Neonatal Idiotypic Exposure Alters Subsequent Cytokine, Pathology, and Survival Patterns in Experimental Schistosoma mansoni Infections

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Summary

Exposure to maternal idiotypes (Ids) or antigens might predispose a child to develop an immunoregulated, asymptomatic clinical presentation of schistosomiasis. We have used an experimental murine system to address the role of Ids in this immunoregulation. Sera from mice with 8-wk Schistosoma mansoni infection, chronic (20-wk infection) moderate splenomegaly syndrome (MSS), or chronic hypersplenomegaly syndrome (HSS) were passed over an S. mansoni soluble egg antigen (SEA) immunoaffinity column to prepare Ids (8WkId, MSS Id, HSS Id). Newborn mice were injected with 8WkId, MSS Id, HSS Id, or normal mouse immunoglobulin (NoMoIgG) and infected with S. mansoni 8 wk later. Mice exposed to 8WkId or MSS Id as newborns had prolonged survival and decreased morbidity compared with mice that received HSS Id or NoMoIgG. When stimulated with SEA, 8WkId, or MSS Id, spleen cells from mice neonatally injected with 8WkId or MSS Id produced more interferon γ than spleen cells from mice neonatally injected with HSS Id or NoMoIgG. Furthermore, neonatal exposure to 8WkId or MSS Id, but not NoMoIgG or HSS Id, led to significantly smaller granuloma size and lower hepatic fibrosis levels in infected mice. Together, these results indicate that perinatal exposure to appropriate anti-SEA Ids induces long-term effects on survival, pathology, and immune response patterns in mice subsequently infected with S. mansoni.

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

Morbidity in human schistosomiasis is associated with a failure to regulate immune responses to parasite antigens. Individuals with the relatively asymptomatic “intestinal” chronic disease form have modulated responses to schistosome soluble egg antigens (SEA),1 whereas individuals who eventually develop severe hepatosplenic disease have unregulated responses to SEA (1). The factors that determine whether patients demonstrate regulated (intestinal) or unregulated (hepatosplenic) responses to parasite antigens have not been definitively established. However, one mechanism that has been associated with less severe morbidity is the expression of certain cross-reactive, immunoregulatory idiotypes (Ids) on anti-SEA Abs (2). Affinity-purified anti-SEA Abs prepared from sera of patients with intestinal schistosomiasis contain Ids that stimulate PBMC responses but those prepared from sera of hepatosplenic patients do not (2). Shared Ids among patients with intestinal disease but not hepatosplenic patients are also demonstrable by serologic cross-reactivity with antiidiotypic Abs (2, 3). The immunoregulatory function of these cross-reactive Ids (CRIs; reference 4) has been shown by their ability to modulate granulomatous inflammation in an in vitro granuloma model (5).

Previously, we have hypothesized that infection status of the mother may determine whether or not individuals who later become infected develop severe disease (1). Upon infection, people born and raised in an area with a high prev-

1 Abbreviations used in this paper: CCA, circulating cathodic antigen; CRI, cross-reactive Id; HSS, hypersplenomegaly syndrome; Id, idiotype; MSS, moderate splenomegaly syndrome; NoHuIgG, normal human Ig; NoMoIgG, normal mouse Ig; SEA, schistosome soluble egg antigen(s); SWAP, schistosome adult worm antigenic preparation.
alence of schistosomiasis generally only develop the intestinal form of chronic disease, with <10% of these individuals progressing to hepatosplenic disease. In contrast, travelers or recent immigrants to an endemic area often present with acute illness, ectopic infection, and/or severe disease if they become infected (6, 7). Age/prevalence and age/intensity curves predict that many of the mothers of people living in endemic areas are likely to have had schistosomiasis at the time they were pregnant with and gave birth to their children. In contrast, mothers of travelers or immigrants to an endemic area would not have had schistosomiasis. Evidence for an immunologic contribution to this observation comes from studies demonstrating that cord blood cells from children born to infected mothers respond to Id prepared from their mother’s serum or from the sera of an intestinal patient pool. Cord blood cells from children born to mothers who did not have schistosomiasis did not respond to these Ids (8).

