Brief Definitive Report

Idiotypes Expressed Early in Experimental *Schistosoma mansoni* Infections Predict Clinical Outcomes of Chronic Disease

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Abstract

In murine *Schistosoma mansoni* infections, schistosome-specific cross-reactive idiotypes (CRI) are present in the sera of mice with moderate splenomegaly syndrome (MSS) at 20 wk after infection. In contrast, sera from animals that have the more severe hypersplenomegaly syndrome (HSS) at 20 wk of infection do not express these CRI in their sera. To examine when these regulatory CRI first appear in mice that eventually develop MSS, sera from infected animals were monitored for CRI from 1.5 to 20 wk of infection. In mice that eventually developed MSS, CRI were detected by 5 to 6 wk after infection, plateaued by 8 to 10 wk, and persisted through 20 wk of infection. Animals that developed HSS pathology or that died before 20 wk of infection never expressed CRI. Moreover, CRI levels present in the sera of mice at 6 wk of infection were inversely correlated with splenomegaly and hepatic fibrosis, but not with parasitologic measures, at 20 wk after infection. These results suggest that critical events occur very early in some schistosome infections that induce the production of regulatory idiotypes and that the presence or absence of these idiotypes predicts, and possibly determines, subsequent morbidity.

Key words: schistosomiasis • idiotypes • splenomegaly • mice • fibrosis

Introduction

The pathology caused by *Schistosoma mansoni* infections results from granulomatous inflammation surrounding parasite eggs in the liver that can lead to periportal fibrosis. Many of the eggs released by adult female worms living in the mesenteric venules of infected hosts beginning 5 to 6 wk after infection are swept by the portal circulation to the liver where they become lodged in the presinusoidal capillaries and invoke an intense immunologic response. The acute phase of schistosomiasis occurs at 8 wk after infection when the magnitude of this granulomatous response peaks. The disease subsequently enters the chronic phase and the intensity of inflammation in response to newly deposited eggs gradually decreases (1). Current hypotheses suggest that failure to appropriately modulate the immune responses associated with granuloma formation after the acute phase of infection results in the increased fibrosis, portal hypertension, and spleen enlargement observed in humans with severe hepatosplenic schistosomiasis (2, 3). Most investigations into the mechanics of immunomodulation in experimental schistosomiasis have focused on the cell subsets and cytokines involved in granuloma formation in the acute and chronic phases of infection (4–8). However, we have also presented strong evidence suggesting that idiotypic interactions also play an important immunoregulatory role in experimental and human schistosomiasis, perhaps functioning through some of the aforementioned mechanisms, such as differential cytokine induction (9–13). Others have reported similar immunoregulatory idiotypic interactions in *Schistosoma japonicum* infections (14–16).

Affinity purified, anti-schistosome soluble egg antigen (SEA) antibodies prepared from sera of patients with the asymptomatic chronic ("intestinal") form of schistosomiasis and 20 wk infected mice with the analogous moderate splenomegaly syndrome (MSS) contain a population of idiotypes (Id) that stimulate proliferation of PBMCs and spleen cells from infected humans and mice, respectively (9, 11). Studies on spleen cells from infected mice have also shown that Id prepared from the sera of MSS mice (MSS Id) stimulates production of IFN-γ, a cytokine that regulates granuloma formation (11, 17–19). Rabbit antibodies

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raised against Id prepared from the sera of patients with intestinal schistosomiasis cross reacts with MSS Id, suggesting certain stimulatory idiotopes are shared across species (10, 20), i.e., are cross-reactive Id (CRI). In contrast, patients with severe hepatosplenic disease and 20 wk–infected mice with the analogous hypersplenomegaly syndrome (HSS) fail to express CRI and antibodies prepared from their sera do not stimulate cellular proliferation or cytokine production (9–11). The association of CRI with less severe pathology combined with the ability of CRI to stimulate production of granuloma-inhibiting cytokines suggested that they may play an important role in regulating the pathology of chronic schistosomiasis.

