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Identification of an Acute-Phase Reactant in Murine Infections with *Trypanosoma brucei*†

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A 42-kDa protein appeared at a much higher concentration in plasma from *Trypanosoma brucei*-resistant (C57BL/6) mice after infection than in plasma from trypanosome-susceptible (C3H/He) mice. This protein was purified by sequential steps of gel filtration, protein A-Sepharose affinity chromatography, isoelectric focusing, and ammonium sulfate precipitation. The purified protein was identified as a subunit of the acute-phase reactant haptoglobin. Causes of elevated plasma haptoglobin and its implications for resistance to trypanosomiasis are discussed.

In humans and domestic animals infection with salivarian or African trypanosomes, if left untreated, commonly leads to death (9, 23). However, certain breeds of cattle, sheep, and goats, as well as some species of wild animals, exhibit a significant degree of reduced susceptibility to the pathologic consequences of trypanosome infection and may even spontaneously recover (15). While trypanosome infection is invariably lethal in laboratory mice, there is wide variation in the resistance of different inbred mouse strains as measured by survival time. Thus, inbred mice have been used to study genetic (11) and immunologic (12) aspects of natural resistance to trypanosomiasis. In an earlier study we examined the regulation of parasite-specific antibody responses in resistant (C57BL/6) and susceptible (C3H/He) mice infected with *Trypanosoma brucei* (5). In the course of performing that study, it was noticed that a 42-kDa protein consistently appeared at a much higher concentration in plasma from *T. brucei*-resistant mice after infection than in plasma from trypanosomiasis-susceptible mice (Fig. 1A, compare lane b with lane d). The high concentration and specific size of this protein suggested that it was not a parasite molecule but rather a host protein, perhaps an acute-phase reactant.

Acute-phase reactants are those protein components of plasma whose concentrations significantly increase in a characteristic pattern in response to many different infections, inflammation, or tissue damage. Although the physiology of the acute-phase reaction is not completely understood, some reactants appear to mediate local inflammatory changes, while others are inflammatory enzyme inhibitors, scavenger-transport molecules, or molecules that may modulate phagocytic cell functions (19). Any of these functions could affect an animal's resistance to pathogenesis from an infecting microorganism.

To characterize the protein detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in trypanosome-resistant mouse plasma, a protocol was developed for its purification. As a first step a specific rabbit antiserum was prepared against the 42-kDa protein by immunization with antigen bands excised from SDS-polyacrylamide gels, according to a procedure described previously (24). Contaminating activity against other mouse plasma proteins was removed from the antiserum by four consecutive overnight incubations at 4°C with increasing concentrations of uninfected C57BL/6 mouse plasma; each incubation was followed by centrifugation for 30 min at 100,000 × *g* to remove antigen-antibody complexes. The absorbed antiserum was demonstrated to be highly specific for the 42-kDa protein by immunoblot analysis of total plasma protein taken from uninfected C57BL/6 mice and C57BL/6 mice infected for 8 days with *T. brucei* GUTat3.1 parasites (5) (Fig. 1B). This antiserum was then used to monitor the presence of the 42-kDa molecule in samples obtained from protein fractionation procedures.