To better understand how neonatal Id exposure affects eventual immune responses and pathology after subsequent infection, we have used a murine model of chronic infection that closely parallels human pathologic and immunologic presentations. Inbred male CBA/J mice with 20-wk Schistosoma mansoni infections present with one of two distinct pathologic syndromes: moderate splenomegaly syndrome (MSS) or hypersplenomegaly syndrome (HSS) (9, 10). HSS affects ~20% of the chronically infected mice and is characterized by massive splenomegaly, ascites, thymic atrophy, severe anemia, and cachexia. The histopathologic features of the HSS syndrome are splenic congestion, lymph node plasmacytosis, and extensive periportal-like liver fibrosis. The remaining majority of mice have MSS chronic infections and develop only moderate splenomegaly. The clinical and pathologic characteristics of MSS and HSS mice closely parallel those features of schistosomiasis patients with the intestinal and hepatosplenic disease forms, respectively. In addition, the parallel mouse and human forms (MSS with intestinal and HSS with hepatosplenic) share idiotypic repertoires on their anti-SEA Abs as shown by cross-reactivity with antiidiotypic Abs (9) as well as T lymphocyte response patterns (11). Like Id from intestinal patients’ sera that can stimulate proliferation of peripheral blood T lymphocytes from schistosomiasis patients, Id preparations from 8 wk-infected mouse sera (8 W kId) or MSS mouse sera (MSS Id) are stimulatory for spleen cells from S. mansoni-infected mice. HSS mice Id preparations, like hepatosplenic human Id preparations, are not stimulatory (2, 11).

To study the influence of perinatal idiotypic exposure on subsequent immunologic responses and development of pathology, morbidity, and mortality, newborn mice were exposed to anti-SEA Abs (Id preparations) from various sources and were subsequently (8 wk later) infected with S. mansoni. The prolonged survival, the enhanced production of IFN-γ, and the downregulation of granuloma formation seen after neonatal exposure to 8W kId or MSS Id suggest that such exposure to CRIs (4) is beneficial during subsequent S. mansoni infections.

### Materials and Methods

Animals, Neonatal Exposure, and Infections. CBA/J mice were obtained from The Jackson Laboratory and housed in the American Association for Accreditation of Laboratory Animal Care-approved animal care facilities of the Centers for Disease Control and Prevention. Newborn (<24 h old) male mice from uninfected parents received transgenic, intraperitoneal injections of 50 μg of immunoaffinity-purified anti-SEA Abs, commercial normal mouse IgG (N O M o l o g / S g i m a ), human anti-SEA mAb E5 (12), or commercial normal human IgG (N o h l u g g / S g i m a ) in PBS. To prepare affinity-purified, polyclonal anti-SEA Abs, pooled sera from mice infected for either 8 or 20 wk (the latter segregated into MSS and HSS) were passed over a column of SEA coupled to cyanogen-bromide-activated Sepharose 4B (Sigma). 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. All Id preparations (i.e., those from different serum sources) used in a given experiment were prepared over the same column. Mice that had been neonatally manipulated were infected at 8 wk of age by subcutaneous injection of 45 cercariae of a Puerto Rican strain of S. mansoni that had been maintained in Biomphalaria glabrata snails. The number of live animals was recorded weekly to give survival proportions.

Cirulating Antigen Detection and Enumeration of Eggs in the Liver. Levels of adult worm circulating cathodic antigen (CCA) were quantified in the sera from infected mice using the 5H11 monoclonal sandwich assay as described previously (13). Absorbance levels from serum samples were compared with those obtained on an adult schistosome worm antigenic preparation (SWAP) standard curve run in parallel to obtain a SWAP equivalent that could be used to compare results from samples run over different days.

