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Major acute phase response of haptoglobin and serum amyloid-P following experimental infection of mice with *Trypanosoma brucei brucei*

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Abstract

Investigation of the pathophysiological role of the systemic cytokines, including interleukin-1, interleukin-6 and tumour necrosis factor α, in the host response to infection with African trypanosomes is hampered by the low and transient concentrations of these cytokines in plasma. One of the actions of these cytokines is the stimulation of hepatocyte production of acute phase proteins such as serum amyloid-P and haptoglobin. These acute phase proteins are more stable in the circulation than the cytokines and can be measured as a means of assessing the systemic cytokine response in the trypanosome-infected host. The plasma concentrations of serum amyloid-P and haptoglobin were measured in an experimental mouse model of *Trypanosoma brucei brucei* infection. Both serum amyloid-P and haptoglobin, increased markedly following infection. Peak concentrations of serum amyloid-P at 125 μg/ml and haptoglobin at 2 g/l were attained 10 to 12 days after infection. Thereafter, serum amyloid-P concentration decreased to approximately 40 μg/ml while the haptoglobin concentration remained elevated at approximately 1.5 g/l. The reactions of the serum amyloid-P and haptoglobin following experimental *Trypanosoma brucei brucei* infection in mice demonstrate that a major acute phase response has occurred indicating that the systemic cytokine network has been activated. Further studies are required to identify whether the response is stimulated by the parasite or indirectly by tissue damage. © 1997 Elsevier Science Ireland Ltd.

Keywords: *Trypanosoma brucei brucei*; Acute phase protein; Trypanosomiasis; Haptoglobin; Serum amyloid-P; Mice

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1. Introduction

Human African trypanosomiasis leads to a severe inflammatory response, extensive tissue damage and ultimately death if untreated [1]. The severity of pathology is related, at least in part, to the tissue invasive nature of the species that infect humans, namely *Trypanosoma brucei gambiense* and *Trypanosoma brucei rhodesiense*. The effectiveness of the specific immune response to trypanosomes is limited by the ability of the parasite to undergo antigenic variation leading to persistence of the infection [2].

In response to bacterial and certain parasitic infections, the host mounts an acute phase response [3,4], which leads to many systemic effects, such as fever, cachexia and stimulation of hepatocyte-derived acute phase proteins (APP) [5,6]. This response is mediated by the release of the cytokines such as interleukin-1 (IL-1), interleukin-2 (IL-6), interleukin-11 (IL-11), tumour necrosis factor (TNF-α) and oncostatin-M [7–9]. The cytokine response can be stimulated either directly by pathogen products, such as Gram-negative bacterial endotoxin [10] or indirectly by tissue damage associated with the invading organism [11].

Experimental approaches to study the role of cytokines in the acute phase response in trypanosomiasis are hampered by the instability and transient presence of these mediators in the circulation. A more effective, although indirect, means of demonstrating that systemic cytokines have been activated during disease is the quantification of their combined activity by measuring the plasma concentration of APP. These proteins are recognised markers of inflammation [12,13] with their concentration in plasma increasing between 2 and 1000 times for several days following infection [4].

Experimental infection with *T. b. brucei* stimulates the production of APP in dogs with increased production of C-reactive protein (CRP) and haptoglobin (Hp) [14], while in rabbits an increase in CRP has also been observed [15]. In mice, serum amyloid-P component (SAP) and Hp are among the most useful acute phase proteins to measure [16,17]. While the serum profile of SAP has not been studied during experimental trypanosomiasis in mice, the Hp concentration in plasma increases following *T. b. brucei* infection [18], but the increase was not quantified or monitored. Furthermore, in experimental *T. b. brucei* infection of mice, induction of mRNA for cytokines capable of stimulating production of the APP has been identified using the polymerase chain reaction [19]. In man, CRP and α-2-macroglobulin, both of which are human APP, are raised during clinical infection with *Trypanosoma cruzi* [20].