Size fractionation of infected C57BL/6 mouse plasma was performed by gel filtration with Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden). Samples of plasma (5 ml) were applied to a Sephacryl S-300 column (2.6 by 88.5 cm) equilibrated with phosphate-buffered saline (PBS) (20 mM sodium phosphate [pH 7.6], 150 mM NaCl). Fractions of 4 ml were collected and assayed for protein by A_{280} (Fig. 2A) and for presence of the 42-kDa molecule (Fig. 2A) by immunoblotting. The 42-kDa molecule appeared to migrate as a 200- to 300-kDa molecule in its nondenatured state. Fractions containing the molecule were pooled, concentrated by ultrafiltration over an Amicon YM10 membrane (Amicon, Danvers, Mass.), adjusted to pH 8.5 with 1 M NaOH, and subjected to affinity chromatography on protein A-Sepharose CL-4B (Pharmacia) to remove contaminating mouse immunoglobulin G (Fig. 2B, lanes d and e). Unbound material eluting from the affinity column was concentrated in a Minicon macrosolute concentrator (B15; Amicon). A sample, containing approximately 10 to 20 mg of protein, was subjected to isoelectric focusing in a standard LKB 110-ml electrofocusing column (type 8100; LKB Produkter, Bromma, Sweden) for 20 h at 1,600 V with pH 3.5 to 10 Ampholines (LKB), using glycerol stabilization. The gradient was fractionated into 4-ml samples which were analyzed for pH and for presence of the 42-kDa protein. The molecule had an apparent isoelectric point between pH 4.2 and 4.5; a doublet band of protein with molecular masses of about 12 and 15 kDa appeared to copurify with the 42-kDa protein (Fig. 2B, lane f). In order to separate the purified protein from low-molecular-weight contaminants appearing after isoelectric focusing (Fig. 2B, lane f), the pooled peak was dialyzed against PBS and then subjected to ammonium

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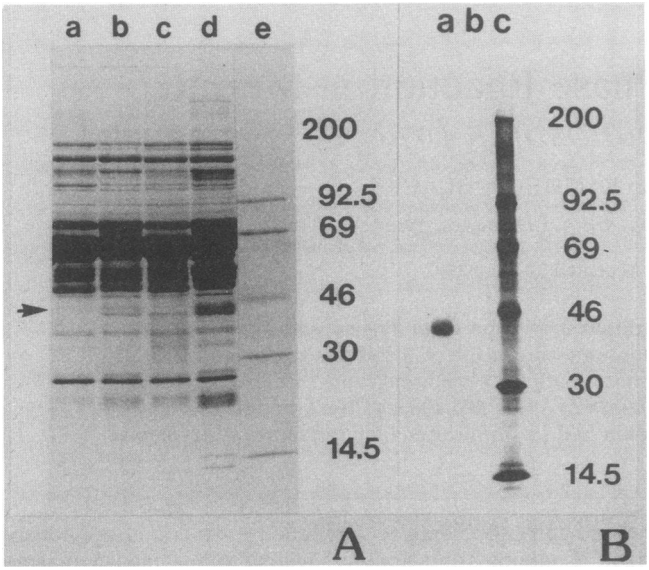

FIG. 1. Detection of 42-kDa protein in acute-phase plasma by SDS-PAGE and immunoblotting. Plasma was collected from uninfected female C3H/He and C57BL/6 mice and from mice 8 days after intraperitoneal infection with 10^3 *T. brucei* GUTat3.1 cells. The plasma proteins were resolved by electrophoresis in a 7.5-to-17.5% gradient polyacrylamide slab gel in the presence of 0.1% SDS (14) and then either stained with Coomassie brilliant blue (A) or transferred to nitrocellulose for immunoblot analysis (B). (A) Proteins present in uninfected C3H/He mouse plasma (lane a), infected C3H/He mouse plasma (lane b), uninfected C57BL/6 mouse plasma (lane c), and infected C57BL/6 mouse plasma (lane d). Molecular weight markers are shown in lane e. Marker protein molecular weights are indicated in thousands to the right of lane e, as follows: myosin, 200; phosphorylase α, 92.5; bovine serum albumin, 69; ovalbumin, 46; carbonic anhydrase, 30; lysozyme, 14.5. The 42-kDa molecule is indicated by the arrow to the left of lane a. (B) Antigen recognized by the antiserum prepared against the 42-kDa acute-phase protein detected in infected C57BL/6 mice. Electrophoretic transfer and immunodetection were performed essentially as described by Burnette (6). Antibody binding to the nitrocellulose was localized with ^{125}I-labelled anti-rabbit immunoglobulin (whole antibody; Amersham International). Lanes: a, infected C57BL/6 mouse plasma protein; b, uninfected C57BL/6 mouse plasma protein; c, ^{14}C-labelled molecular weight markers (CFA 626; Amersham International). To the right of lane c are indicated the molecular weights of the markers as described for panel A.