Further support for this hypothesis came from studies in which neonatal mice were injected with CRI, allowed to mature to adulthood, and infected with *S. mansoni*. Mice exposed to preparations containing CRI (MSS Id or Id prepared from sera of mice infected for 8 wk) demonstrated prolonged survival, smaller granulomas, decreased fibrosis, and enhanced IFN-γ production compared with animals that were neonatally injected with CRI-negative preparations (affinity-purified anti-SEA antibodies prepared from the sera of HSS mice or commercially procured normal mouse immunoglobulin; reference 12). The immunologic effects of neonatal CRI exposure were also investigated by evaluating SEA-induced responses in mice neonatally injected with CRI but that received no exposure to *S. mansoni* antigens or infection. As adults, these mice displayed significant SEA-induced spleen cell proliferation, Th1 cytokine responses, and serum IgG while animals neonatally injected with HSS Id or normal mouse immunoglobulin lacked these SEA-related responses (13). Whether mice that were neonatally injected with CRI were infected with *S. mansoni* or not, their sera as adults contained CRI and anti-CRI, suggesting that neonatal exposure led to the establishment of an idiotypic network with immunoregulatory properties (13, and unpublished data). We have previously shown that serum CRI begins to appear by 6 wk of *S. mansoni* infection (21) but those studies did not document their presence in relationship to eventual clinical outcomes at chronicity.

Because our previous findings suggested a link between serum CRI and decreased schistosomiasis-associated pathology in both humans and mice, we more precisely identified the time points at which regulatory CRIs are first expressed during schistosome infections of mice. In addition, we examined whether the presence or absence of CRI early in infection could predict the pathologic outcome of chronic schistosomiasis in a given mouse or group of mice before 20 wk of infection. We also investigated the relationship between CRI levels and parameters associated with schistosomiasis morbidity, such as splenomegaly, hepatic hydroxyproline levels, and infection intensity.

Materials and Methods

Mice. Male CBA/J mice were obtained from The Jackson Laboratory and housed in the Association for Assessment and Accreditation of Laboratory Animal Care–approved facilities of the Centers for Disease Control and Prevention. The mice were infected by subcutaneous injection with 45 cercariae of a Puerto Rican strain of *S. mansoni* that had been maintained in *Biomphalaria glabrata* snails. Individual mice were tracked by the use of transponders (Bio Medic Data Systems, Inc.). Blood (≤100 μl) was obtained by retroorbital puncture from anesthetized animals at designated weeks after infection. Mice surviving until 20 wk of infection were weighed, killed by CO_2 inhalation, and exsanguinated by cardiac puncture. Spleens and livers were removed and weighed portions (~0.5 g) of liver were frozen at −80°C until analysis for egg and hydroxyproline content were performed.

Enumeration of Eggs and Hydroxyproline Levels in the Liver. Liver tissue that had been collected for egg enumeration was placed in 5 ml of 5% KOH at 37°C for 2 to 4 h until the digestion was complete (22). Duplicate 25-μl aliquots of the digest were placed on a glass slide, and eggs were counted under a microscope. The number of eggs per gram of liver tissue was calculated for each animal.

Hepatic collagen content was determined by measuring hydroxyproline levels in liver hydrolysates. The protocol (23, 24) was modified for microplate analysis and used as an estimate of liver fibrosis. Briefly, weighed liver samples were hydrolyzed in 5 ml of 6 M HCl for 18 h at 110°C in glass tubes, after which 40 mg Dowex/Norit mixture (Sigma-Aldrich) and 5 ml distilled water were added to each hydrolysate. The mixture was centrifuged for 15 min at 2,000 rpm at 25°C, and the supernatant fluid filtered through filter paper into a clean tube. 2 ml of the filtrate was neutralized by adding one drop of 1% phenophthalein and then titrated against 10 M NaOH until colorless. The final volume was adjusted to 4 ml. A small aliquot (12.5 μl) of neutralized liver hydrolysate was pipetted into each well of a microtiter plate and mixed with 25 μl isopropanol. Freshly prepared oxidant solution (chloramine-T/citrate buffer, pH 6.0; Sigma-Aldrich) was added (12.5 μl) into each well with mixing and then incubated at 25°C for 4 min. Freshly prepared Ehrlich's Reagent Solution (150 μl; Sigma-Aldrich) was added, mixed, and incubated at 60°C for an additional 25 min. The plate was allowed to cool and the absorbance read at 570 nm using a microplate reader (Molecular Devices). Hydroxyproline levels were calculated against standard curves of 4-hydroxy-L-proline (Calbiochem) and expressed as μg hydroxyproline/g tissue.