In the present study, SAP and Hp concentrations in plasma were measured to evaluate the status of these proteins as markers of host response to the parasite in a well established animal model system, namely the mouse infected with *T. b. brucei*. *Trypanosoma b. brucei* is a tissue invasive parasite and the mouse develops extensive tissue damage similar to that which occurs in man [21].

2. Materials and methods

2.1. Animals

Female inbred NIH mice, of 28–35 g body weight (bwt) were used in groups of six.

2.2. Trypanosomes

A cloned stabilate, *T. b. brucei* GVR 35, was used to infect the mice as described in [21]; this results in a chronic infection that lasts several weeks if left untreated.

2.3. Infection and treatment

Mice were infected by intra-peritoneal injection (i.p.) with approx. 1×10^4 trypanosomes in phosphate buffered saline (PBS), pH 8.0, containing 1.5% weight per volume (w/v) glucose. Uninfected animals were used as controls.

Blood samples were taken, by tail snip, at 2–3-day intervals and a wet smear was prepared for estimation of parasitaemia. Parasitaemia was scored using the method of Herbert and Lumsden [22]. Blood samples were taken in heparinised

microcapillary tubes and the packed cell volume (PCV) determined after centrifugation. Plasma was expelled into eppendorf tubes for storage at −20°C until the concentration of SAP and Hp could be determined. The mice were euthanised at the end of the experiment after 27 days of infection.

2.4. SAP assay

The plasma concentration of SAP was estimated by ELISA (enzyme-linked immunosorbent assay). Standards were prepared by serially diluting mouse SAP (Calbiochem Novobiochem Corporation, Beeston Nottingham, UK) in 1% bovine serum albumin (BSA) in 20 mM PBS (phosphate buffered saline) (pH 7.4) buffer without Tween–20, to obtain a concentration range of 2.5–160 μg/ml. Plasma samples and standards were diluted 1:100 in coating bicarbonate buffer (pH 9.6) and 100 μl dispensed into the wells of a microtitre plate (Greiner Ltd, Dursley, Gloucestershire, UK) and incubated overnight at room temperature. The plate was washed three times with PBS plus 0.05% Tween–20 (PBST) buffer and blocked with 10% instant dried skimmed milk powder (Marvel®) in PBS buffer (pH 7.4). One hundred microlitres of rabbit antiserum against murine SAP-component (Calbiochem Novobiochem Corporation, Nottingham, UK) at 1:4000 dilution in PBST (phosphate buffered saline with Tween-20) buffer was placed in each well. The plate was incubated for 90 min at room temperature and then washed three times with PBST. One hundred microlitres of secondary antibody (Horseradish peroxidase-conjugated donkey anti-rabbit IgG, Scottish Antibody Production Unit, Law Hospital. Lanarkshire, UK) at 1:2500 dilution in assay buffer was placed in each well and the plate incubated for 90 min at room temperature before washing three times with PBST. The substrate solution (25 ml of the substrate buffer, 10 mM sodium acetate, pH 5.5, 100 μl of 1% H_2O_2 and 400 μl of 0.6% 3,3′,5,5′-tetramethylbenzidine (TMB) in dimethyl sulphoxide (DMSO)) was added (150 μl) to each well of the microtitre plate. The reaction was terminated by the addition of 2 M H_2SO_4 (50 μl) per well and absorbance was read at 450 nm.

The intra-assay coefficient of variation (CV) was calculated for 20 duplicate sample wells. The inter-assay CV of control plasma samples was determined over 18 different SAP assays. The limit of detection was calculated as the zero standard mean ± 2 S.D. [23].

2.5. Haptoglobin assay

Hp levels were estimated using the method by Makimura and Suzuki [24] with modifications by Conner [25]. Standards of purified bovine Hp were prepared from a stock solution of Hp and were diluted in normal saline to cover a concentration range of 0.03–2.14 g/l.