For enumeration of eggs in the liver, a portion (~0.5 g) of weighed liver from infected mice was removed and frozen until digestion. For digestion, 5 ml of 5% KOH (14) was added to each 10 ml RPMI 1640 supplemented with 2% fetal bovine serum and 0.3% Tween 20 (Sigma), plates were incubated with optimized dilutions of infected mouse sera. Anti-SEA Abs bound to the SEA-coated plate were detected using peroxidase-conjugated IgG fractions of isotype-specific goat anti-mouse IgG1, anti-mouse IgG2a, or anti-mouse IgG2b (Southern Biotechnology Associates, Inc.). Assays were developed by the addition of TMB peroxidase substrate solution (Kirkegaard & Perry Laboratories, Inc.) overnight, at 4°C. After a 1-h blocking step of PBS with 0.3% Tween 20 (Sigma), plates were incubated with polyclonal anti-SEA Abs (Id preparations). Bound anti-SEA Abs were detected by an ELISA as described previously (13). Absorbance levels from serum samples were compared with those obtained on an adult schistosome worm antigenic preparation (SWAP) standard curve run in parallel to obtain a SWAP equivalent that could be used to compare results from samples run over different days.

Determination of Anti-SEA Ab Isotypes. SEA isotype analyses were performed by specific ELISAs to detect anti-SEA activity and to evaluate what effects the cytokine environment may have on Ab class switching in these animals (15, 16). SEA (0.25 μg/well in 0.1 M NaHCO3, pH 9.6) was adsorbed onto flat-bottomed microtiter plates (Immunolon II; Dynatech Laboratories, Inc.) overnight, at 4°C. After a 1-h blocking step of PBS with 0.3% Tween 20 (Sigma), plates were incubated with optimized dilutions of infected mouse sera. Anti-SEA Abs bound to the SEA-coated plate were detected using peroxidase-conjugated IgG fractions of isotype-specific goat anti-mouse IgG1, anti-mouse IgG2a, or anti-mouse IgG2b (Southern Biotechnology Associates, Inc.). Absorbance levels from serum samples were compared with those obtained on an adult schistosome worm antigenic preparation (SWAP) standard curve run in parallel to obtain a SWAP equivalent that could be used to compare results from samples run over different days.
stimulated with medium alone, SEA (4 μg/ml), or anti-SEA Id preparations (40 μg/ml). Supernatant fluids were harvested after 48 h and stored at −70°C. Cytokine levels were measured by capture ELISA as described previously (11).

Preparation of tissue for microscopic examination. Upon killing of the experimental animals, livers were removed and a portion of each liver was preserved in 10% buffered formalin. For histologic analysis, tissues were imbedded in paraffin, sectioned (5 μm), and stained with hematoxylin and eosin. Granuloma areas were measured with the aid of computerized morphometrics (ImagePro Plus; Media Cybernetics). Only those granulomas with a single, central egg were measured. Granulomas containing more than one egg or that were contiguous with other granulomas were ignored. A minimum of 25 granulomas was measured per mouse to calculate the average granuloma size for individual mice. The means for all mice in each group were then averaged for statistical comparison of the groups. For evaluation of fibrosis, some liver sections were stained with Gomori’s trichrome stain. Computerized morphometrics were used to calculate the percent fibrotic area of 25 contiguous fields, and the average fibrotic tissue area was calculated for each mouse. As with the granuloma measurements, the means for each mouse in a group were averaged for statistical comparison.

Statistical analyses. Statistical analyses were performed using Instat and Prism software (GraphPad Software for Science). Group means were compared by two-tailed t test for comparison of two groups; two-tailed analysis of variance and multiple comparison tests were used for comparison of three or more groups. Statistical analyses of survival proportions were performed using two-tailed log–rank tests and two-sided Fisher’s exact test. P values ≤ 0.05 were considered to be statistically significant.

