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Induction of the Acute-Phase Protein Serum Amyloid P in Experimental Chagas' Disease

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Serum amyloid P protein (SAP), which shares several structural properties with C-reactive protein, has been recently identified as an acute-phase reactant in mice. In this study, the systemic inflammatory response of mice to infection with *Trypanosoma cruzi* was characterized with respect to induction of SAP as well as to stage-specific alterations on complement C3 and C4 levels. The SAP response depended on the dose and infectivity of the parasites. Kinetic data indicated a close temporal relationship between the onset of parasitemia and induction of SAP. The levels of SAP were maximally enhanced (1,050%) by the time parasitemia started to regress, and the response remained elevated as the infection entered the latency phase. The decline in parasitemia was paralleled by a significant reduction in C3 levels. A reciprocal relationship between the extent of parasitemia and SAP/C3 levels became apparent when these parameters were compared in individual inbred mice during the time of decreasing parasitemia.

Tissue injury and inflammation evoke a systemic acute-phase reaction (APR) characterized by a rapid increase in the plasma levels of certain proteins which normally are trace constituents of blood (16). Although the pathophysiological significance of the APR is not well understood, certain acute-phase proteins may modulate the immune response during states of intense tissue injury: in addition to suppressive effects mediated by the prototypic acute-phase C-reactive protein (CRP) on mixed lymphocyte cultures (23), generation of cytolytic T-lymphocytes (23), antigen-induced proliferative responses (22), and antibody formation (21), there have been reports that acute-phase amyloid A-related serum protein (SAA) (18) also inhibits the specific induction of antibodies to T-dependent antigens (1). It is therefore conceivable that these acute-phase proteins contribute to the state of anergy often associated with certain infections and with tumor growth (15). *Trypanosoma cruzi*, the digenetic trypanosomatid which is the causative agent of Chagas' disease, often sets an early acute phase typified by high parasitemias, hepatosplenomegaly, and fever (2). As with other protozoan infections (30), mice acutely infected with *T. cruzi* develop a state of nonspecific depression of immunological responses (6, 9, 12). Interestingly, it has been reported that the infection elicits the appearance of a serum protein that generates suppressor cells both in vivo and in vitro (10, 11). The present work is the first part of an effort to evaluate the significance of APR to defense and pathogenetic processes which develop in Chagas' disease. Studies of the APR in mice were hampered in the past because mouse CRP is a trace constituent of inflammatory fluids (27). However, Pepys et al. (27) recently found that mice respond to acute inflammatory stimulus with a marked increase in amyloid P component of serum (SAP), a protein which resembles CRP structurally and biochemically: these two molecules share extensive homologies in amino acid sequences, have similar pentagonal configuration and subunit composition (26), and display strong Ca^{2+} dependence on the binding to their respective ligands (14, 28, 29). The above findings suggest that, in addition to their similar acute-phase behaviors in different species, the biological functions of CRP and SAP may be related. The acute inflammatory response of mice to infection with the pathogen *T. cruzi* was characterized in our study with respect to induction of SAP acute-phase reactant, and also with respect to stage-specific fluctuations of C3 and C4 complement levels.

MATERIALS AND METHODS

Animals. Experiments were carried out with 8- to 12-week-old C57BL/10 mice (B10). Swiss albino mice were used only for routine maintenance of parasites. All animals were bred in our own animal facilities.

Parasites. The Y strain of *T. cruzi*, originally isolated from a human patient with acute Chagas' disease (34), was obtained from Z. Brener (Universidade Federal de Minas Gerais). The parasites were maintained by serial passages in albino mice. Two weeks before the

experiments were begun, the parasites were transferred to B10 hosts for two consecutive infection cycles of 7 days. The progression of parasitemia was determined in a few mice to ensure that the characteristic bell shape of the Y strain parasitemia (3) was also developing in the resistant B10 hosts. For purification of bloodstream parasites, heparin (10 U/ml) was added to parasitized blood immediately after bleeding, and the preparation was diluted with 3 volumes of medium 199 (GIBCO Laboratories) containing penicillin (100 μg/ml), streptomycin (100 μg/ml), and 0.5% bovine serum albumin (Sigma Chemical Co.) (M199-BSA). After centrifugation at 118 × *g* for 10 min at 4°C, the preparation was left standing in a water bath for 30 min at 37°C. The trypanosome-rich supernatant was collected, and the parasites were centrifuged at 10,000 × *g* for 30 min at 4°C. The parasites in the sediment were recovered and counted in a hemacytometer, and the concentration was adjusted with M199-BSA. Platelets and erythrocytes were the usual contaminants of the parasite preparations. Purified trypomastigotes or control cells (see Results) were injected intraperitoneally in B10 mice (males). At the proper time, the parasitemia of each mouse was determined, and the plasma was collected and analyzed for SAP, C3, and C4 levels.

