phase proteins (APP) are synthesised in the liver in response to the systemic presence of high levels of pro-inflammatory cytokines. Bacteria are considered to be strong inducers of APP whereas viruses are weak or non-inducers of APP. Very few reports have been published on APP induction by parasites. Here, we report that the tick-borne protozoan parasite of cattle, *Theileria annulata*, induced an atypical acute phase response in cattle. Following experimental infection, serum amyloid A (SAA) appeared first, followed by a rise in α_1 acid glycoprotein (α_1 AGP) in all animals, whereas haptoglobin, which is a major APP in cattle, only appeared in some of the animals, and generally at a low level. All three APP only became elevated around or after the appearance of schizonts in draining lymph nodes and after the first observed temperature rise. Increased α_1 AGP levels coincided with the appearance of piroplasms. The production of SAA and α_1 AGP correlated strongly with each other, and also with some clinical measures of disease severity including the time to fever, development of leucopaenia, parasitaemia and mortality. These results are consistent with the hypothesis that *T. annulata* causes severe pathology in susceptible cattle by inducing high levels of pro-inflammatory cytokines.

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1. Introduction

Theileria annulata, a tick-borne protozoan parasite of cattle causes tropical theileriosis with severe morbidity and mortality and poses a serious constraint to livestock production in large parts of the world. In susceptible European (taurine) breeds of cattle (such as Holsteins), the parasite may overcome the immune system and often proves fatal. The pathology of the disease is associated with the presence of the intra-macrophage stage of the parasite (the schizont) but the pathogenic mechanisms remain unclear (Preston et al., 1999; Glass, 2001; Preston, 2001). The schizont stage induces concomitant host cell division and first appears as foci of blasting cells in the medulla of lymph

nodes draining the tick-bite or experimental inoculation of sporozoites (Campbell et al., 1995). The medulla contains in situ macrophages, and the blasting cells are positive for macrophage markers (unpublished observations). Both in vivo (Forsyth et al., 1996) and in vitro (Glass et al., 1989) schizont-infected cells express macrophage markers. Taken together, the evidence indicates that *T. annulata* sporozoites target and transform bovine macrophages.

Schizont-infected macrophages express mRNA for pro-inflammatory cytokines (interleukin-1 (IL-1), IL-6 and tumour necrosis factor (TNF α)) (Brown et al., 1995) and contain intracellular TNF α protein (Forsyth et al., 1999). In addition, uninfected macrophages from animals recovering from or immune to tropical theileriosis secrete TNF α , at least in vitro (Preston et al., 1993). This would suggest that pro-inflammatory cytokines are released systemically. If so, they probably play a major role in pathology and indeed

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many of the clinical signs of tropical theileriosis resemble those induced by experimental TNF α administration to cattle (Bielefeldt Ohmann et al., 1989).

Although infected cells do not release TNF α as a soluble product in vitro (Sager et al., 1997), infected macrophage lines clonally selected for pro-inflammatory cytokine mRNA expression, showed distinct differences in their ability to polyclonally activate T cells in vitro, implying that expression of mRNA for these cytokines has functional significance (Brown et al., 1995). Importantly, these differences were also shown to have in vivo consequences. Cloned lines selected for low cytokine production, unlike high cytokine-producing lines, induced no associated pathological reactions when inoculated into susceptible cattle (Graham et al., 2001). Thus, it seems likely that pro-inflammatory cytokines play an important role in the host response to *T. annulata* infection. However, little is known about the origin and kinetics of pro-inflammatory cytokines during an in vivo infection, partly because of lack of suitable assays for cattle.

Acute phase proteins (APP) play an important role in innate defence mechanisms (Eckersall, 2000). The principal pathway leading to their production involves initial release of pro-inflammatory cytokines by macrophages at the site of infection or inflammation. This results in a cascade of further release of cytokines by macrophages and other cells. The most important inducers of APP are cytokines of the IL-1, TNF and IL-6 families. If these pro-inflammatory cytokines spill into the circulation, they induce release of APP by the liver into the circulation (Baumann and Gauldie, 1994). The function of APP is not well established but they appear to be involved in controlling inflammation and contribute to innate host defences against a wide range of pathogens. Their circulating levels may also be related to the severity of the response to infection, and thus may provide valuable quantifiable biochemical indicators of the inflammatory response. Bacterial infections induce strong acute phase responses whereas viral infections generally lead to weak or non-detectable acute phase reactions. There are relatively few reports of APP induction by parasite infections. However, infections with *Plasmodium falciparum*, *Babesia canis*, *Leishmania infantum* and *Trypanosoma brucei* might be accompanied by increased levels of APP (Graninger et al., 1992; Shapiro and Black, 1992; Lobetti et al., 2000; Martinez-Subiela et al., 2002; Eckersall et al., 2001).