SEA and Id Preparation. SEA was prepared as described previously (25). Briefly, eggs were isolated by differential centrifugation from homogenized liver tissue of CF-1 mice (Charles Rivers Laboratories) infected with 300 cercariae for 7 to 8 wk. Soluble material from purified eggs was obtained by homogenization in Dulbecco's PBS and subsequent ultracentrifugation. To prepare affinity-purified, polyclonal anti-SEA Abs that expressed CRI (MSS Id), pooled sera from 20 wk infected MSS (10) mice were passed over a column of SEA coupled to cyanogen-bromide-activated Sepharose 4B (Sigma-Aldrich). Bound anti-SEA Abs (Id) were eluted using 0.1 M glycine-HCl (pH 2.8) and collected into 0.025 M borax. The eluates were concentrated and dialyzed against saline, and their protein concentrations were determined.

Rabbit Anti-CRI Production and Competitive ELISA for CRI Levels. Rabbit anti-Id was prepared by immunizing a rabbit (Myrtle's Rabbitry) subcutaneously and intramuscularly with 200 μg MSS Id preparation mixed 1:1 in RIBI adjuvant (RIBI ImmunoChem Research). Three injections were given at 15-d intervals. 15 d after the final injection, the rabbit was bled. Ig was purified from the rabbit serum using a T-Gel purification Kit

(Pierce Chemical Co.). Id-specific antiserum was prepared by exhaustively absorbing the immunized rabbit serum with normal mouse immunoglobulin (Sigma-Aldrich) coupled to cyanogen bromide–activated Sepharose 4B (10 mg Ig/g Sepharose). Repeated absorptions (between 15 and 20) yielded reagent which did not react with normal mouse immunoglobulin while reactivity with the original immunizing MSS Id preparation was maintained. The competitive ELISA using this reagent to measure serum CRI levels has been described previously (9).

Statistical Analyses. Statistical analyses were performed using GraphPad Instat (GraphPad Software).

Results and Discussion

At 20 wk after infection with *S. mansoni*, sera from mice with MSS contain CRI but sera from mice with HSS do not (10, 11). To determine when regulatory CRI first appears in mice that eventually develop MSS, individual infected mice were followed over 20 wk and bled at different times during their infections. Animals that survived until 20 wk were killed and classified as MSS or HSS according to their spleen to body weight ratio and gross pathologic characteristics (10). Serum CRI levels were determined by competitive ELISA using anti-MSS Id rabbit serum and an MSS Id standard curve. Fig. 1 shows the results from three different longitudinal experimental infections. In mice that eventually developed MSS by 20 wk of infection, serum CRI could be detected as early as 5 or 6 wk after infection. Serum CRI levels in these mice rose rapidly by 8 to 10 wk after infection and continued to increase through 20 wk of infection. In contrast, mice that displayed HSS at 20 wk after infection or mice that died before 20 wk expressed little to no detectable CRI in their sera at any time during their infection. Mice that died before 20 wk of infection and were autopsied had characteristics similar to those of mice with HSS at 20 wk of infection: they were anemic, devoid of fat, and had obvious ascites.

Although we have reported that CRI can be detected early in *S. mansoni* infections (21), our previous longitudinal studies of MSS and HSS mice suggested that differences between pathologic groups occurred later in infection as significant differences between anemia and serum triglyceride levels in mice that develop these distinct pathologies are not detected until 10 and 14 wk after infection, respectively (26). The current data indicate that the immunobiologic processes that determine whether or not experimental animals in this model develop severe pathology are in place very early in infection, concurrent with worm maturation and egg production. If this is true, it might be expected that the presence or absence of CRI at 6 wk after infection should be predictive of whether mice will eventually develop MSS or HSS. Regression analyses of individual mice confirmed this and demonstrated significant correlations between CRI levels at 6 wk of infection and spleen percent body weight ratios at 20 wk of infection (Fig. 2).

In addition to splenomegaly, in one experiment (corresponding to the bottom panel from Figs. 1 and 2) we also evaluated the relationship between early CRI production and liver hydroxyproline levels later in infection (Fig. 3).