Quantitation of plasma proteins. Rocket immunoelectrophoresis in agarose plates (5 by 10 cm) was used to assay plasma levels of SAP, C3, and C4. Pooled B10 plasma collected 24 h after intraperitoneal injection of 100 μg of *Salmonella enteridites* lipopolysaccharide W (Difco Laboratories) was used as standard for SAP determinations. Purification of SAP and production of a rabbit antiserum were done as described (27). The monospecificity of the antiserum was confirmed by cross-immunoelectrophoresis of lipopolysaccharide-induced acute plasma. The identification of the acute-phase reactant as SAP was further confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of immune precipitates obtained after treatment of ^{125}I-labeled mouse plasma proteins with antiserum to SAP. Anti-SAA reactivity in this serum was ruled out with aid of reference serum kindly donated by E. Franklin, New York University. Antiserum to C3b was produced in rabbits immunized with zymosan C3b. The antiserum recognized C3 as well as fragments C3b and C3c. Monospecific antiserum to the C4c fragment of mouse C4(Ss) was a generous gift from V. Nussenzweig and A. Ferreira, New York University Medical Center. Normal B10 plasma was used as standards for C3 and C4 determinations.

Statistics. Data are expressed either as percentage of increase or decrease in the plasma levels of infected mice compared with controls or as a ratio between mean levels of the experimental group divided by the mean of control levels. Values represent the arithmetic mean ± standard deviation. Statistical significance was determined by the Student *t* test.

RESULTS

Conditions for development of SAP acute response. The Y strain of *T. cruzi* produces a well-defined curve of parasitemia which starts about 4 to 5 days after inoculation, reaches a peak on day 7, and subsides shortly thereafter (3). In our experiments, *T. cruzi* infection was established by the inoculation of partially purified bloodstream trypomastigotes in B10 mice. The parasites, which are routinely maintained in albino mice, were subjected to two consecutive weekly cycles of infection on B10 hosts immediately before the experiments were started. This precaution was taken to exclude the possibility of host-versus-graft reactions directed to blood cells which usually contaminate the partially purified parasite preparations.

Inoculation of mice with increasing doses of bloodstream trypomastigotes stimulated a steep rise in the plasma levels of SAP when measured at the peak of parasitemia (day 7) (Fig. 1). Depending on the infective dose, SAP levels were 120 to 630% higher than in controls (mice inoculated with homologous blood cells and analyzed 7 days later). No significant alterations of C3 and C4 levels were found in these plasma samples when compared with either base-line or 7-day C3/C4 levels of controls.

The dependence on the parasite load seemed to reflect a requirement of effective tissue parasitism for development of the APR (Fig. 2). In these experiments, bloodstream trypomastigotes were treated with immune serum, washed, and inoculated (5×10^5 cells) into mice. In contrast to treatment with normal serum (Fig. 2a), opsonization of the trypomastigotes significantly limited the development of parasitemia and, correspondingly, the intensity of the SAP response (Fig. 2b). The importance of parasite infectivity in the induction of SAP was further suggested in another experiment (Fig. 2c): when the same number of culture trypanosome forms (93% of which were poorly infective epimastigotes) were inoculated in mice, the SAP response was minor. As expected, epimastigotes failed to induce substantial parasitemia at this dose.

Kinetics of the APR. Infection was established with an infective dose of 10^4 bloodstream trypomastigotes, which was the highest nonlethal dose capable of inducing elevated SAP levels in plasma. Figure 3 shows the temporal relationship between the APR and parasitemia. The earliest noticeable increase in SAP levels (210%, day 3) shortly preceded the onset of parasitemia. By the time parasites started to appear in the circulation (day 5), the concentration of SAP was 520% higher than base-line or control values. Despite the variation seen at day 5, there was a progressive increase in the levels of the acute-phase reactant from days 7 to 9 ($P < 0.005$) at the peak of parasitemia. Maximal concentrations of SAP (1,050%, day 11) were found as the number of bloodstream trypanosomes started to decline. The APR defined by the SAP marker was persistent, since the concentration