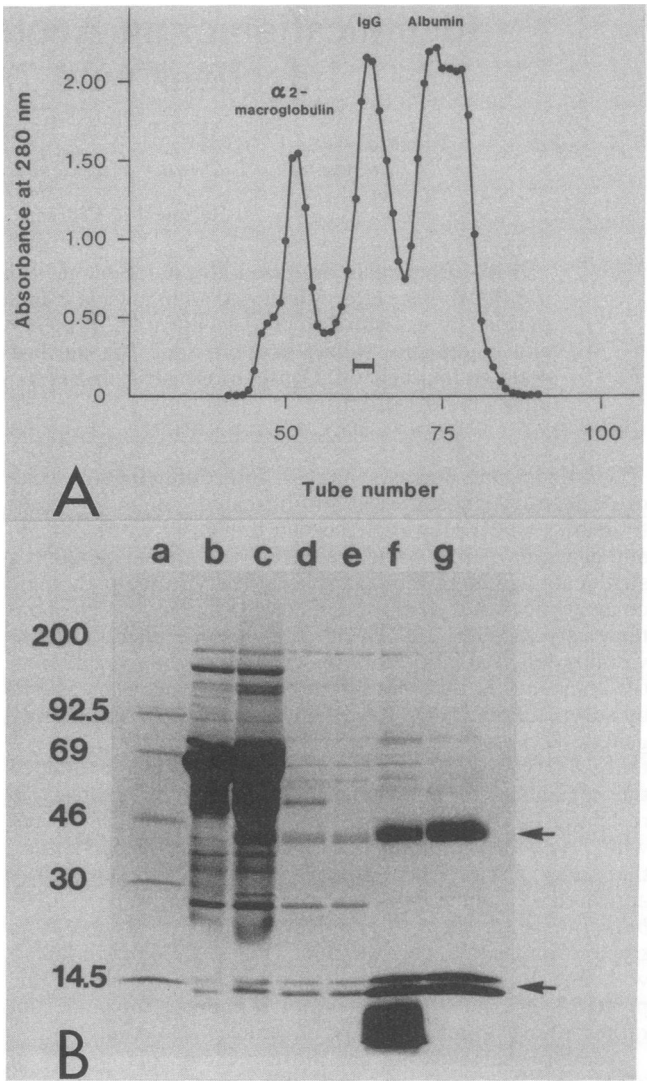

FIG. 2. Purification of 42-kDa protein from acute-phase plasma of C57BL/6 mice infected with *T. brucei*. (A) Elution profile after gel filtration with Sephacryl S-300 of acute-phase C57BL/6 mouse plasma. A 5-ml sample was applied to the column and eluted at a flow rate of 12 ml/h with PBS. The elution positions of three common plasma proteins (α_2-macroglobulin, immunoglobulin G, and albumin) as described by the manufacturer are indicated on the profile. The bar (tubes 61 to 64) indicates the fractions containing the 42-kDa protein that were pooled for further purification. (B) Stagewise purification of the detected acute-phase reactant from whole mouse plasma. Protein at each stage of purification was resolved by SDS-PAGE and stained with Coomassie brilliant blue, as described in the legend to Fig. 1. Lanes: a, molecular weight markers; b, total protein in uninfected C57BL/6 mouse plasma; c, total protein in 8-day trypanosome-infected C57BL/6 mouse plasma; d, protein in fractions pooled after gel filtration on Sephacryl S-300; e, protein in the size column pool not binding to protein A-Sepharose CL-4B; f, protein in the fractions pooled after isoelectric focusing; g, protein after isoelectric focusing precipitated between concentrations of 60 and 70% ammonium sulfate. Marker protein molecular weights (as described in the legend to Fig. 1) are indicated to the left of lane a. The positions of the 42-kDa protein and the 12/15-kDa doublet, discussed in the text, are indicated by arrows to the right of lane g.