The changes in APP levels that occur in response to acute and chronic inflammation vary between different animal species. The profile of APP in cattle, particularly in relation to bacterial infection, has been reviewed recently by Eckersall (2000). Haptoglobin (Hp) is a major APP in cattle with concentrations increasing from undetectable to an average of 1,400 $\mu\text{g/ml}$ in serum during acute inflammation (Horadagoda et al., 1999). In contrast, serum amyloid A (SAA) is a moderate APP in cattle increasing from less than 8.8 $\mu\text{g/ml}$ in normal serum to around

75 $\mu\text{g/ml}$ in inflammatory conditions (Horadagoda et al., 1999), although it appears more quickly than Hp (Horadagoda et al., 1994). α_1 Acid glycoprotein (α_1 AGP) is also a moderate APP and its serum concentration only increases twofold to fourfold in response to acute inflammation i.e. from 200–400 $\mu\text{g/ml}$ in normal cattle to over 1,000 $\mu\text{g/ml}$ during an acute response (Horadagoda et al., 1999). If APP could be detected during an infection with *T. annulata*, it would lend weight to the hypothesis that pro-inflammatory cytokines are released systemically and play a key role in the observed pathogenesis in susceptible cattle. In this paper, we investigated whether APP were detectable following experimental *T. annulata* infection with sporozoites. Hp, SAA and α_1 AGP were all measured together with the clinical, haematological and parasitological responses, and potential correlations with severity of response were investigated.

2. Materials and methods

2.1. Experimental design

2.1.1. Animals

In each of two separate experiments, six Holstein calves (approximately 4–5 months old) were obtained locally and maintained off pasture.

2.1.2. Infection with *T. annulata* sporozoites

Cryopreserved *T. annulata* (Hisar) sporozoites (stabilate) were prepared as described previously (Brown, 1987) from ticks with an average of 145 infected acini per tick (Wilkie et al., 2002). In the first experiment, six calves were infected with 0.2 tick equivalent (t.e.) per animal. This dose was expected to be sub-lethal but still result in clinical signs and allow a kinetic analysis of APP production to be conducted. However, it resulted in higher clinical severity than had been observed with other stabilate batches, and five of six animals were euthanased before day 20. A retrospective analysis of several experiments has in fact shown that the particular batch (Hisar 52) of *T. annulata* used in this study produced more severe disease responses than other apparently similar batches of *T. annulata* (Hisar) (Wilkie et al., 2002). Thus a lower dose (0.1 t.e.) of the same batch of stabilate was used in the second experiment. The reduced effect of this dose was confirmed by finding that none of the calves showed signs of failing general health before day 22; all were euthanased between days 22 and 28 (see Tables 1 and 2). All calves were maintained throughout on commercial food and hay and water ad libitum and their condition assessed as described below.

2.1.3. Clinical, haematological and parasitological monitoring of cattle post infection

Rectal temperatures were measured daily. Haematological parameters, white blood cell count (WBC), total

Table 1
Clinical, haematological and parasitological responses of two groups of six calves infected with *Theileria annulata* (Hisar)

| Dose of sporozoites used for infection (t.e.) | Days to first schizont | Days to $T > 39.5$ °C | Peak T (°C) | Maximum reduction in WBC (%) | Maximum reduction in PCV (%) | Peak parasitaemia (%) | Parasitaemia at day 14 (%) | Days to euthanasia ^a | Clinical outcome ^b |
|--|------------------------|-----------------------|----------------|------------------------------|------------------------------|-----------------------|----------------------------|---------------------------------|-------------------------------|
| Experiment 1: infective dose = 0.2 t.e. Mean \pm SD | 5 \pm 0 | 7.2 \pm 1.3 | 41.7 \pm 0.3 | 65.5 \pm 8.4 | 44 \pm 8 | 45.0 \pm 9 | 37.8 \pm 13.9 | 16 \pm 3.5 | 5/6 |
| Experiment 2: infective dose = 0.1 t.e. Mean \pm SD | 8.5 \pm 1.6 | 8.5 \pm 0.5 | 40.9 \pm 0.4 | 66.3 \pm 7.4 | 50.5 \pm 13.8 | 24.9 \pm 14.5 | 11.1 \pm 5.7 | 23.3 \pm 2.3 | 0/6 |
| Range of response ^c | 5–9 | 5–9 | 40.3–41.8 | 57–80 | 31–73 | 9–55.2 | 3.5–55.2 | 14–28 | – |

t.e., Tick equivalent; T , host temperature; WBC, white blood cells; PCV, packed cell volume.