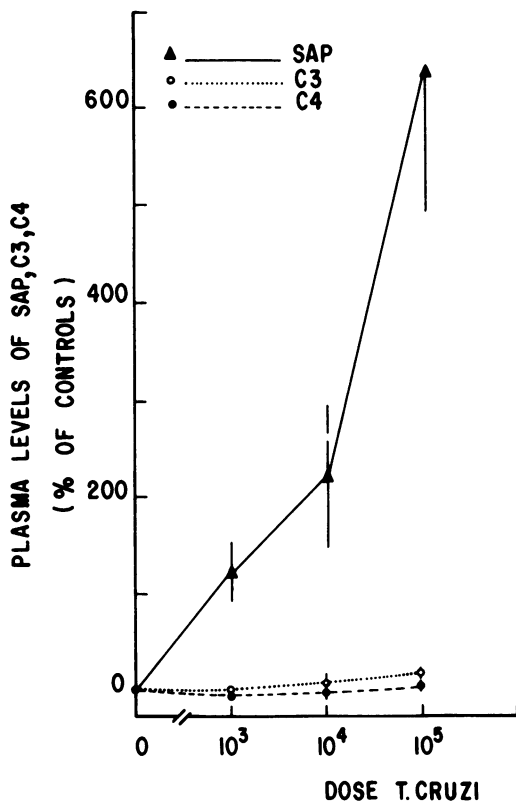

FIG. 1. Plasma levels of SAP, C3, and C4 as a function of *T. cruzi* infective dose (number of parasites). Each point is the mean ± 1 standard deviation of 5 to 11 plasma samples of mice inoculated 7 days earlier either with purified bloodstream trypomastigotes or with homologous blood cells obtained after sham purification (control). Data are expressed as percentage of increase in levels of plasma proteins of infected mice relative to levels found in controls. Control levels were the same as base-line levels.

of this protein remained extremely elevated through the end of the study (821%, day 14).

In contrast to SAP, C3 and C4 levels were not significantly altered during the early phase of infection. A marked rise in the levels of C3 antigen was, however, observed 9 days after infection (94%, $P < 0.005$). This was followed by a sharp drop (−35%, $P < 0.01$) 2 days later, when the parasitemia started to decline. The levels of C3 remained diminished when the study was terminated (−35%, $P < 0.01$). At this stage of infection, C4 levels were slightly reduced (−19%, $P < 0.005$).

Relationship between plasma levels of SAP, C3, and parasitemia in the late phase of acute infection. In a recent study on the natural resistance of inbred mice to *T. cruzi* (Brazil strain) infection, Trischman et al. (35) observed large variations in the parasitemia of individual mice. Accordingly, this heterogeneity was more common at higher parasitemias, when susceptible mice were eventually able to restrict or even clear their parasitemia before dying. A similar phenomenon was occasionally seen in our studies with the Y strain when animals were analyzed during the descending limb of the curve of parasitemia, that is, 10 to 11 days after infection. We found considerable heterogeneity in the parasitemia of individual mice 11 days after infection (Table 1). Thus, animals with less than 10^6 parasites per ml of blood had predominantly elevated SAP levels, whereas the concentrations

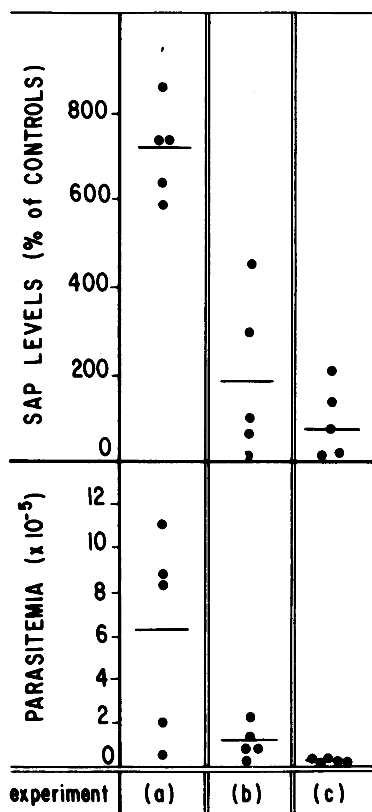

FIG. 2. Dependence of acute SAP response on *T. cruzi* infectivity. Columns include relative SAP levels (top) and parasitemia (bottom) of mice infected 7 days earlier with 5 × 10^5 bloodstream trypomastigotes which were previously treated with (a) normal rabbit serum or (b) antiserum to *T. cruzi* for 20 min in ice. The parasites were subsequently washed with M199-BSA before inoculation; (c) mice were infected with 5 × 10^5 culture-form trypanosomes containing 93% epimastigotes. The horizontal bar in each column indicates the mean of data shown in closed circles (each representing a single mouse). Controls were injected with blood cells subjected to the same treatment as parasites. Levels of the latter did not differ significantly from base-line levels.