sulfate precipitation. The 42-kDa protein, with the smaller doublet, precipitated between 60 and 70% saturation with ammonium sulfate. Figure 2B, lane g, shows the SDS-PAGE analysis of the final product of this purification protocol reduced with 2-mercaptoethanol; SDS-PAGE of the unreduced product is shown in Fig. 3A (lane b).

The molecular weight of the unreduced purified molecule (115 kDa; Fig. 3A, lane b), its isoelectric point (about 4.4), its subunit structure (polypeptide chains of 42 and 12 to 15 kDa), its quaternary structure size (200 to 300 kDa), and its appearance in plasma after infection suggested that the molecule might be the acute-phase reactant haptoglobin (25). This hypothesis was tested in a ligand-binding experiment. Unreduced, purified protein and the proteins of postinfection and preinfection plasma were resolved by SDS-PAGE and transferred to nitrocellulose (6). The blot was incubated at room temperature for 3 h in a solution containing 8 M urea to remove any residual SDS on the protein (21). The nitrocel-

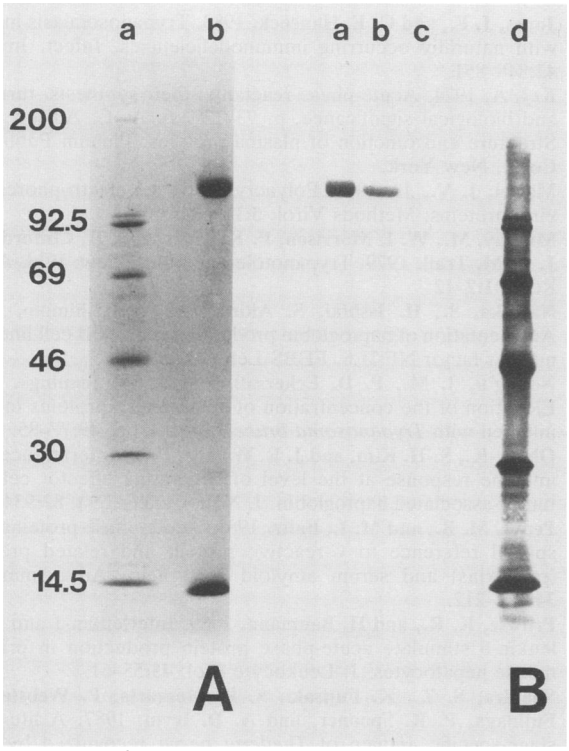

FIG. 3. Identification of the purified acute-phase reactant as haptoglobin. (A) SDS-PAGE analysis of the purified acute-phase protein not reduced with 2-mercaptoethanol (lane b). Lane a contains molecular weight markers. (B) Haptoglobin present in plasma and in the purified preparation localized by binding of radiolabelled hemoglobin. Haptoglobin is present in the purified protein (lane a) and acute-phase mouse plasma (lane b) but absent from uninfected mouse plasma (lane c). Molecular weight markers are shown in lane d. Marker protein molecular weights, as described in the legend to Fig. 1, are indicated to the left of lane a in panel A.

lulose was reequilibrated with PBS, and haptoglobin was then localized by overnight incubation with ^{14}C-methylated hemoglobin (Amersham International, Amersham, United Kingdom). Bound radiolabel was visualized by autoradiography. Hemoglobin was observed to bind to the purified 115-kDa protein (Fig. 3B, lane a) and to a molecule of the same size in the acute-phase plasma (Fig. 3B, lane b).