^a Due to failing health (Section 2).

^b Number of calves that were euthanased prior to day 20/total number of calves in the group (see Section 2).

^c The highest and lowest values measured in all animals are shown.

erythrocyte counts (TEC) and packed cell volume (PCV), were measured at day 0, 5, 9, 12, 14, 16, 19 and 21, 23, 26 and 28. WBC and TEC were measured using a Coulter Counter ZM (Beckman Coulter Ltd) and haematocrit or PCV was measured using a Hawksley Microhaematocrit and reader (Hawksley and Sons Ltd, Lancing, UK). Parasite development was monitored by the appearance of schizont-infected cells in smears of biopsy material from lymph nodes draining the inoculation site and assessed by microscopic analysis. From day 9, thin blood smears were prepared and the percentage of piroplasm-infected erythrocytes (parasitaemia) recorded. Smears were stained with Giemsa's stain. Any calf showing failing general health, cachexia, and a temperature >41 °C for >3 days was deemed to be undergoing lethal infection and euthanased. The time to euthanasia was used to indicate 'clinical outcome' by classifying the calves according to whether they had been euthanased prior to or after day 20 of infection.

2.2. Acute phase protein (APP) measurements

2.2.1. Haptoglobin (Hp)

The concentration of Hp in serum was determined by the haemoglobin binding method that is the basis of the Phase Haptoglobin kit (Tridelta Development Ltd, Ireland) described by Eckersall et al. (1999) using micro-titre plates and reader according to the manufacturer's instructions.

2.2.2. α_1 Acid glycoprotein

α_1 AGP was measured by a radial immunodiffusion assay using kits (supplied by Saikin Kagaku Institute, Sendai, Japan) that were specific for bovine α_1 AGP and following the manufacturer's instructions. The plates were incubated for 48 h at 37 °C in a humidified atmosphere. Each set of plates contained standard and test samples.

2.2.3. Serum amyloid A

SAA was measured by sandwich ELISA using Phase SAA kits (supplied by Tridelta, Ireland) according to the manufacturer's instructions. Reference serum provided was diluted to create a standard curve. Test sera were diluted at least 1/500 to fit the standard curve and incubated together with biotinylated anti-SAA on anti-SAA antibody-coated plates. Incubation was followed by streptavidin-peroxidase and then tetramethylbenzidine substrate (TMB) for colour development, and plates were read at 450 nm.

2.3. Serum samples

These were collected on the same days as the haematological parameters were measured except that in experiment 1, serum was collected on day 10 rather than day 9. Samples were allowed to clot, serum collected and stored at -20 °C until use.

Table 2
Haptoglobin ($\mu\text{g/ml}$) response to *Theileria annulata* infection

| Days after infection | Experiment 1 (0.2 t.e./animal) | | | | | | Experiment 2 (0.1 t.e./animal) | | | | | |
|----------------------|--------------------------------|----------------|----------------|-----------------|----------------|----------------|--------------------------------|-----|-----|----|-----|-----|
| | Animal number | | | | | | | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
| 0 | – | – | – | – | – | – | – | – | – | – | – | – |
| 7 | – | – | – | – | – | – | – | – | – | – | – | – |
| 9 | | | | | | | | 30 | – | – | 36 | – |
| 10 | – | – | 356 | – | 43 | 86 | | | | | | |
| 12 | – | – | 45 | – | 72 | – | – | 168 | 159 | – | 167 | 143 |
| 14 | – ^a | – ^a | 38 | 81 ^a | 61 | – ^a | – | – | 67 | – | – | – |
| 16 | | | – ^a | | | | | | | | | |
| 19 | | | | | | | | | | | | |
| 21 | | | | | | | | | | | | |
| 22 | | | | | | | | a | | a | a | |
| 23 | | | | | – ^a | | | | a | | | a |
| 28 | | | | | | | a | | | | | |

–, not detectable.

^a Euthanased.

2.4. Statistics

The positions and heights of the peak responses to infection with *T. annulata* for α_1 AGP and SAA measurements were estimated by fitting regression curves to the data. α_1 AGP data were log transformed to stabilise the variance and exponential curves were fitted. Fourier curves were fitted to the SAA data. These models provided a good fit to the experimental data for the APP profiles. The data of both experiments were pooled and the relationship between the maximum levels of α_1 AGP and SAA (Fig. 2) and the association of these APP parameters with the clinical, haematological and parasitological parameters (Table 3) were assessed by regression analysis. The association of the maximum levels of APPs with clinical outcome was assessed by Student's *t*-test. The Genstat statistical package

(Genstat 5 Release 4.1, 4th edition, Lawes Agricultural Trust, Rothamsted) was used for all analyses.