Results

Neonatal Exposure to 8WkId or MSS Id Leads to Prolonged Survival of Mice Subsequently Infected with S. mansoni. To determine the long-term effects of neonatal exposure to Id, we monitored the survival of chronically infected mice for 20 wk of infection. Mice were injected with 50 μg of different Id preparations within the first 24 h of life and then infected with 45 cercariae when they became adults. There were no deaths in any group before 8 wk of infection. Mice that received 8WkId lived significantly longer (P < 0.05) than mice that received NoMoIgG (Fig. 1, top and middle). A similar, significant prolongation of survival was also observed after neonatal injection of MSS Id compared with NoMoIgG (Fig. 1, top and middle). All mice that had received 8WkId or MSS Id displayed the less severe characteristics of MSS at 20 wk of infection. The percent survival at 20 wk for each of these experiments is shown in Table I. Interestingly, the mice that received HSS Id showed decreased survival proportions compared not only with 8WkId- and MSS Id–injected animals (P < 0.0001) but also with animals that received NoMoIgG (P < 0.002).

Although 8WkId, MSS Id, and HSS Id were all prepared on the same SEA-Sepharose column, we also tested survival of S. mansoni–infected mice injected neonatally with an mAb (E5) to counter the possibility that the effects observed in the survival studies were a consequence of SEA that had leached off the affinity column into the Id preparations. E5 was produced from a human heterohybridoma that shares CRIs with 8WkId and MSS Id (9, 12, 17). Because this Ab was not passed over the SEA affinity column, there is no chance that the preparation was contaminated with SEA. As with the polyclonal 8WkId or MSS Id preparations, neonatal exposure to E5 resulted in longer survival times than for mice that received NoHuIgG (Fig. 2).

Figure 1. Neonatal injection of mice with CRI (8WkId or MSS Id) confers increased survival upon subsequent S. mansoni infection. Mice neonatally injected with 8WkId, MSS Id, HSS Id, or NoMoIgG were infected at 8 wk of age and monitored for survival over the next 20 wk of infection. The neonatal injection is indicated in the key for each experiment, and the initial number of mice is shown in parentheses.
increased percentage of fibrotic liver tissue at 8 wk after infection (Fig. 3 B) than mice that received NoMoIgG or HSS Id at birth. The histologic appearance of granulomas and fibrotic areas representative of these means is presented in Fig. 4. We also observed that mice receiving 8WkId or MSS Id at birth had decreased 8-wk-infection spleen-to-body weight ratios than animals neonatally injected with NoMoIgG or HSS Id (Table II). To determine whether these differences in pathology were simply an effect of differences in worm burdens, we measured CCA levels in these animals but found no significant difference between mice in any of the neonatal injection groups (Table II).

However, in spite of the comparable worm burdens, mice that received HSS Id as newborns were found to have a significantly higher mean number of eggs per gram of liver than mice that had been injected with MSS Id (Table III), resulting in the fibrosis per egg between these two groups not being significantly different (data not shown). The numbers of eggs per gram of liver for mice neonatally injected with NoMoIgG or 8WkId were not significantly different from each other or from either the MSS Id or HSS Id injection groups (Table II).

Mice Neonatally Injected with 8WkId or MSS Id Present Distinct Cytokine Response Profiles at 8 wk of Infection. To study whether neonatal exposure to Id has an effect on cytokine profiles expressed in response to SEA or Id (11) during subsequent S. mansoni infections, spleen cells were harvested from 8-wk-infected mice that had been exposed to the different Id preparations 16 wk previously, as new...
borns. The spleen cells were stimulated with SEA or Id preparations, and the supernatants were assayed for cytokines. Spleen cells from mice infected for 8 wk that had received 8WkId or MSS Id as newborns produced higher levels of IFN-γ in response to SEA or stimulatory Id preparations (8WkId or MSS Id) than did spleen cells from mice neonatally exposed to NoMoIgG or HSS Id (Fig. 5). Consistent with previous results (11), and similar to anti-SEA Id preparations from hepatosplenic patients (2), HSS Id was not stimulatory for any of the measured responses for cells from any treatment group.