The cause of the much greater increase in plasma haptoglobin in the resistant mice compared with the more susceptible mice is not known. The main function of haptoglobin in the acute-phase response appears to be to bind free hemoglobin released during hemolysis, thus preventing glomerular filtration of hemoglobin which could lead to kidney damage and irreversible loss of iron from the body (13). The synthesis of haptoglobin increases rapidly in response to many types of infection or injury, producing an increased plasma level of 5- to 30-fold in rodents (13). *T. brucei* infection of dogs has been reported to increase plasma haptoglobin about fivefold (17). However, when hemolysis occurs it greatly reduces the plasma half-life of haptoglobin (the $t_{1/2}$ of hemoglobin-bound haptoglobin is about 90 min, while the $t_{1/2}$ of free haptoglobin is around 3 days) (13). Thus, when reduced levels of serum haptoglobin 7 to 10 days after infection were reported for trypanosomiasis in cattle, it was reasoned that bound haptoglobin had been removed from the circulation (10). The lower level in C3H/He mice then might be explained by a much greater amount of hemolysis in those mice, although that has not been observed. Alternatively, the higher level of haptoglobin in resistant mice might be the result of a greater level of synthesis. The synthesis of haptoglobin and other acute-phase reactants can be stimulated by several cytokines (interleukin-1 [IL-1], tumor necrosis factor alpha, IL-6, leukemia inhibitory factor, IL-11) and glucocorticoids, alone or in various combinations (3, 4, 20). Several of these molecules (IL-1, tumor necrosis factor alpha, and IL-6, which is the major inducer of the acute-phase response) are products of activated macrophages. Interestingly, resistant strains of mice have previously been noted to produce more macrophage-activating factors in response to trypanosome infection (8, 22). However, the acute-phase regulation of haptoglobin synthesis is complex, with different stimulatory hormones acting through different *trans*-activating nuclear factors (1, 16) on different *cis*-acting regulatory elements of the haptoglobin gene (2). Thus, increased haptoglobin synthesis in the resistant mice could also have resulted from an increase in any of the other acute-phase response-stimulatory molecules. Measurement of the elevation of IL-6 levels in the sera of the different strains of mice after trypanosome infection and the quantitative relationship between IL-6 level and haptoglobin synthesis in different mouse strains may clarify this situation.

The significance of the greater increase in plasma haptoglobin in mice resistant to trypanosome infection is also unclear. It may be a bystander effect not directly relevant to resistance to trypanosomiasis. In that case, the increased level could reflect less removal of haptoglobin because of much less acute hemolysis or increased synthesis of haptoglobin secondary to increased macrophage activation but could itself not be directly involved in resistance to the parasite infection. However, there are several ways in which increased levels of plasma haptoglobin could affect pathogenesis in trypanosomiasis. First, the long-term consequence of increased haptoglobin production should be improved iron conservation, which would mitigate the chronic anemia which is perhaps the major pathologic consequence of African trypanosomiasis. Also, the peroxidase activity of the hemoglobin-haptoglobin complexes may locally inactivate inflammatory cell products which cause tissue damage in chronic disease (13). While these two mechanisms may affect the chronic course of disease, they could not explain the profound difference observed between C57BL/6 and C3H/He mice in the early susceptibility to disease. Unlike resistant mice, C3H/He mice are not able to control the first wave of parasitemia, apparently because of a suppressed antibody response to the parasite's surface antigen in the first 1 to 2 weeks of infection (5). Much of the effective antibody response against trypanosome surface antigens appears to be T cell independent, occurring efficiently in T-lymphocyte-deficient nude mice (7). In fact, the first peak of parasitemia is better controlled in nude mice than in *nu*/+ controls, suggesting that activated suppressor T cells contribute to immunosuppression in trypanosomiasis (7). Recently, it has been shown that haptoglobin, at acute-phase response levels, can significantly depress T-cell stimulation (18). Thus, increased levels of haptoglobin could function in resistant mice to prevent an early T-cell-mediated immunosuppression that in susceptible mice interferes with control of the first wave of parasitemia.

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