2.6. Statistical analysis

Data are presented as means ± S.E.M. (standard error of mean) and were analysed by repeated measures two-way analysis of variance (ANOVA) using the GLM program on the SAS statistical package (SAS Institute, Cary, North Carolina, USA). Differences were considered significant at $P < 0.05$.

3. Results

3.1. Parasitological findings

Parasites were detected 4 days after infection, at a peak with a median of $10^{8.6}$ (range $10^{8.4}$–$10^{8.7}$) trypanosomes per ml, falling to a level with a median of 10^7 (range $10^{6.3}$–$10^{7.5}$) trypanosomes per ml on day 11, but then remained elevated in the range of $10^{8.5}$–10^9 trypanosomes per ml (Fig. 1) after infection until the end of the experiment on day 27. No parasites were found in the blood of uninfected mice

Uninfected animals maintained a mean PCV of 55–58% throughout the experimental period. Infected animals maintained mean PCV values at pre-infection levels until 10 days after infection. This was followed by a significant decrease ($P <$ 0.05) by day 13 to 47 ± 0.4% a decline that cont-

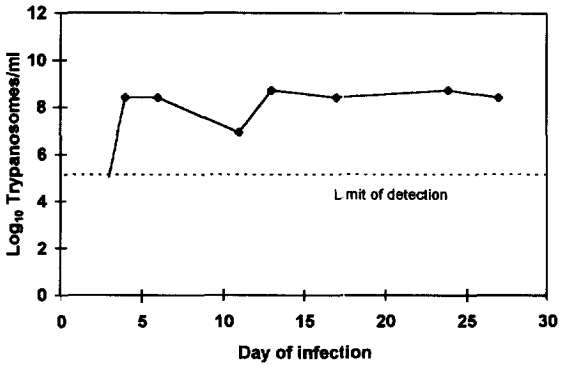

Fig. 1. Parasitaemia (median) in the plasma of mice infected with *Trypanosoma brucei brucei*. Uninfected mice had zero parasitaemia and the dotted line indicates the limit of sensitivity of the assay for parasitaemia ($10^{5.2}$ trypanosomes/ml).

inued to the lowest mean value of 43.5 ± 0.9% by day 27 (Fig. 2).

3.2. Serum amyloid-P

The SAP assay gave an intra-assay CV (coeffecient of variance) of 4.6% and an inter-assay CV of 12% at 30 μg/ml and 11% at 80 μg/ml of SAP. The limit of detection was 5 μg/ml.

The mean SAP concentration changes during infection are shown in Fig. 3. The SAP concentration in the uninfected animals ranged between 25 and 35 μg/ml and did not change during the course of the study. In the infected mice, the mean SAP concentration was significantly greater

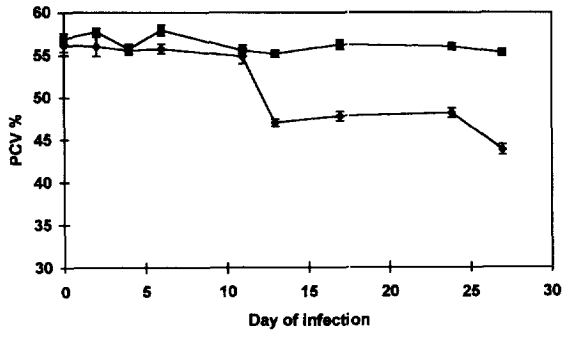

Fig. 2. Packed cell volume (PCV) (mean ± S.E.M.) in the plasma of mice infected with *Trypanosoma brucei brucei* (diamonds) and in control, uninfected mice (squares).