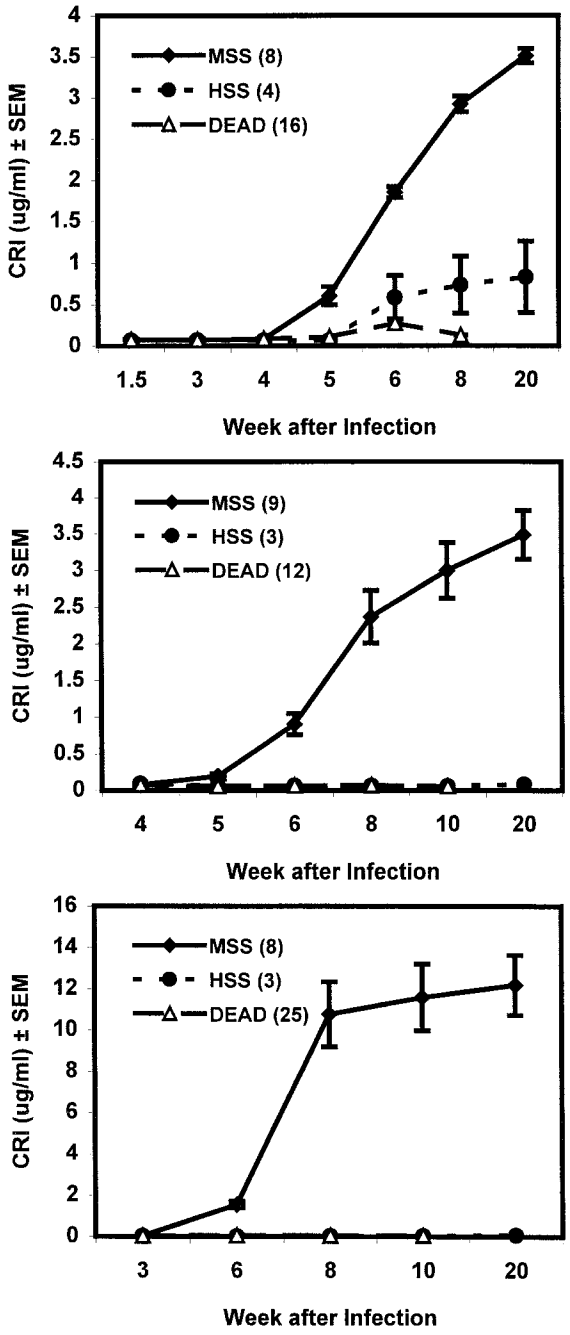

Figure 1. Longitudinal profile of serum CRI levels in *S. mansoni* infected mice. Groups of 30 to 40 CBA/J mice were infected with 45 cercariae of *S. mansoni*. Mice were bled at the indicated weeks and serum CRI levels were determined by competitive ELISA. At 20 wk after infection, surviving animals were killed and classified as MSS or HSS according to the percentage of spleen to body weight ratios. Sera from mice that died before 20 wk of infection were also tested for the weeks they were bled before their deaths. Each panel represents an independent experiment. The status at 20 wk after infection is indicated in the key for each experiment, and the final number of mice is shown in parentheses.

Again, high CRI levels at 6 wk of infection correlated with low hepatic fibrosis and low CRI was associated with increased pathology at 20 wk of infection. We also evaluated the relationship between early CRI and measures of the in-