3. Results

3.1. Clinical, haematological and parasitological responses

The clinical, haematological and parasitological responses following experimental infection with *T. annulata* are shown in Table 1. The lower dose of stabilate resulted in less clinical reactions. The days after infection when the individual animals in each experiment had to be euthanased are shown in Table 2. Although considerable variation in response between animals was observed, all animals became infected and experienced significant increases in

Table 3

Association between APP levels and clinical outcome, and correlations between APP and clinical, haematological and parasitological responses

| Type of APP | Clinical outcome ^a | | Correlation with APP (r^b) | | | | |
|-----------------------------|-------------------------------|----------------------------|--------------------------------|----------------------------------|------------------------------|-----------------------|--------------------|
| | <20 days | >20 days | Days to first schizont | Days to $T > 39.5^\circ\text{C}$ | Maximum reduction in WBC (%) | Peak parasitaemia (%) | Days to euthanasia |
| | Mean \pm SD | | | | | | |
| SAA ^c | 531 ^d \pm 149 | 421 ^d \pm 104 | –0.60* | –0.58* | –0.42 | 0.40 | –0.49 |
| α_1 AGP ^c | 1,020 ^f \pm 284 | 708 ^g \pm 158 | –0.58* | –0.78** | –0.60* | 0.59* | –0.73** |

T, temperature; WBC, white blood cell.

* $P < 0.05$; ** $P < 0.01$: regression analysis between SAA or α_1 AGP levels and responses pooled for both experiments.

^a Animals were classified according to whether they were euthanased prior to (<) or after (>) 20 days (see Section 2).

^b Correlation co-efficient.

^c Maximum level of serum amyloid A (SAA) ($\mu\text{g/ml}$).

^d Values not significantly different from each other.

^e Maximum level of α_1 acid glycoprotein (α_1 AGP) ($\mu\text{g/ml}$).

^{f,g} Values significantly different from each other: $P < 0.05$ (Student's *t*-test).

temperature and decreases in WBC and PCV. Schizont-infected cells were detected in lymph nodes of all animals from day 5 onwards, and piroplasms first appeared from days 9 or 10.

3.2. Acute phase protein responses

The serum concentration of Hp following infection was undetectable (below 40 $\mu\text{g/ml}$, the limit of detection) in four animals (#1, 2, 7 and 10) at all time points (Table 2). However, the other eight animals did show increased levels of Hp from day 9 following infection (Table 2) although the levels were generally low and only detectable at a few time points. Only one animal (#3) displayed a level of Hp (356 $\mu\text{g/ml}$) seen in acute responses in other situations (Horadagoda et al., 1999). The amount of Hp produced did not correlate with the infection dose or with any of the clinical signs in individual animals.

However, the SAA levels became elevated in all animals (Fig. 1A) increasing significantly from day 7 onwards compared to pre-infection levels (day 0) ($P < 0.005$). The rise in SAA occurred around the time that schizonts appeared in the draining lymph nodes and the temperature began to rise (Table 1 and Fig. 1A). All animals showed a peak between days 9 and 14 ($P < 0.001$) (Fig. 1A). Fourier curves were fitted to the data (see Section 2) in order to carry out the following statistical analysis. The mean profiles showed a similar overall pattern for both groups. There was no significant difference between the two groups of calves in the pre-infection levels, timing of peak or height of peak response of SAA. The mean pre-infection level for the group that received 0.2 t.e. *T. annulata* sporozoites/animal was $3.2 \pm 0.5 \mu\text{g/ml}$ SAA and for the group that received 0.1 t.e./ml, the mean pre-infection level was $7.2 \pm 0.6 \mu\text{g/ml}$ SAA. The estimated position and height of the peak were 11.9 ± 1.5 days and $46.2 \pm 10.1 \mu\text{g/ml}$ SAA,