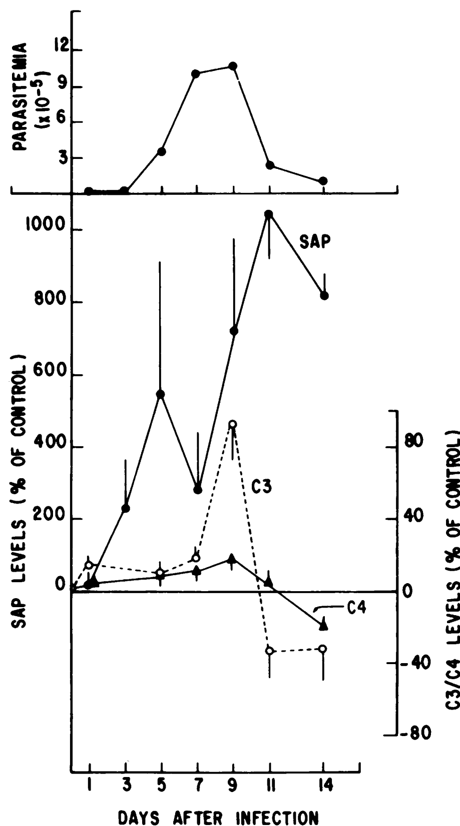

FIG. 3. SAP, C3, and C4 plasma levels during the course of acute infection. Mice were injected with 10^4 bloodstream trypomastigotes or with homologous blood cells (controls) as described in Fig. 1. The results are expressed as percentage of change of each plasma component in infected mice relative to the levels of respective controls. Each point is the mean of 5 to 11 plasma samples ± 1 standard deviation. Note different scales for SAP and C3/C4 relative levels. P values between: SAP, day 7/day 9 < 0.005; SAP, day 9/day 11 < 0.01; SAP, day 11/day 14 < 0.001; SAP, day 5/day 7, not significant; C3, day 7/day 9 < 0.005; C3, day 9/day 11 < 0.001; C4, day 11/day 14 < 0.005.

of C3 were only slightly below normal levels. In contrast, animals with higher parasitemias ($>10^6$) had much lower levels of SAP and C3. The levels of C4 did not change irrespective of the extent of parasitemia. We could not establish any clear relationship between these functions in mice analyzed during the ascending phase of the parasitemia curve (days 5 and 7).

DISCUSSION

The APR, which is recognized as an adaptive response of higher vertebrates to tissue damage and inflammation, is characterized by an increase in the concentration of certain proteins in the plasma. We show here that the induction of the mouse protein SAP is a part of humoral responses to infection with the pathogen *T. cruzi*.

Infection of mice with the Y strain of *T. cruzi* typically produces a parasitemia peak 7 to 8 days after infection. This strain displays preferential parasitism for the phagocytic cells from the spleen, liver, and bone marrow, where large numbers of amastigotes are found as early as 6 days after infection. The presence of parasites in these tissues is substantially reduced on day 9 and thereafter, in contrast to heart and skeletal muscle fibers, where parasitism tends to increase as the infection evolves to the latent phase (19).

Our data suggest that host cell parasitism is a necessary condition for induction of the acute-phase SAP protein. (i) The onset of the reaction (day 3) coincides with the time required for a complete intracellular cycle of parasite replication in vitro (20); the finding that the rise in SAP shortly preceded the emergence of trypomastigotes into the bloodstream is consistent with this view (Fig. 3). (ii) After inoculation of comparable doses (5×10^4) of bloodstream trypomastigotes or culture-form trypanosomes which contained 93% poorly infective epimastigotes, only the former caused severe parasitemia and elicited a correspondingly intense SAP response. (iii) Both the parasitemia and the SAP response were markedly diminished by previous treatment of trypomastigotes with antiserum to *T. cruzi*, but not with normal serum (Fig. 2). These results are compatible with the pioneer histopathological observations of Vianna (36), who reported that the early inflammatory reaction to *T. cruzi* is focal and likely depends on disruption of parasitized cells. Most significant, he observed degeneration of uninfected cells found in the vicinity of ruptured pseudocysts. More recently, evidence was presented that *T. cruzi* antigens released from such cells can bind to different types of cells in vitro, rendering them susceptible to destruction by the antiparasite immune response (31). Whatever the mechanism of acute-phase tissue damage, it is worth pointing out that SAP levels are boosted to maximal values (1,050% increase) when the parasitemia is in decline (day 11). Moreover, the fact that SAP remained extremely elevated through the end of the study (when tissue or blood stage parasites were scarce) emphasizes the sustaining nature of the inflammatory reaction in the infected host. Thus, extensive parasitism may not be as critical for the maintenance of the SAP response as it seems to be for its initiation. The role of products from activated lymphocytes, macrophages, and complement in the maintenance of this late SAP response is presently under study.