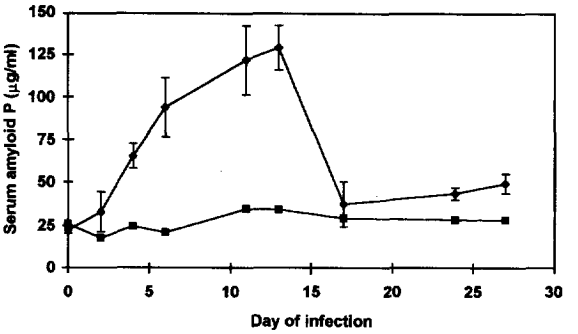

Fig. 3. SAP concentration (mean ± S.E.M.) in the plasma of mice infected with *Trypanosoma brucei brucei* (diamonds) and in control, uninfected mice (squares).

than in the uninfected controls ($P < 0.05$) 2 days after infection and reached a peak concentration of 129.3 ± 13.2 μg/ml at day 13. By day 17 the mean concentration had decreased to 38 μg/ml and remained around this level until the termination of the experiment.

3.3. Haptoglobin

The mean Hp concentrations in infected mice are shown in Fig. 4. Haptoglobin was not detectable in plasma samples collected on the day of infection from infected mice or from the uninfected control mice at any stage throughout the experiment period. Following infection, serum Hp was detectable 2 days after infection and the mean concentration rose to reach a peak level of 2.02 ± 0.19 g/l by day 10. This was followed by a decline to about 1.42 g/l 13 days after infection, remaining at this level until the end of the experiment.

4. Discussion

This study has quantified an acute phase increase in the plasma concentrations of SAP and Hp during *T. b. brucei* infection in mice. In previous studies on the APP response during trypanosome infections, SAP has been shown to increase during infection of man with *T. cruzi* [26], while elevated Hp has been observed in mice [18] and dogs [14] infected with *T. b. brucei*.

Following infection, the initial responses of Hp

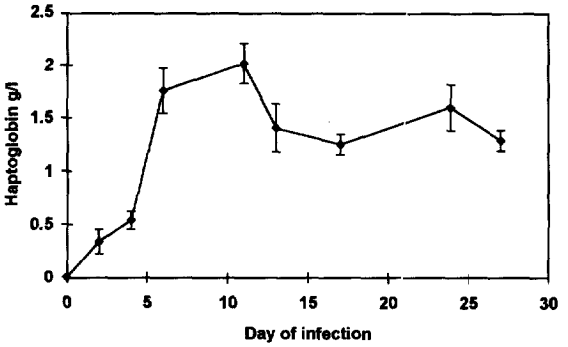

Fig. 4. Hp concentration (mean ± S.E.M.) in the plasma of mice infected with *Trypanosoma brucei brucei* (diamonds). The control, uninfected mice had Hp concentrations below the limit of sensitivity of the assay (0.03 g/l).

and SAP were similar with increases detectable within 2 days of infection, which was 2 days before parasites were observed in blood and several days before the PCV decreased. The concentrations then peaked, on day 11 for Hp and day 13 for SAP, but indicated that, as the production of APPs by the liver is stimulated by systemic cytokines and especially IL-6 [5,6], the infection has caused the production of this cytokine and probably also that of TNF-α and IL-1 three to four days after infection. However, the kinetics of these two APP differed thereafter, as the Hp concentration remained at a high level while that of SAP decreased near to the level observed in uninfected animals despite the persistence of parasitaemia. The reaction of Hp resembled the response of this protein to canine *T. b. brucei* infection where it was found to remain elevated throughout infection although CRP, the more sensitive APP in this species, responded to succeeding waves of parasitaemia [14].

The mechanism responsible for maintaining an elevated Hp concentration in contrast to the greater fall in SAP concentration is not clear, particularly as these proteins are regarded as being in the same class (Type I) of APP in relation to the cytokines which cause their induction in vitro [5]. The production of the APP, after the initial peak, can be compared to studies where chronic inflammation has been induced by repeated stimulation. In mice treated every 2 or 3 days with increasing amounts of inflammatory stimulants, the hepatic mRNA for Hp increased to a peak within 24 h and fell to an intermediate level for the following 14 days [27]. A similar response in hepatic mRNA for Hp would explain the maintained production of serum Hp in the chronic phase of experimental trypanosomiasis which followed the acute phase of initial parasitaemia.