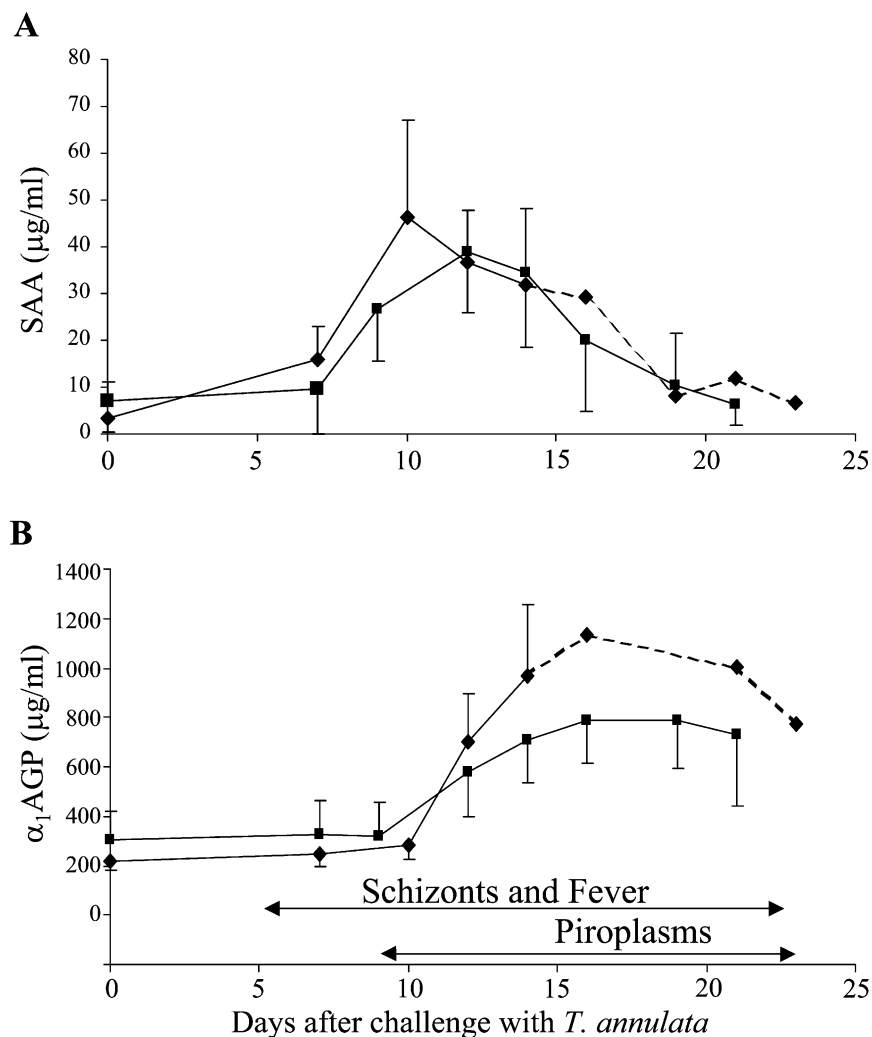


Fig. 1. The kinetics of acute phase protein production following experimental infection with *Theileria annulata*. (A) Serum amyloid A (SAA); (B) α_1 acid glycoprotein (α_1 AGP). Results are shown as mean \pm SD for experiment 1 that received 0.2 t.e. of *T. annulata* sporozoites (◆), and mean \pm SD for experiment 2 that received 0.1 t.e. of *T. annulata* sporozoites (■). Note that only two animals survived beyond day 14 in experiment 1 and the graphs are shown with a dashed line from day 14. The occurrence of schizonts, fever and piroplasms are indicated.

respectively, for the group which received the high dose of sporozoites and 12.4 ± 1.4 days and 42.2 ± 9.4 $\mu\text{g/ml}$ SAA, respectively, for the group which received the low dose.

α_1 AGP levels also became elevated in all animals (Fig. 1B) but in contrast to SAA, α_1 AGP levels remained constant for longer and then rose from day 10 onwards ($P < 0.02$). The rise in α_1 AGP coincided with the appearance of piroplasms in red blood cells (Fig. 1). Critical exponential curves were fitted to the data (see Section 2) in order to carry out the following statistical analysis. As with SAA, the mean estimated baseline levels of α_1 AGP for each group were not significantly different from each other (181 ± 13 $\mu\text{g/ml}$ α_1 AGP for the 0.2 t.e. group; 255 ± 39 $\mu\text{g/ml}$ α_1 AGP for the 0.1 t.e. group). Only one animal infected with the higher dose of sporozoites survived at the end of the experiment (Table 2). The α_1 AGP levels for the other five animals were still rising on their days of death (day 14–day 16). Thus although it was possible to estimate a profile for the group, there was no good estimate of the standard error for the position and height of the mean peak for this group. The mean estimated position and height of the α_1 AGP response was 19.6 days and 1,809 $\mu\text{g/ml}$ α_1 AGP for the 0.2 t.e. group and 18.6 ± 0.5 days and 848 ± 94 $\mu\text{g/ml}$ α_1 AGP and for the 0.1 t.e. group.

3.3. Relationship between APP and clinical, haematological and parasitological responses

The calves showed a wide range of maximum levels of each APP, varying from 29.3 $\mu\text{g/ml}$ to 70.9 $\mu\text{g/ml}$ for SAA and 565 $\mu\text{g/ml}$ to 1,370 $\mu\text{g/ml}$ for α_1 AGP (Fig. 2). There was a significant degree of correlation between the maximum level of SAA produced and the maximum level of α_1 AGP produced by each animal (Fig. 2). In addition,

the amount of α_1 AGP produced between days 9 and 14 correlated with the amount of SAA produced between days 0 and 14 ($r = 0.74$; $P < 0.01$ for all animals).