Because isotype switching to IgG2a in mice is at least in part effected by IFN-γ (15, 16), we investigated the anti-SEA IgG subclasses present in sera of the neonatally manipulated mice (Fig. 6). At 8 wk of infection, SEA- and SWAP-specific IgG2a and IgG2b levels in sera of mice neonatally injected with 8WkId or MSS Id were higher than those of mice neonatally injected with NoMoIgG or HSS Id (P < 0.0001). SEA- and SWAP-specific IgG1 levels were similar among the various neonatal injection groups, indicating that the differences in granuloma and fibrotic regulation were not related to a failure to make schistosome-specific Abs in mice neonatally treated with NoMoIgG or HSS Id.

**Discussion**

Many studies on different human parasitic diseases have shown an effect of maternal infection status on immune responses in the cord blood or early life of the child (8, 18-
In addition, two field site revisitation studies have shown that these effects on the immune system can persist for up to 20 yr (25, 26). However, due to ethical and logistical limitations, it has not been possible to study whether maternal infection status alters a child’s long-term pathology in these chronic infections. Therefore, most of the evidence for maternal infection status (i.e., perinatal exposure to parasite-related stimuli) having a beneficial effect on subsequent clinical form of disease is anecdotal.

This study examines a possible role of maternal idiotypic interactions in experimental S. mansoni infections using idiotypic manipulations of the neonatal immune system. Mice that received appropriate Id injections during the first 24 h of life displayed altered immune responses, decreased pathology, and increased long-term survival upon infection with schistosomiasis. The reduced pathology observed in mice neonatally injected with 8WkId or MSS Id was associated with a Th1-type response. These animals demonstrated increased production of IFN-γ by spleen cells stimulated with either SEA or CRI compared with animals that received NoMoIgG or HSS Id at birth when later stimulated with SEA, 8WkId, or MSS Id.

CCA levels, which reflect relative adult worm burden (13), were similar among all groups of mice. This demonstrates that the observed beneficial effects of appropriate neonatal Id injection are not explained by differences in infection level. We did find that the number of parasite eggs in the livers of animals neonatally injected with HSS Id were significantly higher at 8 wk of infection than in mice neonatally injected with MSS Id. This could be related to the observation that TNF-α potentiates egg production by female worms (31) and our previous observation (32) that TNF-α levels are dramatically elevated in HSS mice. When we divided the relative fibrotic area by the number of eggs in the livers, we did not see a significant difference between the amount of fibrosis per egg between groups, and therefore it is possible that the increased egg burden contributes directly to host mortality. Nevertheless, this difference is still determined by idiotypic manipulations at birth. Furthermore, we observed a difference in patterns of fibrosis in the different groups, as the livers from N oM olgG - or HSS Id - injected animals demonstrated areas of perportal fibrosis whereas the fibrosis in the livers of 8WkId - or MSS Id - injected animals was only associated with eggs in granulomas. Also, animals that received CRI...
as neonates demonstrated significantly less splenomegaly compared with animals injected with NoMoIgG or HSS Id at birth. Although it is not clear whether the decreased mortality in CRI-treated animals is related to the overall level of fibrosis, a difference in distribution of fibrosis, or decreased fibrosis subsequent to fewer eggs in the liver, it is apparent that the differences are related to the neonatal Id treatment and not a result of differences in the infection levels of these animals. The direct cause of mortality is being addressed in ongoing studies.