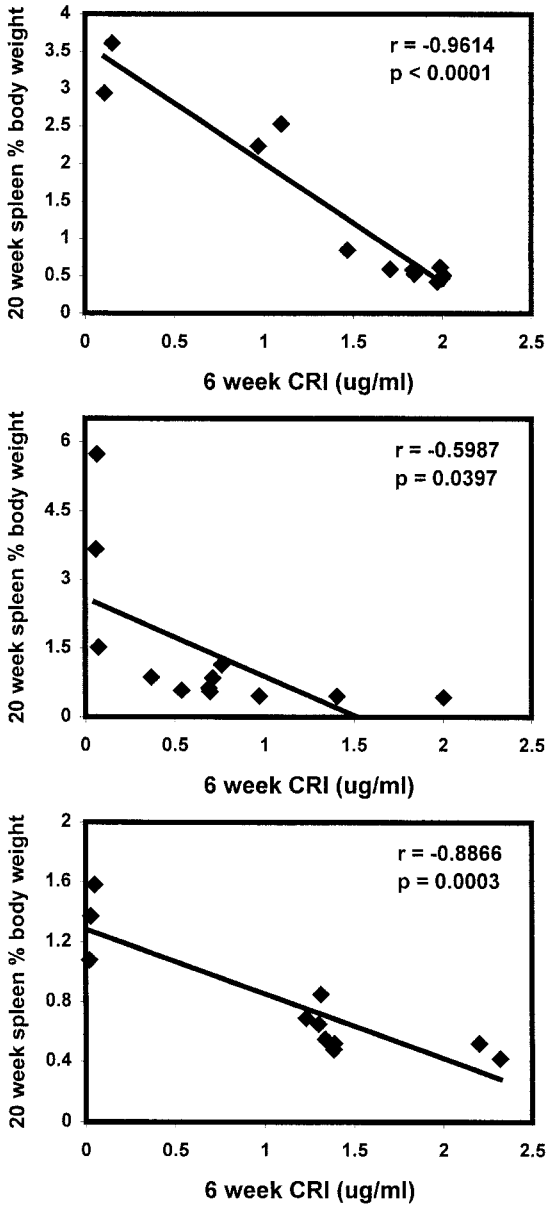

Figure 2. CRI levels at 6 wk of infection predict splenomegaly at 20 wk. Correlation analysis between a mouse's serum CRI at 6 wk after infection and the same animal's spleen percent body weight at 20 wk after infection. Each panel represents an independent experiment corresponding to the experiments in Fig. 1.

tensity of infection. There was no significant relationship between 6 wk CRI levels and the number of eggs in the liver ($r = -0.2765$, $P = 0.4105$) or circulating adult worm cathodic antigen levels ($r = -0.3325$, $P = 0.3177$) at 20 wk of infection (data not shown). These findings corroborated our previous studies demonstrating that severe pathology is not merely a function of increased worm burden (10, 12, 26).

We believe that the strong correlation between early CRI levels and chronic pathology represents a cause and effect of CRI on pathology rather than a simple association

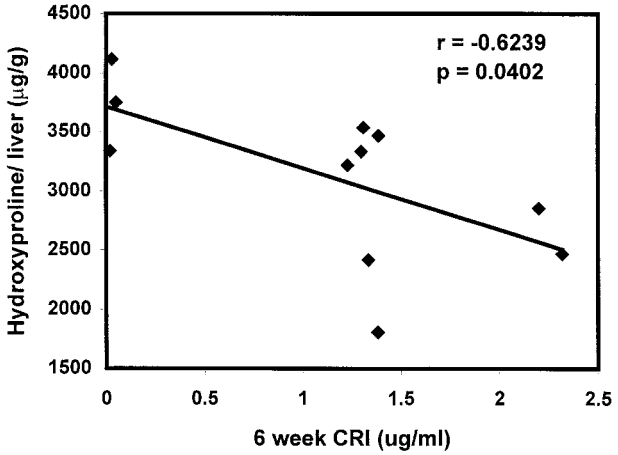

Figure 3. Relationships between serum levels of CRI at 6 wk of infection and hepatic hydroxyproline at 20 wk of infection.

of both measures with some other immunologic measure. This belief is bolstered by our previous findings that a single neonatal injection of mice with CRI leads to high levels of CRI and anti-CRI in the sera of these mice when they become adults and confers humoral and cellular reactivity to schistosome antigens that they have never encountered (13). If these mice are subsequently infected with schistosomes, the animals that received CRI as neonates demonstrate increased IFN-γ production, decreased granuloma size and fibrosis, and increased survival compared with animals that received control immunoglobulin or an Id preparation lacking CRI activity (12). Other studies have similarly demonstrated an important role for antibodies and their Fc receptors in modulation of the granulomatous response (27–30).

The finding that CRI levels early in infection predict chronic pathology is very exciting. We hope to employ this tool to evaluate the dynamic changes that occur in the progression to severe or nonsevere chronic schistosomiasis, identify what immunologic manipulations (in addition to neonatal Id injection) we can employ before or during infection to shift mice toward less severe disease, and perhaps identify which schistosome antigen(s) and/or epitope(s) are important for generation of CRI. It also has the potential for important public health applications. Because mice and humans with low morbidity share idiotopes and display the same pattern of CRI expression during chronic infections, absence of CRI production in humans who have schistosomiasis may be a useful predictor for patients likely to develop hepatosplenic disease. Those individuals could be followed more closely for infection and treated more aggressively than is currently indicated in general schistosomiasis control programs. We hope to verify whether the correlation of serum CRI with low pathology in experimental animals is also true for humans by comparing patients' serum CRI levels with their pattern of hepatic fibrosis detected by ultrasound. We anticipate that schistosome-positive persons with even low levels of fibrosis will

have decreased levels of serum CRI compared with schistosomiasis patients with no detectable hepatic pathology. If this expectation is borne out by clinical studies, we may well be able to develop powerful tools for identifying and preventing severe disease in persons with *S. mansoni* infections.

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