In terms of clinical outcome, the mean maximum levels of α_1 AGP were significantly different between animals that were euthanased before and after day 20, and correlated with the number of days to euthanasia, whereas the mean maximum levels of SAA were not significantly associated with clinical outcome or days to euthanasia (Table 3). However, both SAA and α_1 AGP correlated with some of the clinical, haematological and parasitological responses (Table 3). The most consistent correlations seen for both APP were the first day that schizonts were detected and the first day that fever was greater than 39.5 $^{\circ}\text{C}$. In addition, α_1 AGP correlated with leucopaenia and parasitaemia. No significant correlation was seen with either APP and peak temperature or maximum reduction in PCV or parasitaemia at day 14 (results not shown).

4. Discussion

In this paper, we have shown that an APP response is induced in cattle following infection with the protozoan parasite, *T. annulata*. There are no previous reports on APP responses following parasite infection in cattle and relatively few reports in any other mammalian species. The exact mechanisms whereby *T. annulata* causes clinical responses and pathogenesis in naive susceptible cattle remain unclear (Preston et al., 1999; Glass, 2001; Preston, 2001). However, the induction of APP supports the hypothesis that pro-inflammatory cytokines play an important role.

Assays for the individual pro-inflammatory cytokines, $\text{TNF}\alpha$, IL-1 and IL-6 are not commercially available for

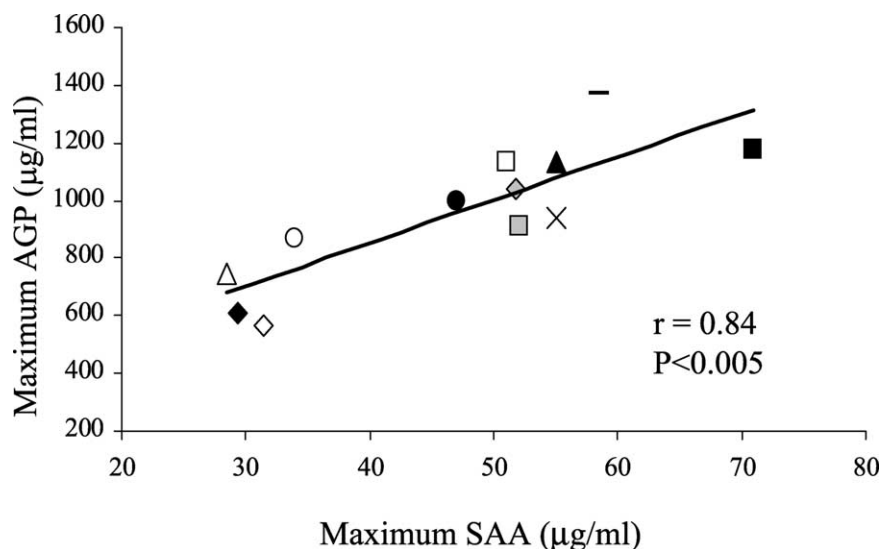


Fig. 2. Correlations between the production of serum amyloid A (SAA) and α_1 acid glycoprotein (α_1 AGP) following experimental infection with *Theileria annulata*. Results are shown as the maximum levels of SAA and α_1 AGP measured. Animals that received 0.2 t.e. of *T. annulata* sporozoites: 1 (◆); 2 (■); 3 (▲); 4 (×); 5 (●); 6 (–). Animals that received 0.1 t.e. of *T. annulata* sporozoites: 7 (◇); 8 (□); 9 (△); 10 (◇); 11 (○); 12 (□). Animal numbers as in Table 2.

cattle. In addition, their appearance in the peripheral circulation is often transitory (e.g. see Horadagoda et al., 1994; Werling et al., 1996). However, they induce APP, which are stable proteins and have relatively long half-lives (Baumann and Gauldie, 1994). In cattle, the APP considered to be most sensitive to inflammation are Hp and SAA whose concentrations increase particularly in response to acute inflammatory conditions, and α_1 AGP which is also increased in chronic inflammatory conditions (Horadagoda et al., 1999). The measurement of these three bovine APP has become relatively simple and straightforward with commercially available kits. The evaluation of APP following experimental infection could provide a valuable, quantitative assessment of the total response to stimulation by the pro-inflammatory pathway (Eckersall, 2000). Thus as a first step in studying the production of circulating pro-inflammatory cytokines, we investigated the presence and kinetics of the bovine APP, Hp, SAA and α_1 AGP.