One common criticism of work using affinity-purified Abs is that antigen has leached from the column and the suggested effect of Ab is actually an effect of the leached antigen. We are confident that Id, and not leached antigen, is the important component of the neonatal injection for a variety of reasons. First, all Id preparations for a given experiment were made using the same SEA affinity column. If SEA were leaching off of the column and were the "active" component in the neonatal Id injections, there would be no differences between the effects observed between HSS Id and MSS Id or 8WkId injections. Second, mice injected with MSS Id or 8WkId, but not HSS Id or NoMoIgG, within the first 24 h of life have CRI and anti-CRI present in their sera at 8 wk of life, even in the absence of subsequent infection or exposure to schistosome antigens (our manuscript in preparation). These levels of CRI far exceed the amount of CRI initially injected (and that would have decayed by this time; reference 33) and indicate that neonatal Id injection induces animals to produce Id. Finally, work by Hang et al. (34) demonstrated that neonatal injections of SEA were ineffective at altering subsequent immune responses to schistosome infection unless at least 600–900 μg SEA were injected. Therefore, even if a small amount of SEA (which is undetectable on silver-stained gels of Id preparations [35]) did leach from the affinity column, it would not be sufficient to confer the results observed in these studies. Furthermore, a similar survival curve as seen with 8WkId and MSS Id was obtained using the CRI-expressing mAb, E5 (9, 12, 17).

Recent studies by Jankovic et al. (36) have also indicated a role for Ab in the immunoregulation of morbidity and mortality during experimental schistosomiasis. Mice lacking B cells exhibited decreased granuloma modulation and increased mortality compared with wild-type controls (36). FcγR knockout mice also exhibited decreased granuloma modulation (but no increase in mortality) upon infection, suggesting an essential role for FcR signaling in granuloma modulation (36). Our studies indicate that not every schistosome-reactive Ab is sufficient to initiate regulatory responses, but that specific anti schistosome Ids on certain anti-SEA Abs are required for the development of the immunoregulation of disease and protection from mortality during schistosomiasis. For example, HSS Id and sera from animals with HSS have strong reactivity to SEA (11; Fig. 6) but lack the CRI found in MSS Id or sera from MSS Id–infected animals (9). Not only do neonatal HSS Id injections fail to confer the immunoregulation and enhanced survival conferred by neonatal exposure to MSS Id or 8WkId, but animals neonatally injected with HSS Id actually have a significantly decreased survival proportion compared with mice that received NoMoIgG at birth (Fig. 1, middle and bottom). This result suggests the interesting possibility that in addition to idiotypic specificities that are beneficial for host immunoregulation of disease, there may also be other anti-SEA idiotypic specificities that promote morbidity and mortality.

Based on proliferative and cytokine responses of spleen cells to MSS Id but not HSS Id (11), we believe that Id is effecting responses through cross-linkage of T cell receptors. Although we have yet to directly demonstrate this in mice, studies on idiotypic stimulation of PBMCs from schistosomiasis patients suggest that this is the case (35, 37). Soluble, divalent whole Id or F(ab′)2 fragments of Id stimulate T cell proliferation, but monovalent Id Fabs do not unless they are covalently bound to a solid support. Processing by APCs is not required, and the need for adherent accessory cells can be overcome if IL-1 is provided (35, 37). The contention that the Id effect is receptor mediated is also supported by many similar studies demonstrating that even one low level perinatal exposure to Id-expressing Abs and/or anti-Id Abs profoundly alters the generation of B and T cell repertoires expressed later in life (38–46).

In most areas endemic for schistosomiasis, the majority of people infected with schistosomes were born of mothers that had schistosomiasis (1). Furthermore, few individuals born and raised in such areas exhibit acute, symptomatic disease upon their early schistosome infections, and only 5–8% go on to develop severe chronic disease. Based on the Id/anti-Id interactions reported here and observations in endemic areas, it might be fruitful to examine the possibility of these circumstances and their effects in a variety of chronic infections that exhibit endemicity and high prevalence in the child-bearing years (1).

The authors would like to thank Drs. Alfredo Goes and Barbara Doughty for the gift of the E5 mAb. The authors would also like to thank the Infectious Disease Pathology Activity, Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention for preparation of the liver tissues for histologic evaluation.

This work was supported in part by National Institutes of Health grant AI-11289.

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