The simultaneous assessment of C3 and C4 levels during the progression of infection has led to the identification of some stage-specific changes. C3 levels, which remained stable throughout the entire ascending limb of parasitemia, were significantly increased (94%, $P < 0.005$) shortly before deflection of the parasitemia curve. The decline in parasitemia was accompanied by a sharp drop in C3 antigenic levels (−32%, $P < 0.01$), in support of previous observations that *T. cruzi*-infected animals develop progressive hypocomplementemia (8). A small but significant ($P < 0.005$) fall in C4 levels was also detected at the end of this study. These results are interesting in the light of evidence that complement is implicated in host defense against *T. cruzi* infection (4). As a matter of fact, our data could be explained by the finding of complement-fixing immunoglobulin G antibodies bound to bloodstream trypomastigotes during the descending limb of parasitemia (17). Determination of the relative contribution of C3 degradation products such as C3d to the antigenic fluctuations described here should help to evaluate whether excessive C3 catabolism, perhaps secondary to antibody-dependent complement activation, is responsible for these patterns. It is, however, worth keeping in mind that other immunological pathways may likewise affect serum complement levels: the control of C3 (13) and C4 (33) concentrations in normal mice is determined by *H2*-linked genes, and it is conceivable that C3/C4 fluctuations may also depend on *H2*-controlled functions associated with immunological activation. The finding that C2 synthesis by macrophages is markedly stimulated by exposure to lymphokines (B. H. Littman and S. Ruddy, Abstr. J. Immunol. **120**:1783, 1978) illustrates how these functions may be interrelated. Although these processes are most likely confined to local inflammatory sites, the possibility of systemic consequences should also be considered. For example, mononuclear phagocytic cells have been recently shown to regulate the synthesis of other circulatory proteins in the liver: the synthesis of acute-phase protein SAA is markedly stimulated by a monokine (32) that is possibly identical to leukocyte pyrogen (K. P. W. J. McAdam and C. A. Dinarello, Abstr. 4th Int. Congr. Immunol., 1980). Similar mechanisms may underlie our observations in experimental Chagas' disease, since resistance to *T. cruzi* seems to depend on macrophages and T cells both in vivo (5, 35) and in vitro (24, 25). Circumstantial evidence to support this hypothesis was the detection, late in infection (day 11), of an inverse relationship between SAP and C3 levels and the extent of parasitemia of individual mice (Table 1). These findings were apparent in experiments where a wide variation of parasitemia was detected among mice of the same experimental group during the descending phase of parasitemia. This heterogeneity may reflect the contribution of nongenetic elements to natural resistance to acute *T. cruzi* infection (35) as well as to the control of serum C3 (13) and SAP levels. We did not find any relationship between these variables during the ascending limb of parasitemia. Additional studies are in progress to establish the significance of these findings in host defense to infection with *T. cruzi*.

TABLE 1. Distribution of SAP, C3, and C4 levels according to parasitemia late in acute infection[a]

Parasites/ml of blood	R(SAP)	R(C3)	R(C4)
$>10^6$	3.5 ± 1.6	0.35 ± 0.23	1.27 ± 0.15
$<10^6$	11.1 ± 2.0	0.71 ± 0.19	1.08 ± 0.18
P	<0.001	<0.001	NS

[a] B10 mice infected 11 days earlier with 10^4 bloodstream trypomastigotes were ranked in two groups of 11 mice each according to whether their parasitemia was higher or lower than 10^6 parasites per ml of blood. The relative levels (R) of plasma proteins were calculated by dividing SAP, C3, and C4 plasma levels by the control values. The data shown are from a pool from two separate experiments. The results are expressed as means of relative levels ± 1 standard deviation. NS, Not significant.

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