A rise in the APP, SAA and α_1 AGP was observed in all cattle following experimental infection with the protozoan parasite, *T. annulata*. The total production of these two APP were highly correlated with each other in all infected cattle. However, Hp was only detectable in a few animals and generally at low levels. The pre-infection levels of these APP were within the normal ranges (Horadagoda et al., 1999).

The particular stabilate used in the experiments described here, resulted in exceptionally severe responses at the relatively low doses used, compared to other studies. A retrospective analysis of the infectivity of this stabilate showed that it also resulted in more severe disease in cattle in earlier studies (Wilkie et al., 2002), suggesting that the stabilate was either more virulent or was obtained from ticks with a higher infection level than other similar stabilates. This may have also accounted for the differences in outcome resulting from the two different doses. However, as the two experiments were conducted at different times and the number of animals in each group was limited to six per group, it is not possible to conclude definitively that the apparent differences in APP responses were related to dose of parasite. Further experiments with a wider range of doses and animals would provide more definitive evidence.

The rise in SAA was not seen until around day 7 which coincided with temperature rise and appearance of schizonts. The peak level of SAA was similar to that induced by infusion with lipopolysaccharide (Werling et al., 1996) or by experimental infection with bacteria (*Pasteurella haemolytica*, now renamed *Pasteurella manheimia*) (Horadagoda et al., 1994) or viruses (bovine respiratory syncytial virus (BRSV)), (Heegaard et al., 2000).

The α_1 AGP response was slower and more prolonged than the SAA response. As this APP is associated with chronic inflammatory conditions (Horadagoda et al., 1999), its kinetics may be an indication of prolonged production of pro-inflammatory cytokines in response to *T. annulata* infection. There are very few reports of α_1 AGP presence

during inflammatory conditions in cattle, particularly, its induction following experimental infection (Hirvonen et al., 1996; Horadagoda et al., 1999). One of the functions ascribed to α_1 AGP is immunosuppression (Logdberg and Wester, 2000) and its presence in cattle has been correlated with suppression of lymphocyte blastogenesis (Motoi et al., 1992). In susceptible cattle undergoing a lethal infection with *T. annulata* infection, the presence of the parasite has been associated with a non-cytolytic CD2⁻CD8⁺T cell population (Nichani et al., 1999a) as well as developmentally immature B cells (Campbell et al., 1997). In this present study, α_1 AGP was significantly associated with the leucopaenia that followed *T. annulata* infection. The prolonged presence of α_1 AGP following *T. annulata* infection may be one explanation why immunity did not develop in these cattle.

The molecular basis for the difference in profile between SAA and α_1 AGP is not clear, although it could be due to variations in the balance of activating cytokines, growth factors and corticosteroids (Suffredini et al., 1999) during disease progression. However, the finding that there was a significant correlation between the maximum levels of SAA and AGP suggests that similar control mechanisms are involved. If the production and secretion of these APP were under different control, it is unlikely that there would be such a significant correlation. Further investigation of the experimental infection described here could provide valuable insights into the pathophysiological mechanism for the differing APP kinetics.

In contrast to the induction of SAA and α_1 AGP, the presence of Hp was only seen sporadically and generally at a relatively low level compared to that induced by bacteria or viruses. This suggests that there may be differences in the way *T. annulata* triggers APP in cattle, compared to APP stimuli such as bacteria (e.g. *P. manheimia* (Horadagoda et al., 1994), *Salmonella* spp (Deignan et al., 2000), and *Escherichia coli* (Salonen et al., 1996)) or viruses (BRSV, (Heegaard et al., 2000)). The related protozoan parasite, *P. falciparum*, induces an APP response in humans with increased levels of C-reactive protein and α_1 AGP among other APP, whereas, in contrast, Hp is significantly depleted (Graninger et al., 1992). This may be because of haemolysis. Hp binds free haemoglobin and the complex is cleared by the reticulo-endothelial system resulting in depletion of serum Hp. However, in response to experimental infection in mice with the protozoan *T. brucei*, Hp is increased even though simultaneous anaemia occurs (Eckersall et al., 2001). Thus it is possible that the apparent low Hp response to *T. annulata* infection may be because it is cleared faster than synthesised. Anaemia does occur in the later stages of *T. annulata* infection apparently in association with developing parasitaemia (Preston et al., 1992; Preston, 2001). Investigation of the turnover rate of the APP in vivo is warranted to elucidate the mechanisms that lead to elevated levels of SAA and α_1 AGP but not Hp.