The SAP mRNA response to repeated stimulation was not determined in the study of Glibetic and Baumann [27] but the continuing stimulation of cytokine by the parasite in our study may have caused the establishment of a tolerant state in relation to SAP production. This has been observed in the CRP response of rabbits to repeated turpentine injection (5 times in 20 days) which resulted in a progressively reduced response and eventually no response to a sixth injection [28]. However, in a contrasting study, mice treated with turpentine/arachis oil at three to four day intervals did not develop tolerance to repeated injections in their ability to produce SAP [29]. It is also possible that the in vivo response to infectious disease produced a cytokine and cytokine inhibitor mix which resulted in the continuing production of Hp, while the necessary stimulation for prolonging SAP production was absent. The use of this experimental model of infection where an acute infection leads into a chronic condition provides a fascinating experimental model for investigating the mechanisms for the alteration in the host response as the infection progresses.

The major biochemical role of Hp is to bind haemoglobin following haemolysis to prevent loss of the haem residue from the internal environment, whereupon the Hp–haemoglobin complex is removed from circulation. In the infected mice the plasma Hp concentration was maintained at approx. 1.5 g/l after the initial peak, similar to the finding in dogs infected with *T. b. brucei* [14]. The mice, at this stage of the infection, were developing anaemia which is believed to be of haemolytic origin [30] which might be expected to greatly reduce the concentration of Hp in the circulation. The maintenance of plasma Hp dur-

ing the fall in PCV could result from a sufficiently high hepatocyte production rate replacing the Hp molecules removed following haemoglobin binding. Alternatively, the maintenance of plasma Hp during the development of anaemia could be explained if the mechanism of anaemia is by erythrophagocytosis by the expanded and active mononuclear phagocytic system which develops soon after infection [31] and in which haemoglobin is not released into the circulation. Clarification would require further investigation of the turnover rates of Hp and haemoglobin in plasma during this phase of infection.

It is known that APP have important roles in defence against bacterial infection, with functions related to anti-protease activity, scavenging for cell breakdown products and in opsonisation. There have been recent indications that the APP also have important roles to play in protozoan parasite infections. Thus it has been shown that human CRP, which shares many molecular characteristics with SAP, is able to bind to lipophosphoglycans on the surface of *Leishmania donovani*, increasing uptake into human macrophages [32]. A most interesting recent finding is that human Hp can inhibit the trypanosome lytic factor which protects man from infection with *T. b. brucei* [33]. Human trypanosome lytic factor is a haptoglobin-related apolipoprotein associated with high density lipoprotein [33]. Presumably mice and other animal species which are susceptible to infection with *T. b. brucei* do not have an analogous apolipoprotein.

In this experimental *T. b. brucei* infection of mice, the cytokine-induced acute phase response possibly resulted directly from the parasite by release of mediators with endotoxin-like activity [34,35] or indirectly by tissue damage caused by the tissue-invasive parasite. It is also possible that endotoxin from secondary bacterial infections or from the bacteria in the gastrointestinal tract might be the original cause of stimulation [34]. Identification of the precise mechanism responsible for cytokine and APP stimulation will require further investigation which should determine whether cytokines can be detected in the circulation prior to production of the APP and whether induction of the mRNA for the proteins in liver cells during the course of infection has occurred.

Use of APP analysis as a means of assessing the systemic cytokine reaction will be of particular benefit when monitoring the effects of agents such as cytokine antagonists on disease progression and therapy. Furthermore, it would be of great interest to investigate whether the acute phase response persists after sub-curative treatment with the trypanocidal drug diminazine aceturate. This clears the parasite from the circulation but leads to a reactive encephalopathy in the central nervous system during which inflammatory cytokine transcripts have been detected in association with activated astrocytes [19]. In addition monitoring infection with a species of trypanosome, such as *T. congolense*, which does not invade the tissues or the central nervous system [36] and which is completely cleared from the host by treatment with diminazine aceturate would give further insight into the mechanism of stimulation of the acute phase response following trypanosome infection.

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