The acute phase response is regulated by a complex network of interactions (Baumann and Gauldie, 1994; Suffredini et al., 1999). It is primarily stimulated by the up-regulation of the pro-inflammatory cytokines, IL-1, IL-6 and TNF α in response to infection. These cytokines are primarily produced locally by macrophages in response to infection. If a strong stimulus is present, these cytokines are amplified through both feedback action on macrophages and activation of other cells and these cytokines enter the systemic system. This cascade leads to the synthesis of APP among other sequelae. It has been reported that the cytokine-induced activation of SAA, α_1 AGP and Hp genes in the liver may be different in that IL-6, the principal regulator of most APP, induces the production of Hp, whereas IL-1 and TNF α induce expression of SAA and α_1 AGP and Hp, depending on the mammalian species (Baumann and Gauldie, 1994). All three cytokines have been shown to induce an acute phase response in cattle (Nakajima et al., 1993; Godson et al., 1995; Watanabe et al., 2000) but the precise pathways may be different to other species and there are some apparently conflicting reports. Hp was up regulated in vitro by IL-6 or TNF α , but not IL-1, in one situation (Nakagawa-Tosa et al., 1995) whereas in another situation, both IL-6 and TNF α were required (Alsemgeest et al., 1996). However, in vivo administration of recombinant IL-1 (Godson et al., 1995), IL-6 (Nakajima et al., 1993) or TNF α (Watanabe et al., 2000) led to the production of Hp. This may reflect the fact that these three cytokines can also influence each other and act in an autocrine fashion.

All three pro-inflammatory cytokines (IL-1, IL-6 and TNF α) are transcriptionally expressed at high levels in *T. annulata*-infected cells. However, their individual roles in *T. annulata*-induced pathology in naive cattle is unknown, although there is indirect evidence to suggest that this pathology is pro-inflammatory cytokine induced. The clinical signs of anaemia, cachexia, leucopaenia and fever (Uilenberg, 1981) are similar to those induced by recombinant bovine TNF α (Bielefeldt Ohmann et al., 1989) and high IL-1 and TNF α expressing *T. annulata*-infected macrophage clones-induced pathological responses in cattle unlike low expressing clones (Graham et al., 2001). The elevation in APP following *T. annulata* infection indicates very strongly that pro-inflammatory cytokines are released into the peripheral circulation. The precise identity of these pro-inflammatory cytokines is yet to be determined. The poor production of Hp compared to SAA and α_1 AGP following *T. annulata* infection suggests that IL-6 may not play an important role in *T. annulata*-induced pathology.

The origin of the pro-inflammatory cytokines is, however, unclear. *Theileria annulata*-infected macrophages proliferate rapidly in draining lymph nodes (Campbell et al., 1995) but do not appear in efferent lymph and thence, the peripheral circulation until day 6 (Nichani et al., 1999b). The rise in the first APP seen, SAA, coincides with the appearance of schizonts in the peripheral circulation and

with the development of fever. This would suggest that although infected macrophages up regulate mRNA for IL-1, TNF α and IL-6, these cytokines are at first only generated locally if at all. However, in vitro neither IL-1 (unpublished observation) nor TNF α (Sager et al., 1997) are secreted from infected cells. Possibly in vivo interaction of infected cells with other cells of the immune system may lead to further and more extensive release of pro-inflammatory cytokines. Activated lymphocytes as well as macrophages are an important source of pro-inflammatory cytokines (Abbas et al., 2000). Non-infected macrophages in animals recovering from *T. annulata* infection do secrete TNF α , albeit at a low level (Preston et al., 1993). In addition, infected cells polyclonally activate and induce proliferation of autologous naive T cells, both in vitro and in vivo (Glass and Spooner, 1990; Brown et al., 1995; Campbell et al., 1995). Indeed, the levels of IL-1 and IL-6 cytokine mRNA in isolated macrophage-infected clones correlates with the degree of T cell activation and proliferation (Brown et al., 1995).

Both SAA and α_1 AGP were associated with disease severity, although only α_1 AGP levels were related to clinical outcome. The most consistent correlations between APP production and the clinical, haematological and parasitological parameters, were with appearance of schizonts and the time to temperature rise above 39.5 °C. However, only α_1 AGP was associated with maximum reduction in WBCs, parasitaemia and time to euthanasia. Since APP reflect disease severity, quantitation of APP responses may provide an additional objective score for assessing the efficacy of vaccine candidates or determining disease susceptible or resistant cattle breeds.

In conclusion, this paper provides evidence that the severe disease in susceptible European breeds of cattle following *T. annulata* infection is associated with pro-inflammatory cytokine production. The presence of circulating APP is indicative of macrophage activation and the production of high quantities of these cytokines. Further studies are now required to determine which pro-inflammatory cytokines are involved in pathogenesis, as well as their origin and kinetics.

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